Biological Activity of *Bordetella pertussis* in Lipopolysaccharide-Resistant Mice

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Effects of *Bordetella pertussis* organisms, such as adjuvanticity, induction of hypersplenia, and leukocytosis as well as modification of nonspecific resistance to infection and typical morphological response of lymphatic organs, were studied in the lipopolysaccharide-resistant C3H/HeJ mouse strain. It was shown that *B. pertussis* exerted all of these effects in C3H/HeJ mice, although the morphological response, hypersplenia, and modification of resistance to infection with *Listeria monocytogenes* in such animals were less pronounced than those in lipopolysaccharide-sensitive mouse strains. This indicated that the biological activity of *B. pertussis* as determined in the present studies, is due partly to structural components other than lipopolysaccharide.

Killed *Bordetella pertussis* organisms possess a variety of biological activities; adjuvanticity, induction of hypersplenia, leukocytosis, and increase of nonspecific resistance against infection are the most prominent activities (5, 16). However, until now it has remained obscure whether the endotoxic moiety of killed *B. pertussis* organisms alone, as described by Malkiel and Hargis (15), or other structural components in conjunction with endotoxin, as suggested by other workers (9), are responsible for the biological effects of killed *B. pertussis* organisms. Very recently it has been reported (1) that a nontoxic component of *B. pertussis* endotoxin is able to increase nonspecific resistance against infection. Therefore, the C3H/HeJ inbred mouse strain, which, due to a single gene defect (25), is not susceptible to bacterial lipopolysaccharide (LPS) (3, 17, 18, 20, 22, 24) was chosen to test whether killed *B. pertussis* organisms are able to produce adjuvanticity, hypersplenia, leukocytosis, and modification of nonspecific resistance in this strain of mice.

**MATERIALS AND METHODS**

Mice. C3H/HeJ and C3HeB/FeJ mice were purchased from Jackson Laboratories, Bar Harbor, Maine. NMRI/Han mice were obtained from the Central Institute for Laboratory Animals, Hannover, Federal Republic of Germany. Throughout these experiments, male animals weighing between 22 and 26 g were used. The mice were fed ready-made pellets (Ssniff; Intermast, Soest, Federal Republic of Germany) and water ad libitum.

*B. pertussis* organisms. Suspensions of killed *B. pertussis* organisms were received from Boehringer AG, Marburg, Federal Republic of Germany. Suspensions contained $3 \times 10^{10}$ bacteria per ml (phase I, not absorbed, and killed at 56°C) and Merthiolate (1:10,000). Shortly before use, bacterial suspensions were appropriately diluted in 0.9% phosphate-buffered saline, pH 7.2.

Antibody plaque technique. The numbers of direct and indirect plaque-forming cells (PFC) were determined by methods originally reported by Jerne et al. (13) and Wortis et al. (26), with slight modifications as described earlier (8). Direct PFC are generally regarded as 19S antibody producers, whereas indirect PFC primarily represent 7S antibody-producing cells.

Serological tests. Agglutinating and hemolyzing antibodies against sheep erythrocytes (SRBC) were measured in test sera without (19S and 7S antibodies) and with (7S antibodies) 2-mercaptoethanol treatment as described earlier (5).

Antigen. SRBC suspensions were diluted with phosphate-buffered saline to a final concentration of $5 \times 10^7$ SRBC or $4 \times 10^8$ SRBC/0.2 ml. These erythrocyte suspensions were administered intraperitoneally (i.p.).

Leukocyte count. Blood was taken from the tail vein of mice and appropriately diluted in Isotonic (Coulter Electronics). Leukocyte counts were determined after hemolysis with saponin in a Coulter Counter, model P.

Infection with *Listeria monocytogenes*. Infection with *L. monocytogenes*, a facultative intracellular parasite, was performed by the method of Mackaness (14), employing *L. monocytogenes* serotype 4b as published elsewhere (10). At certain intervals after the intravenous infection, the germ numbers in the spleen were evaluated by culture of homogenized splenic tissue in a 10-fold dilution in tryptose agar (Difco Laboratories, Detroit, Mich.).

Histomorphological studies. Immediately after sterile removal of the spleen, a portion of this organ
was fixed in 3.5% buffered Formalin for 24 h. After fixation, tissues were embedded in paraffin, and corresponding histological sections were stained with hematoxylin-eosin. Morphological analysis was focused on the detection of follicular hyperplasia, lymphoblasts, and plasma cells. The findings were evaluated semiquantitatively as described earlier (11).

Statistical tests. Differences between mean values were tested for significance using Student’s t-test after analysis of variance.

RESULTS

Adjuvanticity. To test whether killed B. pertussis organisms can serve as immunological adjuvant in LPS nonresponders (C3H/HeJ), such mice were immunized i.p. with 5 × 10⁷ SRBC and then divided into two groups, one of which received an additional i.p. injection of 3 × 10⁹ killed B. pertussis organisms. The same immunization schedule was applied to LPS-sensitive C3HeB/FeJ mice and to NMRI mice, the latter known to respond particularly strongly to B. pertussis. Four weeks after primary immunization, all animals were given a booster of 4 × 10⁹ SRBC i.p. On days 5, 10, 14, and 28 after primary immunization and on day 4 after secondary immunization, numbers of direct and indirect PFC in the spleens were counted, and circulating antibodies against SRBC were measured in the sera of all animals. As shown in Fig. 1, killed B. pertussis organisms exhibited a distinct adjuvant activity in both C3H strains. Furthermore, the typical course of the adjuvant action of killed B. pertussis organisms, with delayed onset during the primary response and a profound effect during the secondary response, was evident in all experimental animals. Especially in respect to the indirect PFC, primarily representing 7S antibody producers, the action of killed B. pertussis organisms was significant (2P < 0.05) throughout the period of observation. This has likewise been found in other mouse strains (4, 8). The estimation of the overall adjuvant effect, i.e., the increase in PFC irrespective of cell type (indirect or direct PFC) and time after priming, revealed that killed B.
pertussis organisms induced a 3.6-fold increase of PFC in C3H/HeJ mice as compared with a 5.5-fold increase in C3HeB/FeJ mice and a 5.4-fold increase in NMRI mice.

The histological evaluation of the effects of killed B. pertussis organisms on spleen morphology revealed findings closely resembling those observed earlier (2). In contrast to animals receiving only SRBC, a profound hyperplasia of the lymphoid follicles with extensive germinal center formation was present in C3H/HeJ mice (Fig. 2) as well as in C3HeB/FeJ animals after application of killed B. pertussis organisms. Furthermore, spleens of mice treated with SRBC

![Figure 2](http://i.imgur.com/3jB.png)

**Fig. 2.** Histomorphology of spleens of C3H/HeJ mice 10 days after immunization with SRBC (a) or SRBC and killed B. pertussis organisms (b). a, Lymphoid follicles with small and relatively inactive germinal centers. b, Lymphoid follicles with large, highly active germinal centers and numerous "nuclear fragment" macrophages. x160.
and killed *B. pertussis* organisms showed significantly greater numbers of lymphoblasts and plasma cell precursors. The results of a semiquantitative histological evaluation of the morphological reactions observed are presented in Tables 1 and 2. From the data shown it is obvious that the follicular hyperplasia (Table 1) and the numbers of lymphoblasts and immature and mature plasma cells (Table 2) were considerably greater in animals treated with killed *B. pertussis* organisms than in control animals of the same mouse strain. However, these differences were more marked after primary immunization than after secondary immunization (Tables 1 and 2). On day 4 after the booster injection, all experimental animals exhibited numerous plasma cells in the red pulp of the spleen (Fig. 3). Nevertheless, differences in the intensity of the morphological reactions to SRBC or SRBC plus killed *B. pertussis* organisms between the three mouse strains were evident (Tables 1 and 2), indicating responses in NMRI > C3HeB/FeJ > C3H/HeJ mice. Thus, LPS-resistant mice appeared to respond slightly less intensely to the application of killed *B. pertussis* organisms.

**Hypersplenia.** To test the induction of hypersplenia by killed *B. pertussis* organisms in LPS nonresponders, C3H/HeJ and NMRI mice (controls) received a single i.p. injection of $3 \times 10^9$ killed *B. pertussis* organisms without any other antigenic stimulus. Untreated animals of the same strain served as normal controls. At days 1, 6, and 14 after injection of killed *B. pertussis* organisms, the spleens were removed and weighed. In C3H/HeJ animals, the injection of killed *B. pertussis* organisms induced a 2.5-fold increase in spleen wet weight (Fig. 4). As is also evident from the data presented in Fig. 4, hypersplenia, similar to the adjuvant activity of killed *B. pertussis* organisms, was more pronounced in NMRI mice than in C3H/HeJ animals.

**Leukocytosis.** The promotion of leukocytosis by killed *B. pertussis* organisms was tested in C3H/HeJ and C3HeB/FeJ mice by counting peripheral leukocytes before injection and on days 1, 3, 6, and 10 after i.p. injection of $3 \times 10^9$ killed *B. pertussis* organisms. As is obvious from Fig. 5, killed *B. pertussis* organisms increased the leukocyte count in both mouse strains in an almost identical manner.

**Non-specific resistance to infection.** Since it is known from previous studies (12) that the modification of non-specific resistance to infection by killed *B. pertussis* organisms is time dependent, different experimental schedules were employed. In the first experiment, one group of C3H/HeJ and one group of C3HeB/FeJ mice received an i.p. injection of $3 \times 10^9$ killed *B. pertussis* organisms 7 days before the intravenous infection with *L. monocytogenes.*

**Table 1.** Histological findings (follicular hyperplasia) in spleens of mice after immunization with SRBC or SRBC and killed *B. pertussis* organisms*

<table>
<thead>
<tr>
<th>Days post-immunization</th>
<th>C3H/HeJ mice</th>
<th>C3H/FeJ mice</th>
<th>NMRI mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SRBC</td>
<td>SRBC</td>
<td>SRBC</td>
</tr>
<tr>
<td></td>
<td>plus B. pertussis</td>
<td></td>
<td>plus B. pertussis</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>++ (+)</td>
<td>++ (+)</td>
</tr>
<tr>
<td>10</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>14</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>28 (0) &lt;sup&gt;b&lt;/sup&gt;</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>32 (4) &lt;sup&gt;b&lt;/sup&gt;</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>

*Grading system: (+) to ++++, very weak to very marked. Results represent mean values of five mice each.

<sup>b</sup> Days after secondary immunization.

**Table 2.** Histological findings (lymphoblasts or immature or mature plasma cells) in spleens of mice after immunization with SRBC or SRBC and killed *B. pertussis* organisms*

<table>
<thead>
<tr>
<th>Days post-immunization</th>
<th>C3H/HeJ mice</th>
<th>C3H/FeJ mice</th>
<th>NMRI mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SRBC</td>
<td>SRBC</td>
<td>SRBC</td>
</tr>
<tr>
<td></td>
<td>plus B. pertussis</td>
<td></td>
<td>plus B. pertussis</td>
</tr>
<tr>
<td>5</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>10</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>14</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>28 (0) &lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>32 (4) &lt;sup&gt;b&lt;/sup&gt;</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>

*Grading system: (+) to ++++, very weak to very marked. Results represent mean values of five mice each.

<sup>b</sup> Days after secondary immunization.
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Fig. 3. Histomorphology of the spleen of a C3H/HeJ mouse 4 days after the secondary immunization with SRBC. Primary immunization was performed with SRBC and killed B. pertussis organisms. Dense accumulation of lymphocytes, lymphoblasts and immature and mature plasma cells in the red pulp. ×640.

Fig. 4. Change of spleen wet weight induced by killed B. pertussis organisms. Symbols: ●, C3H/HeJ mice, 3 × 10⁹ killed B. pertussis organisms i.p.; ■, NMRI mice, 3 × 10⁹ killed B. pertussis organisms i.p.; ○, C3H/HeJ mice, untreated; □, NMRI mice, untreated. Five or six mice were used per point. Mean values and standard error of the mean are indicated.

This timing is known to provide optimal conditions for an increase of nonspecific resistance by killed B. pertussis organisms. At days 3, 4, and 7 after infection, the numbers of viable bacteria in the spleens were counted. Significant differences in numbers of L. monocytogenes between groups pretreated with killed B. pertussis organisms or untreated were found in both mouse strains (Table 3). No differences in the activity of killed B. pertussis organisms could be observed in C3H/HeJ and C3HeB/FeJ mice. Injection of killed B. pertussis organisms simultaneously with viable L. monocytogenes is known to decrease resistance to the parasite (12). Thus, in a second experiment, C3H/HeJ and C3HeB/FeJ mice received an i.p. injection of 3 × 10⁹ killed B. pertussis organisms simultaneously with the intravenous infection of 4 × 10⁵ viable L. monocytogenes organisms. Controls consisted in HeJ and FeJ mice without injection of killed B. pertussis organisms and in NMRI mice with and without application of killed B. pertussis organisms together with viable L. monocytogenes. At days 1, 2, and 5 after infection the spleens of the experimental animals were removed, and the numbers of viable bacteria were counted. Figure 6a shows the results obtained in C3H/HeJ, and Fig. 6b shows the results in C3HeB/FeJ mice. As is obvious from Fig. 6a and b, killed B. pertussis organisms, when given simultaneously with the infection, induced a significant decrease in resistance to L. monocytogenes. Differences between the various strains were found in regard to mortality; 90% of NMRI mice and 100% of C3HeB/FeJ mice pretreated with killed B. pertussis organisms died within the period of observation, whereas only 10% of similarly treated C3H/HeJ mice succumbed to the infection.
FIG. 5. Induction of leukocytosis by killed B. pertussis organisms. Symbols: O, C3H/HeJ mice, $3 \times 10^6$ killed B. pertussis organisms i.p.; C3HeB/FeJ mice, $3 \times 10^6$ killed B. pertussis organisms i.p. Five mice were used per point. Mean values and standard error of the mean are indicated.

TABLE 3. Influence of killed B. pertussis organisms on the course of infection with L. monocytogenes in C3H/HeJ and C3HeB/FeJ mice*

<table>
<thead>
<tr>
<th>Mouse strain and pretreatment</th>
<th>Log on day after infection:</th>
<th>3</th>
<th>4</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H/HeJ mice with B. pertussis</td>
<td></td>
<td>0.50 ± 0.45</td>
<td>0.69 ± 0.59</td>
<td>0.40 ± 0.20</td>
</tr>
<tr>
<td>C3H/HeJ mice without B. pertussis</td>
<td></td>
<td>4.12 ± 0.17</td>
<td>4.08 ± 0.32</td>
<td>1.93 ± 0.46</td>
</tr>
<tr>
<td>C3HeB/FeJ mice with B. pertussis</td>
<td></td>
<td>1.59 ± 0.59</td>
<td>0.50 ± 0.40</td>
<td>1.04 ± 0.40</td>
</tr>
<tr>
<td>C3HeB/FeJ mice without B. pertussis</td>
<td></td>
<td>5.24 ± 0.48</td>
<td>5.44 ± 0.52</td>
<td>2.74 ± 0.43</td>
</tr>
</tbody>
</table>

* Results are expressed as log of L. monocytogenes ± standard error of the mean. Five or six mice were used per group per day.

DISCUSSION

The C3H/HeJ mouse strain has been shown to be refractory to the adjuvant activity of LPS (20, 21). The experiments described here show that killed B. pertussis organisms are active as an adjuvant in this mouse strain, although the adjuvant action is slightly less profound than that in LPS-sensitive C3HeB/FeJ animals. This is consistent with previous studies that demonstrated that the LPS of B. pertussis as well as other structural components, such as peptidoglycan, possess less adjuvanticity than whole killed B. pertussis organisms (6, 9).

The single gene defect of C3H/HeJ mice, responsible for LPS nonresponsiveness (25), also modifies the macrophage response to endotoxin,
a mechanism thought to be crucial for the increase of nonspecific resistance to infection after injection of LPS (19). Thus, LPS failed to increase resistance against *Klebsiella pneumoniae* in C3H/HeJ mice (3). Our studies show that killed *B. pertussis* organisms significantly increased nonspecific resistance in HeJ as well as in FeJ mouse strains. In contrast, the decrease in resistance observed after simultaneous infection and with killed *B. pertussis* organisms was much more pronounced in LPS-sensitive strains. Analogous with these findings are previous studies which demonstrated that LPS can only partially substitute for killed *B. pertussis* organisms in overcoming dextran sulfate-induced loss of resistance to infection (10).

Further characteristics of the biological activity of killed *B. pertussis* organisms are the induction of splenic hyperplasia, leukocytosis, and a morphologically distinct response of the lymphatic organs (2, 7).

Previous studies (9) indicated that LPS of *B. pertussis* is unable to elicit leukocytosis, a finding confirmed by our experiments. Hypersplenia and the typical response of the lymphatic organs were also present in LPS-nonresponding HeJ mice, although to a slightly lesser extent than in mice susceptible to LPS.

Further studies will be necessary to test whether the endotoxin protein, described as mitogenic for C3H/HeJ lymphocytes (23), or other distinct structural components of killed *B. pertussis* organisms are responsible for these effects. According to the studies described here, such
structures participate more or less in adjuvanticity, splenic hyperplasia, leukocytosis, and modification of resistance to infection mediated by killed *B. pertussis* organisms.

**LITERATURE CITED**


