Differences in Coughing and Other Responses to Intrabronchial Infection with *Bordetella pertussis* among Strains of Rats

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Four strains of rats were each infected intrabronchially with approximately $10^8$ CFU of *Bordetella pertussis* 18-323 encased in fine agarose beads. After 8 days, Sprague-Dawley rats developed the highest incidence of coughing paroxysms, as monitored with voice-activated tape recorders; Brown Norway, Lewis, and Hooded Lister rats coughed significantly less frequently. Marked leukocytosis, with counts up to four times the normal levels, and retardation of normal weight gain occurred in all four rat strains. Both coughing and leukocytosis were greater in animals that were infected at 4 weeks of age than in those infected at 6 weeks of age. Total serum immunoglobulin E (IgE) rose in all four rat strains 9- to 244-fold by day 8 after infection and returned to near preinfection levels at 6 weeks. Sprague-Dawley and Lewis rats, which had the lowest basal levels of total IgE in serum, showed the greatest degrees of elevation. All four rat strains had IgG to *B. pertussis* whole-cell sonicate and to filamentous hemagglutinin in 6-week-postinfection sera. However, the strains differed in production of IgG to pertussis toxin, with Sprague-Dawley rats having the highest titers and Hooded Lister and Lewis rats being nonresponders. These studies highlight the importance of rat strain as a variable in the coughing-rat model of pertussis and validate the choice of the Sprague-Dawley rats in previous studies.

In unimmunized humans, particularly infants, *Bordetella pertussis* causes the distressing respiratory symptoms of whooping cough. The uncomplicated disease is generally not fatal, although respiratory complications and central nervous system disturbances can occur and are due mainly to the pressure effects and anoxia resulting from severe coughing paroxysms (2, 20, 29). Marked leukocytosis is also a characteristic clinical sign. Despite the detailed molecular and biological characterization of pertussis toxin (PT) and other virulence factors of *B. pertussis*, their role in causing the paroxysmal cough is unknown (21). Such studies have long been hampered by the lack of a coughing-animal model of this human disease. The mouse, which has been much used for experimental infection with *B. pertussis*, does not cough when infected via the respiratory route. The rabbit and various monkey species may also be infected experimentally with *B. pertussis*, but only some primates have been reported to develop paroxysmal coughing (26).

In a very early but apparently overlooked paper, Hornibrook and Ashburn (12) first reported that young rats coughed after respiratory tract infection with *B. pertussis*. Nearly 50 years later, Woods et al. (33) infected Sprague-Dawley (SD) rats intrabronchially with *B. pertussis* encased in microscopic agar beads and found that the animals “coughed with whooping sounds similar to humans” and that the infection was nonlethal. In our previous studies (6, 22, 30), these findings were confirmed and the coughing-rat model was further developed. Low-gelling-temperature agarose was used instead of agar to confirm and the coughing-rat model was further developed.

**MATERIALS AND METHODS**

**Animals.** Outbred female SD and HL rats and inbred BN (Brown Norway/Sa) and Lewis rats were purchased at 3 and 5 weeks of age from Harlan Olac Ltd. (Shaws Farm, Blackthorn, Bicester, United Kingdom). The animals were barrier reared, and each group was certified as being free from a range of viral, protozoal, and bacterial pathogens, including *Bordetella* and *Pasteurella*. They were allowed to acclimatize for 1 week before use.

**Bacterial suspension and bead preparations.** The organisms were grown and maintained as described previously (6). Briefly, *B. pertussis* 18-323, phase I (ATCC 9797), was grown on Bordet-Gengou agar (Difco Laboratories) containing 20% (vol/vol) defibrinated horse blood (E & O Products, Bonnybridge, Stirlingshire, United Kingdom). Growth from plates which had been heavily inoculated and incubated overnight at $37^\circ$C was suspended in a solution containing 1% (wt/vol) Casamino Acids (CAA) (Difco), and the suspension was standardized by visual comparison with the fifth international reference preparation of opacity developed by the World Health Organization (24). This opacity
standard, of 10 opacity units, is equivalent to approximately $2 \times 10^9$ CFU of B. pertussis per ml. Dilutions of this suspension were plated out onto Bordet-Gengou agar to obtain viable counts.

In our previous studies, the microscopic beads of bacteria in agarose were prepared by depositing B. pertussis suspension at low speed at 15 s. The final bacterium-agarose bead suspension contained approximately $10^4$ CFU/ml in 1% (wt/vol) agarose in CAA and 0.1% (wt/vol) MeCD.

Infection procedure. Rats were infected intratracheally as described previously (6, 30). Each animal was given 0.1 ml of suspension previously standardized to approximately $4 \times 10^9$ CFU/ml was warmed to 38°C. Molten agarose (4 ml) was mixed with an equal volume of low-speed at 38°C for equilibration. Likewise, the bacterial suspension previously standardized to approximately $4 \times 10^9$ CFU/ml was warmed to 38°C. Molten agarose (4 ml) was mixed with an equal volume of


to each well. After 1 h and a further washing, streptavidin-peroxidase (Sigma) was added to the wells and after 1 h at 37°C for 1 h, followed by three washes in WB. Serial dilutions of samples and each of the readings at 492 nm were plotted against log10 dilutions of the samples, and the end point at an A492 of 0.5 was taken as the titer of the serum. Each test included 10-fold dilutions of normal rat serum and a positive reference preparation of B. pertussis (6, 30). The reference serum had been assigned ELISA titers of antibodies to B. pertussis whole-cell sonicate, FHA, and PT antigens of 20,000, 80,000, and 500, respectively, and enabled allowance to be made for day-to-day variation between tests.

**RESULTS**

**Coughing.** Previous studies had shown that the peak of the leukocyte response to B. pertussis infection in SD rats occurred at around day 8 and coughing occurred from day 5 onwards, with a peak around days 8 to 14 (6, 30). In this study, only the SD animals coughed to any appreciable extent during the period of observation, and those that were 4 weeks old at the time of infection were more prolific coughers than animals infected at 6 weeks of age. Figure 1 summarizes the quantitative observations on paroxysmal coughing at day 8 after infection from three independent experiments involving a total of 96 infected rats of the four strains. The 4-week-old HL rats gave a few paroxysmal coughs but markedly fewer than the SD rats, while BN and Lewis animals gave too few coughs to be of any use in the coughing-rat model of pertussis. No paroxysmal coughing was heard in tapes from a total of 50 control, uninfected animals of the four strains.

**Mortality and weight gain.** Before day 8 after infection, there was an overall 18% mortality (17 of 96) in the infected animals (Table 1). There were no deaths within 24 h of the operation, and the median day of death was day 5 for the 17 animals that died (9 of which were sacrificed due to ill health, i.e., when there was a change in their appearance or behavior such as a ruffled coat and hunched stance). Fourteen of these
were 4 weeks of age when infected, while only three deaths occurred among animals that were 6 weeks old when infected. Thus, there were more deaths among the younger animals, but the difference in proportion of deaths was not significant by the $x^2$ test ($x^2 = 1.53$). No deaths occurred among control, untreated rats.

The rats were weighed individually on the day of infection and on days 1, 3, and 7 thereafter. The day 0 mean weights, in grams, for each strain at 4 weeks of age were as follows: SD, 115; BN, 85; HL, 114; and Lewis, 105. The day 0 mean weights, in grams, for each strain at 6 weeks of age were as follows: SD, 168; BN, 121; HL, 156; and Lewis, 150. Figure 2 shows that control animals had weight gains of between 10 and 20% during the period of observation, the younger animals having the greater increases. In contrast, infected animals of all four strains had lost weight at 24 h but subsequently gained weight at approximately the same rate as the controls but not catching up with them during the period of observation. Exceptions were the Lewis rats of both ages, which failed to gain any weight during the 7 days postinfection or even to regain their preinfection weights. The SD, BN, and HL rats were similar in their weight changes after infection, despite the marked differences in their coughing responses.

**Leukocytosis.** Leukocytosis is one of the characteristic features of severe pertussis in the human infant, and leukocyte counts were therefore investigated in the infected rats. The geometric mean leukocyte counts and their 95% confidence limits for the 16 groups of rats are detailed in Table 1. All four strains of rat had developed leukocytosis at day 8 after infection with *B. pertussis*, and animals that were 4 weeks old when infected had higher levels of leukocytosis than animals infected at 6 weeks of age.

The highest mean level of leukocytosis occurred in 4-week-old infected Lewis rats and was 6.5-fold over that in the uninfected controls. In 4-week-old SD rats the figure was 5.5-fold. In contrast, 4-week-old BN and HL animals showed leukocytosis levels only 2- and 2.8-fold, respectively, higher than that in the uninfected controls. Analysis of variance of the data summarized in Table 1 confirmed that there were highly significant differences ($P < 0.001$) between the strains and ages of rat and between infected and control animals. For the four interaction terms generated by the three independent variables (strain, age, and infection), there was highly significant interaction between strain and infection and between age and infection but not between age and strain or among the three variables.

**IgG responses.** Detailed studies were performed by ELISA of the IgG responses to *B. pertussis* antigens in sera taken on days 8, 21, and 42 after infection. Since there were no significant differences between titers in 4- and 6-week-old animals, the results for the two age groups were pooled (Table 2). A positive serum was one having a titer of 10 ($\log_{10} = 1$) or higher. Because many of the sera had titers below the limit of detection, it was not possible to summarize group results as the means. Instead, the median values with the range and the 95% confidence limits were used.

On day 8 after infection there were no detectable IgG responses to any of the three *B. pertussis* antigens in sera from SD or Lewis rats. However, a low response to FHA was detected in BN and HL animals, with the former also producing a modest response to antigens in sera taken on August 28, 2017 by guest
consistent than those to FHA: only 10 of 15 SD rats, the best-responding strain, had detectable levels of anti-PT IgG. The median titer of 95 (log$_{10}$ 5 1.9) was drawn from the wide range of <10 to 400,000 in individual animals. Only 3 of 15 BN animals contained detectable levels of anti-PT, while none of the HL or Lewis rats responded at all.

By day 42, the antisonicate titer was higher than on day 21 in BN rats, whereas among the other three strains the proportion of responders declined, although the ranges increased slightly. The anti-FHA responses, in contrast, were higher on day 42 than on day 21, with every single animal in each of the four strains having a high titer. The order of median anti-FHA titers in the day 42 sera was SD = BN > Lewis > HL. Unlike the responses to FHA, the IgG responses to PT had fallen by day 42 in SD animals and similarly in BN rats, while there was still no detectable response to this antigen in Lewis or HL rats. Sera from untreated control animals all gave titers of <10 in ELISAs specific to each of the three antigens (results not shown).

**Total serum IgE levels.** Pre- and postinfection levels of total serum IgE are presented in Fig. 3. As there were no significant differences between the levels obtained in the 4- and 6-week-old animals, the results were pooled for convenience. A marked increment in total serum IgE on day 8 after infection was apparent in all four rat strains. Mean levels in Lewis rats increased from 0.5 to 122 ng/ml (244-fold), while in SD rats there was a 100-fold increase, from a mean basal serum IgE level on day 0 of 1.5 ng/ml to a mean level of 150 ng/ml. A 26-fold increment was seen in HL rats, in which the level increased from 7 to 185 ng/ml, while the serum IgE level in the BN strain increased 9-fold, from a basal value of 250 ng/ml to a mean level of 2,240 ng/ml. By day 21, total serum IgE levels had dropped by various degrees in most of the groups with the exception of the Lewis rats, in which the elevation was maintained, while the day 42 levels had returned nearer to preinfection readings in Lewis, SD, and BN rats (1.3, 5.6, and 375 ng/ml, respectively), with an elevation still apparent in HL rats (42 ng/ml). Levels of total serum IgE in untreated controls...
we were measured on each of the above-mentioned days and did not differ significantly from preinfection levels (results not shown).

**Antigen-specific IgE ELISA.** Antigen-specific IgE ELISAs were performed on the same sera as used in the above-described experiments. All experimental samples had undetectable (<10) titers of antibodies against the three antigens tested, i.e., *B. pertussis* whole-cell sonicate, FHA, and PT. To overcome any possible interference from IgG antibodies, these were removed from eight representative sera, taken on day 21 after infection, by passage through an affinity column of anti-rat IgG and then reassayed on both IgG and IgE antigen-specific ELISAs. Before absorption, the group of eight sera from two experiments on days 0, 8, and 21 and from one experiment on day 42.

**DISCUSSION**

The main objective of this work was to compare the coughing responses of four different strains of rat after intrabronchial infection with *B. pertussis* encased in agarose beads. We previously showed that SD rats coughed following infection and that this response was accompanied by leukocytosis and retardation of weight gain (6, 30). We report here that, of all strains compared, SD animals are indeed the only rats to cough consistently, regardless of age, although the other symptoms of infection, namely, leukocytosis and retardation of weight gain, were demonstrated in all four rat strains.

Age-related susceptibility to infection was apparent, with appreciable mortality, during the first week postinfection in all rats infected at 4 weeks of age. Retardation of weight gain was particularly evident in Lewis rats infected at either 4 or 6 weeks of age. The degree of weight gain retardation was paralleled by susceptibility to leukocytosis, which was most pronounced in Lewis and SD rats in both age groups. In comparison, the leukocyte count in HL rats was significantly altered when the rats were infected at 4 weeks of age and in BN rats less so, but neither showed leukocytosis when infected at 6 weeks of age.

**TABLE 2. Summary of log_{10} IgE ELISA titers in sera of rats of different strains infected with *B. pertussis* and tested with coating antigens**

<table>
<thead>
<tr>
<th>Strain and day</th>
<th>BP</th>
<th>FHA</th>
<th>PT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median and range (95% CL)</td>
<td>No. of sera positive/ no. tested</td>
<td>Median and range (95% CL)</td>
</tr>
<tr>
<td>SD 8</td>
<td>&lt;1</td>
<td>0/15</td>
<td>&lt;1</td>
</tr>
<tr>
<td>21</td>
<td>&lt;1–1.2 (NC)</td>
<td>4/15</td>
<td>3.8, 2.7–5.1 (3.1–4.3)</td>
</tr>
<tr>
<td>42</td>
<td>&lt;1–1.2 (NC)</td>
<td>1/7</td>
<td>5.0, 4.6–7.3 (4.9–7.0)</td>
</tr>
<tr>
<td>BN 8</td>
<td>&lt;1, &lt;1–1.3 (NC)</td>
<td>2/15</td>
<td>&lt;1, &lt;1–2.4 (&lt;1–1.4)</td>
</tr>
<tr>
<td>21</td>
<td>1.1, &lt;1–2.3 (&lt;1–1.9)</td>
<td>9/15</td>
<td>4.6, 3.5–5.6 (4.4–5.0)</td>
</tr>
<tr>
<td>42</td>
<td>2.6, &lt;1–3.8 (&lt;1–3.0)</td>
<td>5/7</td>
<td>5.1, 3.9–5.9 (4.6–5.0)</td>
</tr>
<tr>
<td>HL 8</td>
<td>&lt;1</td>
<td>0/14</td>
<td>&lt;1, &lt;1–2.9 (&lt;1–1.1)</td>
</tr>
<tr>
<td>21</td>
<td>1.7, &lt;1–2.6 (&lt;1–2.1)</td>
<td>9/13</td>
<td>3.5, 2.4–4.5 (2.6–4.2)</td>
</tr>
<tr>
<td>42</td>
<td>&lt;1–1.4 (&lt;1–3.1)</td>
<td>4/7</td>
<td>3.6, 4.3–4.9 (4.4–4.7)</td>
</tr>
<tr>
<td>Lewis 8</td>
<td>&lt;1</td>
<td>0/14</td>
<td>&lt;1</td>
</tr>
<tr>
<td>21</td>
<td>&lt;1–3.0 (&lt;1–1.3)</td>
<td>2/8</td>
<td>3.0, 2.8–4.1 (1.9–3.5)</td>
</tr>
<tr>
<td>42</td>
<td>&lt;1–1.3 (&lt;1–1.2)</td>
<td>1/5</td>
<td>4.8, 3.6–5.3 (NC)</td>
</tr>
</tbody>
</table>

*BP, B. pertussis whole-cell sonicate; CL, confidence limit; NC, noncalculable.*
Without better knowledge of the time course of bacterial persistence in the lungs, we cannot be certain that the cause of deterioration in young animals was the multiplication of pertussis organisms or even their persistence. In the original study by Woods et al. (33) and in a preliminary investigation of our own (unpublished data), B. pertussis was cleared fairly rapidly from rat lungs and could not be recovered from lung homogenates by day 10, although in the former study, the bacteria reappeared at day 21. Since the deaths occurred in the younger rats and during the first week, it is possible that the infecting dose was directly toxic for that age group.

It is well established that rat strains vary in their abilities to produce IgE, from the poorly producing Lewis rats to the very highly producing BN rats, and our pattern of basal serum IgE measurements obtained on day 0 confirm these earlier distinctions (1). An elevation in total serum IgE was evident by day 8 after infection in all strains, and the degree of increment was inversely proportional to the basal serum IgE levels of the strain, with the greatest increases occurring in the low-level-IgE-producing Lewis and SD rats. The levels gradually declined, returning close to normal in Lewis, SD, and BN rats, but an elevation was still apparent in HL rats 6 weeks after infection. There are some inconsistencies we cannot explain, such as why the HL rat day 42 level was higher than the day 21 level, but these measurements were repeated, with the same result.

The mechanisms of regulation of IgE production have yet to be fully characterized. A series of in vitro experiments with helminth-infected rodent lymphocytes highlighted soluble factors with modulatory effects specific for IgE (14). The fact that helminth-infected rodent lymphocytes highlighted soluble factors affecting IgE synthesis and presumably reflects some of the IgE-specific enhancement mechanisms described above.

One of the characteristic features of pertussis is leukocytosis, which is evident in humans, mice, and rats. PT is responsible for this leukocytosis-promoting activity by causing leukocytes, particularly lymphocytes, to digress from their normal migratory route to lymph nodes and to persist in the blood (16, 18). This supports the hypothesis that the IgE adjuvant action of PT may be due to the depletion of lymphocytes (which would include suppressor populations) in spleen and lymph nodes, allowing IgE production to proceed unrestrained (27). Our comparison of rat strains following infection has given some conflicting results, namely, that leukocytosis occurred in the absence of an anti-PT response (Lewis and HL) and that increments in total IgE have occurred in the absence of significant leukocytosis (BN and HL). The rise in IgE without concomitant leukocytosis occurred in the groups more resistant to infection, as seen from their weight gains, and it is possible that the bacteria were eliminated from these animals before leukocyte levels were affected. However, even if PT is not being actively secreted, some lingering traces may have an effect on IgE levels, which are known to be sensitive to minute amounts of this toxin (19). It has been suggested that the symptoms of pertussis may not be due directly to live organisms but to the long-lasting effects of released toxins (25). That PT had eventually provided an antigenic stimulus in BN rats is borne out by the detectable IgG responses in some of these rats at 3 and 6 weeks after infection.

The IgG antibacterial responses to infection were measured by ELISA for up to 6 weeks after infection and generally developed with time (Table 2). The response to FHA was particularly strong and persistent, with some exceptionally high titers recorded for the SD rats at day 42. The tendency for the anti-B. pertussis whole-cell sonicate titers to be lower than those of antibodies to FHA, which was reported in our previous studies (6, 22), presumably reflects the heterogeneous mixture of antigens present in this preparation. With regard to the anti-PT responses, the wide variation in IgG responses in SD rats was also noted previously (6, 22). There was a conspicuous absence of an IgG response to PT in Lewis and HL rats. This suggests that some rat strains are unable to respond, or are poor responders, to PT, but this should be confirmed with larger numbers of rats of different strains. As PT is regarded as a major virulence factor in B. pertussis, the results highlight the interesting point that the greatest number of responders to PT (at day 21) were of the rat strain (SD) heard to cough most consistently. These strain differences presumably reflect genetic differences which may also occur in humans and contribute to variations in disease severity and vaccination responses in infants. However, the role of PT in cough production is uncertain. B. parapertussis and B. pertussis mutant strains lacking PT did not produce coughing in the rat model (22), whereas some human parapertussis patients have been reported to show coughing paroxysms similar to those in pertussis patients (10, 32).

A summary of some of the key responses to B. pertussis infection in 6-week-old rats is presented in Table 3. This study has highlighted that the ability to cough varies with the strain of rat, as does responsiveness to PT, both as an antigen and as a toxin, and that the normally concomitant symptoms of pertussis, namely, leukocytosis, retardation of weight gain, and a rise in total serum IgE, can occur independently.

**B. pertussis-specific IgE antibodies were not detected in any of the four strains of rats during the 6-week period after infection. This was not due to interference from high levels of specific IgG antibodies already present, because their removal from representative sera did not alter the results of the specific IgE ELISA. There was, however, a reduction in the EA-specific IgE titer of the RAST reference preparation from 10,000 to 5,000, which implies that there was some contribution to the measurement of IgE titer by the IgG antibodies present. A rise in total IgE has been reported to occur in convalescent-phase human sera following whooping cough (28), and a low incidence of IgE antibodies to B. pertussis whole cells has been detected (31). IgE antibodies with specificity for B. pertussis whole cells and PT have been reported to be present in children after immunization (8, 9). Investigations with experimen-
tal animals often use pertussis vaccine as adjuvant when the subsequent IgE response to the accompanying antigen is measured. However, in two studies, Pauwels et al. (23), using RAST, detected IgE antibodies to various pertussis vaccines in BN rats, and Lindsay et al. (15), using purified components, induced low titers of mouse anti-FHA and anti-PT IgE measurable by passive cutaneous anaphylaxis. In the present study, IgE antibodies specific to *B. pertussis* whole-cell sonicate, FHA, or PT did not appear to contribute to the raised levels of circulating IgE in the serum of *B. pertussis*-infected rats.

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REFERENCES


