Prophylaxis of *Bordetella bronchiseptica* Infection in Guinea Pigs by Intranasal Vaccination with Live Strain ts-S34

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The temperature-sensitive mutant strain, ts-S34, of *Bordetella bronchiseptica*, which cannot grow at or above the temperature 34°C, has been studied in guinea pigs. This strain grew to moderate numbers in the nasal turbinates but did not grow in the lungs. Guinea pigs given strain ts-S34 developed moderate levels of serum antibody. This strain also induced very high resistance to subsequent intranasal challenge with 4,000× the 50% lethal dose of virulent strain S1 of *B. bronchiseptica*. In these studies, it appeared that the ts-S34 strain had favorable properties for potential use as a live attenuated vaccine.

*Bordetella bronchiseptica* is known to cause infectious atrophic rhinitis of swine (13) and rabbits (9), to cause respiratory diseases in dogs (1), and to bring about death due to sepsis of animals such as mice (5), guinea pigs (5), and swine (M. Maeda and T. Shimizu, 83rd Meet. Jpn. Soc. Vet. Sci., Abstr. No. 66).

Hitherto, killed vaccines were mainly studied for prophylaxis of *B. bronchiseptica* infection in swine (4, 6, 7, 11; Y. Nakase, M. Kimura, and K. Shimoda, Abstr. Proc. Int. Pig Vet. Soc. Congr. 1976, p. 8), and in guinea pigs (10). The experimental results including those of live vaccine (3) indicated that the protective effect of these vaccines was suboptimal.

In 1974, Maeda and Shimizu (8) reported that the growth of *B. bronchiseptica* inoculated intranasally was revealed by the fluorescent-antibody technique on the surface of the nasal, tracheal, and bronchiolar epithelium of swine. On the other hand, the temperature of nasal turbinate mucosa is known to be approximately 32 to 34°C. Therefore, I developed the temperature-sensitive (ts) mutant strain of *B. bronchiseptica* which cannot grow at or above 34°C and studied it in guinea pigs for use as a live attenuated vaccine.

The present paper deals with the characteristics of the strain and patterns of infection, immune response, and induction of resistance to subsequent challenge with a virulent strain of *B. bronchiseptica*.

**MATERIALS AND METHODS**

Guinea pigs. Male and female guinea pigs of Hartley strain, weighing 250 to 300 g, were used throughout the present study. All the guinea pigs were obtained from a breeding colony which had been proved to be free of *B. bronchiseptica* contamination by repeated examinations. The animals were placed in metal cages on bedding of straw and fed with commercial pellets and tap water ad libitum and sometimes supplied with green feed. Groups of animals receiving different bacterial strains were kept in separate cages within the same isolation room.

**Strains.** Two cultures of *B. bronchiseptica*, S1 and A-19, were used in this experiment. These strains were isolated from the natural cases of atrophic rhinitis of swine. Two additional strains, ts-S1 and ts-S34, which had been derived from strain S1 by exposure to mutagen (described below) were used.

**Exposure to mutagen and recovery of ts mutant.** The culture of strain S1 was diluted in a freshly prepared solution of N-methyl-N-nitro-N-nitrosoguanidine in 0.15 M phosphate-buffered saline, pH 7.2 (PBS), which had a final concentration of 1,000 μg/ml and a final pH of 7.2. The mixture (4 ml) was incubated with shaking at 37°C for 1 h. After washing three times with PBS, the treated cells were resuspended in PBS and were cultured on Bordet-Gengou medium (Difco) with 10% defibrinated sheep blood (B-G medium) at 37, 34, or 32°C. By using the replica method, the ts-S1 mutant, which cannot grow at 37°C but can grow at 34°C, was isolated. After a similar treatment, the ts-S34 mutant, which cannot grow at or above 34°C, was obtained.

**Inoculation of the test organisms.** Each test strain was cultured on B-G medium at 37 or 32°C for 24 h and was suspended homogeneously in 10 ml of PBS. Serial 10-fold dilutions were prepared from this bacterial suspension, and each 0.2 ml of a given dilution was instilled into each guinea pig's nostril by using a syringe with a small vinyl tube (1.5 mm in diameter).

**B. bronchiseptica** recovery. When guinea pigs died, they were autopsied immediately, and all surviving guinea pigs were autopsied at week 1, 2, 4, or 6 after inoculation. In autopsied animals, the nasal mucosa of turbinates and lungs were cultured quantitatively.

**Quantitative culture.** Three animals from each study group were sacrificed. The nasal turbinates and lungs were removed from the animal and were weighed. These organs were ground with PBS. Each plate of DHL agar medium (Eiken, Tokyo, Japan) was
inoculated with 0.1 ml of 10-fold dilutions of the organ suspension and was cultured at 37 or 32°C for 48 h. The mean colony count of three plates was used to calculate the number of bacteria.

**Agglutinin titers.** The modified method (12) with Formalin-killed antigen, approved by the government for use (Kitasato Laboratories, Ltd.), was applied to sera at autopsy. A positive titer was certified by a ++ grade of agglutination.

**Vaccine.** Strain ts-S34 was used for vaccine preparation. Each 0.4 ml of freshly prepared bacterial suspension as described above, containing $1.5 \times 10^9$ organisms per ml, was used as vaccine.

**Challenge inoculation.** Prophylactic value of the ts mutant vaccine was assessed by intranasal challenge inoculation at week 4 after immunization with $3.6 \times 10^9$ organisms (i.e., $4,000 \times 50\%$ lethal dose [LD50]) of virulent strain S1. Animals were observed for 5 weeks after challenge, and at death they were autopsied immediately, and the organs were cultured.

**RESULTS**

The growth curve of strain ts-S34 in the Trypticase soy broth (Baltimore Biological Laboratory) is shown in Fig. 1. This mutant was able to grow at 32°C but unable to grow at 34°C.

A $10^{-6.2}$ reversion frequency was detected with strain ts-S1; however, a $10^{-10.3}$ or lower reversion frequency was detected with strain ts-S34.

In the nasal turbinates, strain A-19 grew consistently to high numbers, but strain ts-S34 grew to high numbers by week 1 and then decreased (Fig. 2). In the lungs, strain A-19 grew consistently to high numbers, but strain ts-S34 did not grow (Fig. 3).

As shown in Fig. 4, serum agglutinin titers rose to a level of 1:160 at week 6 after inoculation in both groups inoculated with either strain ts-S34 or A-19.

Strain S1's LD50 for guinea pigs by intranasal inoculation was $8.8 \times 10^5$ viable cells per guinea pig (Table 1). All dead guinea pigs inoculated with strain S1 showed hemorrhagic pneumonia, and inocula were recovered in every case.

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**FIG. 1. Growth curves of strain ts-S34.**

**FIG. 2. Growth of strains ts-S34 and A-19 in the nasal turbinates of the guinea pigs.** Symbols: O, Individual guinea pigs given strain ts-S34; △, individual guinea pigs given strain A-19; □, ▲, geometric mean titers.
Challenge inoculation of $3.6 \times 10^8$ organisms (i.e., $4,000 \times LD_{90}$) was performed to all the animals 4 weeks after vaccination. All guinea pigs immunized with the vaccine survived without manifesting clinical symptoms for 5 weeks after challenge, but all unvaccinated animals died with hemorrhagic pneumonia within 7 days after challenge (Table 2).

**DISCUSSION**

The reversion frequency of strain ts-S34 was the lowest among those of strains ts-S1 ($10^{-6.3}$), ts-S34 ($10^{-10.3}$) and respiratory syncytial virus ts1 ($10^{-6.3}$) (2). The extent of the genetic change which occurred in the ts mutants cannot be inferred from available data. However, this low frequency of reversion to the wild strain, $10^{-10.3}$, suggests that strain ts-S34 had undergone more than one change in nucleotide sequence, either in the same or different cistrons.

When about 2,000 strains of *B. bronchiseptica* isolated from guinea pigs and other small animals were inoculated intranasally, they did not cause any death in guinea pigs (M. Nakagawa, personal communication). However, of the two strains isolated from natural cases of swine atrophic rhinitis, strain S1 caused death but strain A-19 did not in this experiment. Therefore, the virulence of strain S1 may be exceptionally high.

Nakagawa et al. (10) reported that, although there was a general correlation between protection against challenge inoculation and agglutinin response (over 1:2,560) in the guinea pigs inoculated intramuscularly with killed vaccine, discrepancies were observed in some animals.

In this experiment, agglutinin titers were very low (less than 1:160 at week 4 after immunization).

<table>
<thead>
<tr>
<th>Guinea pig group</th>
<th>No. dead/no. challenged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunized</td>
<td>0/10</td>
</tr>
<tr>
<td>Control</td>
<td>10/10</td>
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</tbody>
</table>

*a Immunization dose, $6 \times 10^9$ organisms; challenge dose, $3.6 \times 10^6$ organisms ($4,000 \times LD_{90}$); challenge time, week 4 after immunization; observation period, 5 weeks after challenge.

**TABLE 1. Experimental intranasal infection with strain S1 of *B. bronchiseptica* in guinea pigs**

<table>
<thead>
<tr>
<th>Infection dose</th>
<th>No. dead/no. infected</th>
<th>LD$_{50}$</th>
<th>Time of death (days) after infection$^a$</th>
<th>Autopsy finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>$2.8 \times 10^7$</td>
<td>4/4</td>
<td></td>
<td>2, 2, 3, 3</td>
<td>Hemorrhagic pneumonia</td>
</tr>
<tr>
<td>$2.8 \times 10^6$</td>
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<td>3, 7, —, —</td>
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<tr>
<td>$2.8 \times 10^5$</td>
<td>2/4</td>
<td>$8.8 \times 10^4$</td>
<td>4, 12, —, —</td>
<td>Hemorrhagic pneumonia</td>
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<tr>
<td>$2.8 \times 10^4$</td>
<td>0/4</td>
<td></td>
<td>—, —, —</td>
<td>—</td>
</tr>
<tr>
<td>$2.8 \times 10^3$</td>
<td>0/4</td>
<td></td>
<td>—, —, —</td>
<td>—</td>
</tr>
</tbody>
</table>

$^a$ —, Survivor. Observation period: 2 weeks after infection.
tion), but the degrees of protection against challenge inoculation in guinea pigs were very high. So, hereafter, the antibody in nasal secretions should be investigated thoroughly.

In conclusion, it appeared that strain ts-S34 had favorable properties for potential use as a live attenuated vaccine.

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LITERATURE CITED


