Immunoglobulin and Histamine-Sensitivity Response of Mice to Live Bordetella pertussis

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Mice injected intranasally (i.n.) and intraperitoneally (i.p.) with a nonlethal dose (2.5 × 10⁴ colony-forming units) of live Bordetella pertussis were examined for 50 days for infection, respiratory tract immunoglobulins (Ig), changes in serum Ig, and histamine sensitivity. With mice infected i.t.n., respiratory infection markedly declined between day 20 and day 30. Ig classes (A, G₁, G₂a, G₂b, but no M), which had specificity for B. pertussis, were present in tracheobronchial wash (TBW) by day 15; by day 50, TBW immunodiffusion and immunoelectrophoretic precipitin bands were more intense. A sharp rise in serum IgA after day 30 was the only significant change relative to controls among the five serum Ig examined. A high degree of histamine sensitivity developed by day 15 to 20 and persisted for the 50 days. With mice inoculated i.p., no bacteria were recovered, no Ig or only traces were found in TBW and IgA only was specific, and no significant changes in the serum Ig relative to controls occurred. Histamine sensitivity developed somewhat more slowly and to a lesser degree than in i.t.n.-injected mice but persisted for the 50 days. A similar small number of killed bacteria (pertussis vaccine) injected i.t.n. or i.p. likewise induced slowly developing histamine sensitivity in contract to published reports of 4 to 5 day peak sensitivity and decline following i.p. injection of 10⁴ or more killed bacteria.

Whooping cough (pertussis), the disease caused by Bordetella pertussis, has been effectively controlled by intramuscular or subcutaneous immunization with standardized pertussis vaccine (14). The immunological mechanism involved in prophylaxis against this local respiratory infection is not understood. Pittman (14) has postulated that, although the disease does not develop, infection may not be prevented. If permeability of the respiratory tract, like in the experimental mouse intracerebral infection, is altered after infection, and if protective antibodies gain access to and kill the bacteria, then the characteristic symptoms of the disease do not develop. Iida et al. (6) found that, with the use of immunofluorescence, anti-pertussis serum (equine) inoculated intraperitoneally (i.p.) into intracerebrally infected mice did not contact the bacteria, largely localized among the cilia of the ependymal cells lining the ventricles, until vascular permeability had been increased. Bacteria had to reach a certain concentration before effecting the change in the brain barrier.

As in prophylaxis, the nature of the mechanisms of recovery from the disease has not been fully explained. A number of papers on passive protection in the mouse support the role of immunoglobulins. Dolby and Dolby (4) reported that 7S and 19S globulins mixed with challenge culture were equally effective against lung infection in the mouse, whereas 7S was 100 times more effective than 19S against brain infection. Kendrick et al. (7) likewise found that the protective activity against the brain infection was in the immunoglobulin class G (IgG) fraction of rabbit Ig. The effectiveness of the different classes of Ig in man has not been studied adequately.

In an approach to a better understanding of active protection in pertussis, we studied, with the use of the mouse respiratory infection model (1, cf. 14), the classes of respiratory and serum Ig present during infection and recovery. Comparisons were made with Ig that were present after i.p. injection of live B. pertussis. The development and duration of histamine sensitization was also followed. The mouse model

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resembles the human infection in several aspects. The duration of infection is similar, and the onset and duration of histamine sensitivity in the mouse parallels paroxysmal coughing in the child (13).

**MATERIALS AND METHODS**

**Mice.** N: NIH(SW) strain female mice, approximately 1 month old and weighing 14 to 17 g, were received from the Rodent and Rabbit Production Section, Division of Research Services, National Institutes of Health. They were housed 6, 10, or 16 per cage and given food and water ad libitum.

**Bacterial techniques.** The *B. pertussis* culture, strain no. 18333, was prepared by the method described for the challenge in the pertussis-vaccine potency assay (17). A 17- to 20-h culture grown on Bordet-Gengou (B-G) agar slants containing about 15% defibrinated rabbit blood was suspended in a solution of 1.0% Protolyse (a pancreatic enzyme digest of casein, Mead Johnson & Co., Evansville, Ind.) and 0.6% NaCl, filtered through cotton and adjusted by spectrophotometry to 10 U.S. opacity units (OPU) (17), then diluted to provide an inoculum of $10^{-2}$ OPU in a volume of 1 minin (approximately 0.06 ml) for either intranasal (i.n.) or i.p. injection. (The U.S. Opacity Standard has an assigned value of 10 units. It was originally adjusted to be equivalent in turbidity to $10 \times 10^8$ [direct count] bacteria in aged pertussis vaccine. Suspensions of freshly harvested live bacteria of equal turbidity have around $6 \times 10^8$ bacteria, and colony counts are lower.) The inoculum contained approximately $2.5 \times 10^9$ colony-forming units (CFU; about $6 \times 10^6$ bacteria) and was nonlethal.

One drop of a tracheobronchial wash (TBW), a cut surface of a lung lobe, and peritoneal fluid were cultured on plates of B-G agar containing no peptone. The lung fragment was rubbed on the surface and left in situ. Peritoneal fluid was transferred with a cotton swab. Cultures were incubated for 3 to 5 days. Identity of *B. pertussis* was determined by typical colonial and bacterial morphology.

**Sera.** Blood obtained from the tail vein after a brief warming of the mouse under an incandescent lamp was collected in polyethylene tubes (400 $\mu$liters) and spun for 5 min. Serum was withdrawn and stored individually or in pools of equal volumes per mouse at $-15^\circ$ C. The reference normal mouse serum consisted of a pool from N: NIH(SW) mice. It was distributed in small samples before storage. A freshly thawed portion was used for each test. Residues were discarded. The levels of Ig classes M, A, G, $G_m$, and $G_m$ (IgM, IgA, IgG, $IgG_m$, and $IgG_m$) in this reference were determined by radial immunodiffusion by R. Asofsky, of the National Institute of Allergy and Infectious Diseases.

Goat antisera to mouse myeloma proteins, made monospecific for IgM, IgA, IgG, $IgG_m$, and $IgG_m$ by adsorption, were used in the identification tests for specific globulins. They were kindly supplied by R. Asofsky.

**TBW.** After bleeding, the mouse was anesthetized with sodium pentobarbital. The trachea was exposed, hemisected, and intubated with fine-gauge polyethylene tubing (PE 10) which was tied in place with suture. By use of a tuberculin syringe fitted with a 26-gauge needle and filled with 1 ml of cold saline, the tracheobronchial tree was washed by gently injecting and withdrawing the saline. Slight tension exerted on the trachea via the tubing insured a maximal return of the injected fluid. In experiment I, one drop of the TBW from each animal was cultured for *B. pertussis* and the remaining TBW per group were pooled. Each pool was concentrated in a collodion bag to approximately one-tenth the original volume and examined for presence of the five subclasses of Ig. In experiment III, individual unconcentrated TBW were tested for presence of Ig.

**Ig determinations.** The presence of Ig in serum and TBW was determined by immunodiffusion and immunoelctrophoresis. The quantitation of the immunoglobulins was by radial immunodiffusion (RID) on cellulose acetate (18) or on agar (9), or both. In addition, individual TBW in experiment III were examined by immunofluorescent microscopy as follows.

Smears of a suspension of a 20-h culture of *B. pertussis* were made on microscope slides, air-dried, and heat-fixed. The TBW from each mouse was added to five slides. The slides were incubated for 20 min in a moist chamber, then washed twice in 200 ml of fresh saline containing 0.2% gelatin for 10 min per wash. Next, monospecific goat antiserum IgA, IgM, IgG, $IgG_m$, or $IgG_m$ was added to one of the five slides. The slides were incubated for 20 min and washed twice. Normal goat serum was applied to a control culture-smeared slide. Finally, diisothiocyanate-labeled anti-goat serum (rabbit; Cappel Laboratories, Inc.) was added to each slide. Slides were incubated for 20 min and washed three times. They were examined by use of a Zeiss ultraviolet microscope fitted for reflected-light fluorescent microscopy.

**Histamine sensitization.** In experiment I, groups of 16 mice per study group at the respective bleeding times were tested for histamine sensitivity by i.p. injection of 100 mg of histamine diphosphate per kg of mouse weight (0.36 mg of histamine base/10 g of mouse weight). In two subsequent experiments, the sensitizing effect of the small dose of living bacteria and the same number of killed bacteria were compared. Pertussis vaccine lot 7b was used. Deaths usually occurred within 2 h. Final results were recorded at 24 h and were reported as the percentage of deaths per group of 16 mice.

**Experiment I.** Two groups of 124 mice each were inoculated i.n. or i.p., respectively, with the dose of culture described above. A control group of 156 mice was not inoculated. Before inoculation and 5, 10, 15, 20, 30, and 50 days thereafter, four mice per group were bled. The five classes of Ig of the pooled sera per group were determined by RID, the TBW were cultured individually, and pools per group were tested for presence of Ig classes by immunodiffusion and immunoelctrophoresis. At day 5 and at each subsequent period, 16 additional mice from each group were tested for sensitivity to histamine.

**Experiment II.** Three groups of five mice each
were bled. Then one group was inoculated with the culture i.t.n., another group was inoculated i.p., and the third group was left untreated. Each animal was bled weekly for 7 weeks. The Ig level of the serum pool per group per bleeding time were determined as in experiment I. Since mice were not sacrificed after bleeding, no cultures were made.

Experiment III. Five mice per group were inoculated either i.t.n. or i.p. with the culture. On day 15, TBW were obtained and examined individually without concentration by immunodiffusion, immunoelectrophoresis, and immunofluoroscopy for presence of Ig classes.

RESULTS

B. pertussis infection. In experiment I, intranasal instillation of the nonlethal dose of B. pertussis caused a high incidence of respiratory tract infection which remained for 20 days, then declined (Table 1). On day 30 only a few organisms were recovered from one of four mice. On day 50 all cultures were negative. At no time was B. pertussis recovered from i.p.-inoculated or control mice.

Serum Ig. In experiment I the measured levels of five serum Ig classes of the three study groups of mice from 0 to 50 days are shown in Fig. 1. Although there were rather wide variations between the readings of each Ig class per group, there was an upward trend in the amounts of each class in the sera of the mice in all test groups. The differences between the respective Ig levels of i.t.n., i.p., and control mice were not significant except for the IgA level of the i.t.n. mice. With the latter, the level rose from 0.89 mg/ml on day 30 to 2.61 mg/ml on day 50 (Fig. 1 and Table 2).

In experiment II, instead of using separate groups of mice per bleeding as in experiment I, the same five mice per group were bled weekly throughout the experiment. The measurements of Ig classes (Fig. 2) varied more widely than those in experiment I. In general, however, the results of the two experiments were in agreement. The rise of the IgA level of the i.t.n. mice of experiment II from 0.34 mg/ml on day 28 to 2.34 mg/ml on day 42 was the significant finding (Fig. 2 and Table 2).

TBW Ig. In experiment I, the immunoelectrophoretic and immunodiffusion tests demonstrated the presence of Ig in the pooled concentrates of TBW from i.t.n.-infected mice only. The results given in Fig. 3 illustrate the presence and absence of Ig in the TBW from i.t.n. and i.p. mice, respectively. In both types of tests the bands for each of the four Ig classes were more intense with day-50 than day-15 TBW. At no time was IgM detected. Tests of TBW from control mice (not shown) were negative.

In experiment III, five individual unconcentrated TBW collected on day 15 from i.t.n. mice had the same four classes of Ig as did the pooled TBW in experiment I. In contrast, in experiment III the TBW from three of five i.p. mice showed faint bands for IgG2a and IgG2b, and one showed a band for IgG, and for IgA. Although the same technique was used in each experiment, serum source of the small amounts of Ig in TBW from the i.p. mice in experiment III cannot be excluded. There were marked differences in the numbers and intensities of the precipitin bands in the immunoelectrophoretic patterns of the TBW from individual i.t.n. and i.p. mice (Fig. 4).

The immunofluorescent test of the individual TBW from i.t.n. mice confirmed the presence of the four classes of Ig and also their specificity for B. pertussis and the lack of IgM (Table 3). With the TBW from i.p. mice, IgA only was bound to B. pertussis and in trace amount.

Histamine sensitzation. The i.t.n. mice of experiment I gradually developed histamine susceptibility which was at a peak of 94 to 88% between days 15 and 30 and then declined to 69% by day 50 (Fig. 5). Sensitization of the i.p. mice developed more slowly and reached a lower peak of 56% on day 20. Thereafter sensitivity remained almost constant through day 50. This

<table>
<thead>
<tr>
<th>Route of inoculation</th>
<th>Source of culture</th>
<th>Cultures positive/4 mice cultured*</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0*</td>
</tr>
<tr>
<td>Intranasal</td>
<td>Tracheobronchial wash</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Lung smear</td>
<td>0</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td>Swab of peritoneal cavity</td>
<td>0</td>
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</tbody>
</table>

* Cultures of control mice caged in close proximity to inoculated mice were always negative.

a Day after inoculation.

c Number in parentheses is number of individual cultures that showed a mixture of B. pertussis and other bacteria.

d Only two colonies were present in the culture.
sensitization of the i.p. mice was unexpected.

Induction of histamine sensitivity in mice usually has been demonstrated by i.p. injection of about 10^9 or more killed bacteria. Sensitivity reaches a peak in 4 to 5 days and then declines (8). Sensitization with a later peak by a small number of live bacteria given it.n. has been attributed to multiplication of the bacteria to a sensitizing concentration (13). Because there is little or no multiplication of B. pertussis in the peritoneal cavity, the relatively small dose of 2.5 x 10^4 CFU given i.p. had not been expected to induce sensitization, much less sensitization of prolonged duration.

Two experiments were performed to compare the sensitizing action of the same number of live and dead bacteria (Fig. 6). The live bacteria injected it.n. induced a response that was similar to the one shown in Fig. 5, with peak on day 20, whereas sensitization induced by live bacteria injected i.p. was lower than shown in Fig. 5: the peak of 31% was on day 28 to 32 when the test was terminated. A similar pattern was induced by the vaccine injected it.n. However, the vaccine given i.p. induced a level of sensitivity (53%) similar to the level (56%) induced by live bacteria injected i.p. (Fig. 5), but the decline appeared to be more rapid. Further testing would be required to determine whether the difference observed were due to chance selection of mice of different susceptibility to sensitization. It is definite, however, that a small number of live or killed B. pertussis cells are capable of inducing late histamine sensitivity by either i.p. or it.n. route of injection.

**DISCUSSION**

The B. pertussis infection of the respiratory tract of mice followed the course described previously for this model (e.g., 13, 15). The bacteria were recovered in cultures of the TBW and lungs through 20 days, then the bacteria declined markedly by day 30. The duration of infection resembled that of human pertussis.

**TABLE 2. Serum immunoglobulins of mice 49 to 50 days after B. pertussis inoculated intranasally (it.n.) and intraperitoneally (i.p.)**

<table>
<thead>
<tr>
<th>Immuno-globulin class</th>
<th>Expt I</th>
<th>Expt II</th>
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<tbody>
<tr>
<td></td>
<td>it.n.</td>
<td>i.p.</td>
</tr>
<tr>
<td>IgM</td>
<td>1.7 ± 1.1</td>
<td>0.47</td>
</tr>
<tr>
<td>IgG2b</td>
<td>2.2 ± 2.0</td>
<td>1.30</td>
</tr>
<tr>
<td>IgG1</td>
<td>1.1 ± 1.1</td>
<td>1.80</td>
</tr>
<tr>
<td>Total IgG</td>
<td>5.00 ± 5.10</td>
<td>4.20</td>
</tr>
<tr>
<td>IgA</td>
<td>2.61 ± 0.95</td>
<td>1.05</td>
</tr>
<tr>
<td>IgM</td>
<td>0.55 ± 0.55</td>
<td>0.57</td>
</tr>
<tr>
<td>Total Ig</td>
<td>8.16 ± 6.60</td>
<td>5.82</td>
</tr>
<tr>
<td>IgA (%)</td>
<td>32.0 ± 14.4</td>
<td>18.0</td>
</tr>
</tbody>
</table>

* Measured in milligrams per milliliter.
* Significantly different from IgA levels of i.p. and control mice (P < 0.01).
* Day 42 serum.
antibodies were specifically bound to *B. pertussis*. IgA gave the most intense reaction. In contrast, no Ig was demonstrated in pooled TBW concentrates from the i.p.-inoculated mice in experiment I, but in experiment III some individual TBW had traces of one or two IgG subclasses and IgA. Only IgA was specifically bound to *B. pertussis*.

Both culture-inoculated and control mice showed an upward trend in the amounts of five classes of Ig in the serum. Maturation was one influential factor. Mice were about 4 weeks old at the beginning of the experiments. At 50 days the measured quantities of the respective Ig classes did not differ significantly between the it.n., i.p., and control mice except for IgA (Table 2). Differences in the levels between experiment I and experiment II may have been influenced by the repeated weekly bleedings. In both experiments the most striking and significant observation was the rise in serum IgA in the it.n. mice after day 30; it constituted more than 30% of the total Ig. Although antibody activity of the serum globulins was not determined, the high postinfection rise in serum IgA appears to be correlated with the increase in serum IgA antibody in cholera patients. Northrup and Hossain (12) observed more than a 30-fold increase in serum IgA antibody against *Vibrio cholerae* after disappearance of the vibrios from the intestines of the patients. Increase in total serum IgA was minimum (Northrup, personal communication). Characteristic of both diseases is the localization of the infectious bacteria on a membrane surface which is capable of producing IgA. In each infection the stimulus for the marked rise in serum IgA was no doubt related to the site of the infection.

Information on the antipertussis specificity of serum globulins of both mouse and man is needed. Wiedermannová and Wiedermann (19) measured by immunoelectrophoresis the serum IgG, IgM, and IgA levels in children 3, 5, and 60 weeks after beginning of infection. No significant differences relative to time or to agglutinin titers were found. However, *B. pertussis* antibody activity of the classes of Ig was not determined.

In our study the time sequence of the disappearance of *B. pertussis* and the presence of apparent increasing concentrations of respiratory immunoglobulins suggest that Ig played an antibacterial role. North (11) found that serum from it.n.-infected mice bled on days 30 to 50 passively protected mice, whereas 14-day serum was ineffective. IgG antibody is antibacterial and passively protective for mice against *B.
FIG. 3. Experiment I: Immunoelectrophoretic precipitin bands of four classes of Ig in pooled tracheobronchial wash (TBW) collected 15 days (bottom) and 50 days (top) after intranasal (itn) injection of live B. pertussis. No Ig precipitin bands developed with the TBW from the intraperitoneally (ip)-injected mice. Troughs contained anti-mouse serum (rabbit).

FIG. 4. Experiment III: Immunoelectrophoretic patterns of individual tracheobronchial washes from five intranasally (ITN) and five intraperitoneally (IP) injected mice collected on day 15. Troughs were filled with anti-mouse serum (rabbit).
pertussis (4, 7). The mechanism of IgA action has not been fully explained. IgA inhibits growth of viruses (16). Less is known about its antibacterial function (21). Very recently Williams and Gibbons (20) presented evidence of action through inhibition of bacterial adherence to the mucosal surface.

Our study has not answered the question of how parenterally administered pertussis vaccine prevents the development of whooping cough. However, trace amounts of Ig in the respiratory tract of some i.p. mice suggests that serum Ig may pass the lung barrier. The same question has been raised in regard to the prevention of cholera by parenterally administered cholera vaccine (10). The explanation in each case may be analogous. Recently Fubara and Freter (5) observed the entry of serum antibodies in the intestinal lumen of animals immunized with V. cholerae. Earlier Crabbe et al. (3) reported the development of IgA antibodies in the intestinal mucosa following parenteral as well as oral immunization with ferritin. A study of the antibody immunoglobulin response in mice challenged i.t.n. after recovery from respiratory infection and after i.p. vaccination of live and dead bacteria should provide applicable information. Although not considered here, the direct intervention of cells by phagocytosis or delayed hypersensitivity must not be ignored.

As observed previously (13), the gradual development and persistence of histamine susceptibility after subsidence of infection in the mouse paralleled the onset and duration of paroxysmal coughing relative to infection in the child. A new observation was the late peak development (20 days) and persistent susceptibility induced by a small number of bacteria. This was in contrast to 4- to 5-day peaking and decline following the customary use of 10^8 or more bacteria for induction of histamine susceptibility. This difference suggests that the mechanisms of action of large and small numbers of bacteria may be different. One aspect to investigate would be the relative amounts of IgE induced by the different amounts of bacteria (2).

LITERATURE CITED