Development of a Rat Model for Respiratory Infection with \textit{Bordetella pertussis}

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Considerable effort directed toward designing a safe and effective vaccine for \textit{Bordetella pertussis} in which the cellular and/or acellular antigens necessary to confer immunity are known has been hampered by lack of information on the pathogenesis of the natural infection. The study presented here describes an animal model of lung infection by \textit{B. pertussis} enucleated in agar beads in adult (200- to 220-g) male Sprague-Dawley rats. At 3 and 7 days after inoculation with phase I \textit{B. pertussis}, organisms could be recovered from the lungs of rats; however, organisms were not recoverable at days 10 and 14 but reappeared in lung homogenates at day 21. Histopathological examination revealed findings similar to those seen in human disease. At day 3, a mild lymphocytic infiltrate was present in the bronchi, with progressive lymphoid hyperplasia peribronchially. By day 7, a necrotizing inflammation of the tracheobronchial mucous membranes, characterized by both mononuclear and polymorphonuclear cells, was noted. Phase III \textit{B. pertussis} organisms were not recoverable from the lungs of inoculated rats at day 3 after inoculation, nor were histological changes noted in these animals. Clinical findings in phase I \textit{B. pertussis}-infected rats included hypoglycemia, circulating lymphocytosis, and paroxysms in which air was forcibly expelled from the mouth or nose.

\textit{Bordetella pertussis} causes a localized infection of the ciliated epithelium of human bronchi and the trachea, resulting in the disease whooping cough. The introduction of vaccines composed of inactivated \textit{B. pertussis} organisms reduced dramatically the incidence, morbidity, and mortality of pertussis (11). Interest in the disease has reemerged in recent years because of two interrelated factors: (i) a decreased public acceptance of the current vaccine as a result of reports of side effects (the vaccine causes local reactions in most recipients and, rarely, serious systemic reactions, including brain damage and death), and (ii) a subsequent increase in the incidence of the disease (3, 15).

Considerable effort directed toward designing a safe and effective vaccine in which the cellular and/or acellular antigens necessary and sufficient to confer immunity are known has been hampered by lack of information on the pathogenesis of the natural infection. Furthermore, studies on the pathogenesis of \textit{B. pertussis} respiratory infection have not advanced because of the lack of a totally satisfactory animal model. A mouse respiratory infection model has helped to identify \textit{B. pertussis} antigens that promote protection by active and passive immunization (4, 10). However, the model differs from human disease in several respects, including lack of cough, symptomatic upper respiratory infection, whoop, and the consistent production of a pertussis pneumonia. Thus, the pulmonary infection produced is not analogous to the most upper respiratory manifestations of human infection (6).

By using an approach that we originally developed to establish a chronic respiratory infection by \textit{Pseudomonas aeruginosa} (1), we have developed an animal model in rats for \textit{B. pertussis} which closely parallels the disease as seen in humans with respect to the clinical, pathological, and physiological changes seen with human infections.

\textbf{MATERIALS AND METHODS}

\textbf{Bacterial strains.} The Tohama I and III strains of \textit{B. pertussis} were used in this study. Tohama I is a phase I strain; it produces high yields of pertussis toxin and is virulent for mice when injected intraperitoneally. Tohama III, a phase III organism, is avirulent for mice.

\textbf{Growth conditions.} Organisms were initially grown on the surface of Bordet-Gengou plates at 37°C for 48 h. After incubation, heavy inocula of Tohama I (hemolytic colonies) and Tohama III (nonhemolytic colonies) were transferred to Stanier-Scholte medium (14) and incubated with shaking (200 rpm in a gyratory shaker; New Brunswick Scientific Co., Inc., Edison, N.J.) for 18 h at 37°C.

\textbf{Preparation of inocula.} Beads were prepared by a modification of the method of Cash et al. (1). Melted 2% (wt/vol) ionagar no. 2 (Oxoid Ltd., London, England) in phosphate-buffered saline (PBS), pH 7.0, was kept at 50°C, and a PBS dilution of overnight bacterial culture was added to give a final concentration of approximately 10^7 bacteria per ml of melted agar. Heavy mineral oil (200 ml; Fisher Canada, Inc.) warmed to 50°C was vigorously stirred with a magnetic stir bar. To this, 10 ml of melted agar, with bacteria, was added. The oil-agar mixture was cooled rapidly by placing crushed ice around the vessel while stirring continued for approximately 5 min. During this time, agar droplets solidified into beads. After three washes in PBS (10,000 × g, 4°C) to remove excess mineral oil, the loosely packed beads were suspended in an equal volume of PBS to form a bead-buffer slurry, and the material was placed on ice before inoculation of animals.

\textbf{Experimental animals.} A total of 200 adult (200- to 220-g) male Sprague-Dawley rats were used. Of these, 100 were inoculated with Tohama I and 100 were inoculated with Tohama III by deposition of 0.05 ml of the above-described inocula into the left lower lobe of anesthetized and tracheotomized rats, using a bead-tipped curved needle as previ-
ous injury to the heart and lungs en bloc. Ten percent buffered formalin was instilled via the trachea under 10 cm of hydrostatic pressure, while the tissue was immersed in the same preservative. Slices of the fixed lungs, approximately 5 mm thick, were dehydrated in graded alcohols, embedded in paraffin, and cut into sections 6 μm thick. Mounted sections were stained for light microscopy with hematoxylin and eosin.

Electron microscopic examination of fixed lung tissues was performed on tissues washed with cacodylate buffer (0.05 M, pH 7.4), postfixed in osmium tetroxide (1% in cacodylate buffer) for 2 h, washed with cacodylate buffer, and dehydrated in increasing concentrations of acetone. Specimens were further dehydrated in propylene oxide and embedded in Epon 812. Thick sections (1 μm) were stained with toluidine blue and then examined by light microscopy to evaluate embedding and condition of tissues and to select areas for subsequent electron microscopic examination. Thin sections (80 nm) were stained with uranyl acetate and lead citrate. Thin sections were examined in a Hitachi H-600 electron microscope.

For quantitative bacteriological studies, the entire lung was removed, and lobes were cut from the hilus and placed in 3 ml of sterile PBS. The tissue was homogenized with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, N.Y.). Serial dilutions of the homogenate in PBS were plated on Bordet-Gengou agar plates.

Physiological measurements. Total circulating leukocytes were counted by hemacytometer, using the Unopette system (Beckton Dickinson and Co., Paramus, N.J.). The percentage of lymphocytes was determined by differential counting of whole-blood smears after Wright staining. Serum glucose levels were determined by the glucose oxidase method (procedure 510; Sigma Chemical Co., St. Louis, Mo.).

Serum antibody levels. Serum antibody levels to pertussis toxin and lipopolysaccharide (LPS) (7) were determined by enzyme-linked immunosorbent assay (5). Purified pertussis toxin and LPS were obtained by published procedures (8, 12). Blood obtained by cardiac puncture from 50 noninfected rats was used to obtain control values for the physiological and antibody measurements. Blood for the physiological and antibody measurements in infected animals was obtained at the time of sacrifice.

RESULTS

Quantitative bacteriological findings. The Tohama I and III strains of B. pertussis have been used for our initial studies of B. pertussis pathogenesis in the rat lung model. Tohama I was capable of establishing an infection in the rat lung model, whereas Tohama III was completely unable to do so (Table 1). Initial quantitative counts in the Tohama I-infected lungs were at a mean level of 6.24 × 10⁷ at day 3. By day 7, counts decreased to 3.1 × 10⁶. We were unable to recover organisms at days 10 and 14 after inoculation; however, 7 × 10⁴ organisms were recovered from Tohama I-infected animals at day 21 after inoculation. No mortality was seen before sacrifice, and no organisms were recoverable from blood.

Our current hypothesis regarding the inability to recover organisms at days 10 and 14 after inoculation is that these organisms were residing intracellularly in the lungs at low levels. This hypothesis was tested by examining the lungs of Tohama I-infected animals by electron microscopic techniques (Fig. 1). Figure 1A demonstrates B. pertussis organisms present inside an agar bead in a large airway, Fig. 1B demonstrates organisms residing intracellularly, and Fig. 1C is a higher magnification of Fig. 1B showing that these organisms were electron dense and most likely viable. Cells that contain intracellular organisms have not been identified to date. Immunofluorescent-antibody treatment of Formalin-fixed lung tissue from these animals, using antibody to B. pertussis Tohama I LPS, demonstrated positive fluorescence (not shown), which indicated that the intracellular organisms were most likely B. pertussis. Interestingly, increases in antibody to LPS correlated with disappearance of cultivatable organisms from the lungs (Table 2). Antibody levels to pertussis toxin, while increasing significantly after inoculation, did not seem to correlate with the inability to culture organisms from the lungs.

Histopathological findings. Histopathological examination of lung tissue from rats infected with the Tohama I strain of B. pertussis (Fig. 2) revealed findings similar to those seen in early autopsy studies (8). At day 3, a mild lymphocytic infiltrate appeared in the bronchi, with progressive lymphoid hyperplasia peribronchially. By day 7, a necrotizing inflammation of the tracheobronchial mucous membranes was noted, characterized by both mononuclear and polymorphonuclear cells which spread to involvement of the terminal bronchioles and alveoli by day 21.

Physiological findings. Rats infected with the Tohama I strain of B. pertussis became hypoglycemic. These animals exhibited a significant reduction in blood glucose levels at day 7 after inoculation, which continued throughout the 21-day study period (Table 2). In addition, Tohama I-infected animals exhibited a significant circulating lymphocytosis in comparison with noninfected control animals (P < 0.05, paired t test; Table 2). This lymphocytosis was present at day 3 after inoculation and persisted throughout the 21-day study period. Additional clinical symptoms noted in B. pertussis-infected rats included paroxysmal cough with whooping similar to that heard in humans.

DISCUSSION

One of the greatest limitations to the study of microbial virulence factors is the availability of relevant animal models (16). This is particularly important for pertussis, which appears to be a uniquely human disease. The rat model described here may prove to be of particular significance because of the marked similarities between the model and human disease.
In the prototypical case, pertussis is characterized by three phases (16). After exposure to the organism, it is generally 5 to 7 days before symptoms occur, and these initial symptoms of the catarrhal phase are nonspecific and similar to those of the common cold. During this period, B. pertussis can be isolated from nasopharyngeal cultures at a rate as high as 90%. The catarrhal phase becomes the paroxysmal phase as the dry, nonproductive cough evolves to the characteristic paroxysmal cough, with mucous production and vomiting. Patients exhibit other pathophysiological changes, including lymphocytosis, weight loss, occasional hypoglycemia, and, rarely, encephalopathy (9). The isolation rate for B. pertussis drops progressively after the onset of the paroxysmal phase, and the inability to culture the organisms during the most severe stage of the disease has led to the hypothesis that the majority of the clinical findings

<table>
<thead>
<tr>
<th>Time (days)*</th>
<th>Median titer by ELISA*</th>
<th>Blood glucose* (mg/100 ml, mean ± SD)</th>
<th>Leukocyte count* (mean ± SD)</th>
<th>% Lymphocytes* (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (uninfected controls)</td>
<td>1:2 1:2</td>
<td>172.5 ± 16.3 0.90 ± 0.20</td>
<td>77 ± 6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1:2 1:2</td>
<td>165.0 ± 22.2 2.1 ± 0.81</td>
<td>89 ± 4*</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1:8 1:16</td>
<td>123.3 ± 10.3f 2.8 ± 0.90f</td>
<td>92 ± 6f</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1:8 1:128</td>
<td>121.2 ± 5.1f 2.5 ± 0.93f</td>
<td>90 ± 5f</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>1:64 1:512</td>
<td>89.8 ± 19.4f 2.0 ± 0.85f</td>
<td>94 ± 7f</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>1:512 1:512</td>
<td>140.5 ± 42.2 1.9 ± 0.77f</td>
<td>92 ± 5f</td>
<td></td>
</tr>
</tbody>
</table>

* For the time 0 determinations, 50 uninfected control rats were used; for determinations at all other time points, 20 rats were used.
* ELISA, Enzyme-linked immunosorbent assay.
* Measured by the glucose oxidase procedure.
* From Wright-stained smears.
* Significantly different from uninfected control value (P < 0.05, paired t test).
FIG. 2. Light microscopic examination of histopathological changes observed during infection with *B. pertussis* Tohama I. Early changes seen at day 3 after inoculation were characterized by a marked mononuclear cell infiltrate surrounding the airways (A). Higher magnification of the enclosed area of panel A demonstrated that the cellular response was primarily lymphocytic in nature (B). *B. pertussis* organisms in agar beads in large airways were surrounded by lymphocytes (C). Histopathological findings at 21 days demonstrated a significant cellular response, both mononuclear and polymorphonuclear in nature, to the presence of *B. pertussis* in the airways for an extended time period (D). Bars, 100 μm.
are due to toxins released by the bacteria and disseminated systemically, since the organisms do not enter the bloodstream (9, 10).

The Tohama I and III strains of *B. pertussis* have been used for our initial studies in the rat lung model. Tohama I is a phase I strain that produces high yields of pertussis toxin and is virulent for mice when injected intraperitoneally. Tohama III is a phase III organism and is avirulent for mice. The finding of intracellular organisms in the lungs of Tohama I-infected animals is of particular interest, since it may explain our inability to recover organisms from the lungs of these animals at days 10 and 14 after inoculation, which was a reproducible finding. Furthermore, this finding could explain the human situation in which organisms are nonrecoverable during the paroxysmal stage of pertussis, when symptoms are the most severe. Increases in antibody to LPS correlated with disappearance of culturable organisms from the lungs. Antibody levels to pertussis toxin did not correlate with the ability to culture organisms from the lungs. Previously investigators have reported that *B. pertussis* may reside intracellularly in macrophages (2). Studies are ongoing to determine the cell population in the rat lung which harbors intracellular *B. pertussis* organisms.

Clinical findings of pertussis have included descriptions of lymphocytosis and hypoglycemia, both of which are present in the animal model described here and are presumably due to the action of pertussis toxin. Examination of islet cells by fluorescent-antibody procedures using antibody to pertussis toxin should provide some information on whether islet cell damage is pertussis toxin mediated.

Support for the classical view of pertussis in humans is for the most part derived from a limited number of autopsy studies. In 1912, Mallory and Horner (8) examined three cases at autopsy and found the bacteria growing among the cilia. This was an important finding in that it provided a connection between the organism and the lesion, i.e., the cough. Smith described two cases in which the bronchopneumonia in whooping cough was due to *B. pertussis* alone (13). He identified organisms in the alevoli and concluded that infection in this area could be the most important lesion in disease. G. M. Lawson (Ph.D. thesis, Harvard School of Public Health, Cambridge, Mass., 1932) examined 27 patients at autopsy; in patients who died before week 5 of illness, the alveoli were almost invariably culture positive for *B. pertussis*, whereas the trachea or bronchi were less apt to be culture positive. The information from these studies provides strong support for the suggestion of *B. pertussis* as a direct cause of bronchopneumonia and indicates that the lung may be a major site of infection. Certainly, our own data from the animal model studies strongly support the concept that *B. pertussis* can cause significant pneumonia. By day 7 of the infection, a necrotizing inflammation of the tracheobronchial mucous membranes was noted, characterized by both mononuclear and polymorphonuclear cells which spread to involvement of the terminal bronchioles and alveoli by day 21.

In summary, the rat lung model of *B. pertussis* infection described here closely resembles human disease with respect to clinical, histopathological, and pathophysiological changes. It should prove useful in studies of *B. pertussis* pathogenesis as well as in tests of current and future vaccine candidates for the prevention of pertussis.

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LITERATURE CITED