Vaccination of Lewis Rats with Temperature-Sensitive Mutants of *Mycoplasma pulmonis*: Adoptive Transfer of Immunity by Spleen Cells but Not by Sera

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Temperature-sensitive mutant vaccines protect rats against *Mycoplasma pulmonis* infection. The role of the humoral or cellular immune response in resistance to mycoplasma infection was investigated by adoptive-transfer experiments. Spleen cells from Lewis rats vaccinated but not challenged with wild-type organisms (vaccinated) and spleen cells from rats vaccinated (or not) and challenged were effective in preventing syngeneic recipients from developing respiratory disease. There was also a significant reduction in the incidence and number of challenging organisms in the respiratory system. In contrast, sera from the same donors had no detectable effect on the number of mycoplasmas recovered or on lesion development in the respiratory tract. We conclude that cellular immunity rather than humoral immunity generated in vaccinated rats confers protection against subsequent infection.

*Mycoplasma pulmonis* is one of the most important diseases in both conventionally maintained and specific-pathogen-free rodent colonies. Studies of the immune mechanisms in experimentally induced murine respiratory mycoplasmosis (MRM) should provide useful information for the control of MRM infections. To develop suitable vaccines, it is necessary to understand the mechanisms involved in resistance to reinfection. The interaction of circulating antibodies, local secretary antibodies, natural killer (NK) cells, and cell-mediated immunity in mycoplasma infections is not known.

When an animal is exposed to mycoplasmas, the organisms often persist in the respiratory tract for prolonged periods. For example, carriage of *M. pneumoniae* in the upper respiratory tracts of humans may persist for several weeks (13), while in mice inoculated intranasally (i.n.) with *M. pulmonis*, both the pneumonic lesions and the mycoplasmas persist for several months (21). This suggests that the host's immune response is inefficient. However, persistently infected animals are resistant to subsequent i.n. challenge with the same organism. Thus, mice inoculated i.n. with small numbers of *M. pulmonis* organisms do not develop lung lesions, although mycoplasmas can be isolated from their lungs for at least several weeks postinoculation. Following i.n. challenge with a large dose of *M. pulmonis*, which produces severe pneumonia in control animals, mice previously inoculated with small numbers of mycoplasmas develop much less severe pneumonia and fewer organisms are isolated from their lungs than from those of controls (26). Thus, recovery from naturally occurring or experimentally induced MRM results in immunity. Although reinfection of previously infected animals can occur, such animals are generally protected against development of subsequent MRM. Rats, like mice, can be effectively protected by immunization (3, 15). The immunopathologic character of the respiratory disease caused by the same *M. pulmonis* in rats and mice suggests that the immune mechanisms opera-

tive in the two species differ. In infected rats, bronchial tract-associated lymphoid tissue hyperplasia is predominant, whereas in mice a plasma cell response is predominant (22). In contrast to the case in mice (25–27), preexisting antibodies are not protective in rats. Levels of passively acquired maternal antibody are quite high in offspring, and antibody production in pups begins within the first 6 weeks of life. However, this antibody does not protect against colonization and subsequent development of disease (6). Furthermore, immune spleen cells, but not immune serum, can confer protection in normal syngeneic naive rats (2).

We have developed a vaccination protocol for rats by using a temperature-sensitive mutant (TSM) of *M. pulmonis* (15). Therefore, we felt that it was important to understand more fully the importance of serum antibody and cellular immunity in resistance to MRM, especially after vaccination. We compared the abilities of spleen cells and serum from rats vaccinated with the TSM to immunize syngeneic Lewis rats passively.

MATERIALS AND METHODS

Experimental animals. Specific-pathogen-free, 5- to 6-week-old Lewis rats of both sexes (Harlan Sprague Dawley, Indianapolis, Ind.) were used in all experiments. They were housed in groups of five in filter-topped, sterile polycarbonate cages in a barrier facility and allowed free access to sterilized rodent chow and water. The animal rooms were maintained at 20 to 22°C with a 12-h light-dark cycle. Animals were allowed to stabilize for 1 week before initiation of the experiments. The pathogen-free status of animals was determined before and at the end of experiments. Sera were tested for murine viruses, *M. pulmonis*, and *M. arthritidis* antibodies by enzyme-linked immunosorbent assay (ELISA). Oropharyngeal samples were cultured for *M. pulmonis* and pathogenic bacteria. Ammonia levels were measured at least once a week with a Drager gas detector pump (Dragerwerk, Lübeck, Federal Republic of Germany) and maintained at 25 to 50 ppm (19 to 38 mg/liter) by changing the bedding as needed.

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**Adoptive Transfer of Immunity by Spleen Cells**

\[ M.\ \text{pulmonis} \] strains. Wild-type \( M.\ \text{pulmonis} \) UAB-6510, originally isolated from rats by Gail Cassell at the University of Alabama at Birmingham, was cloned twice in our laboratory and kept at \(-70^\circ\text{C}\). TSMs of \( M.\ \text{pulmonis} \) were produced by treating the wild-type strain with \( N\)-methyl-\( N\)′-nitro-\( N\)′-nitrosoguanidine. Two TSMs were selected at \(38^\circ\text{C}\) as a restrictive temperature and at \(34^\circ\text{C}\) as a permissive temperature (15). These organisms were thawed and diluted on the day on which they were used.

**Vaccination schedule.** Age- and sex-matched rats were divided into four groups and treated as follows. Group 1 rats \((n = 48)\) were not vaccinated but were challenged in a manner with \(10^7\) CFU of wild-type \( M.\ \text{pulmonis} \). Group 2 rats \((n = 51)\) were vaccinated i.n. three times with \(10^8\) CFU of a TSM at 2-week intervals and then challenged with \(10^7\) CFU of wild-type \( M.\ \text{pulmonis} \) 4 weeks after the last vaccination. Group 3 rats \((n = 40)\) were neither vaccinated nor challenged but were injected with mycoplasma medium. Group 4 rats \((n = 44)\) were vaccinated i.n. with a TSM but not challenged. Group 2 and 4 rats were kept in a separate room from group 1 and 3 rats when initially vaccinated. At the time of infection with wild-type \( M.\ \text{pulmonis} \), groups 1, 2, and 4 were housed separately from group 3 (not vaccinated or challenged). All animals were humanely killed by intraperitoneal injection of sodium pentobarbital 4 weeks postchallenge. The sera and spleens were collected.

**Passive immunization.** Sera and spleen cells from rats of groups 1 and 2 were mixed and pooled, but the sera and spleen cells from group 3 and 4 rats were kept separate. Antibody titers were measured by ELISA (15). Immune spleen cell suspensions were prepared by gently teasing chopped pieces of spleen through a 100-mesh stainless steel sieve into RPMI 1640 medium. Cell suspensions were filtered through sterile gauze and rendered monodisperse by rapid aspirations through pipette tips. The cell suspensions were filtered over columns of nylon wool to remove most macrophages and B lymphocytes (18). The cells were sedimented at 300 \( \times \) 10 \( \text{g} \) for 10 min, washed twice with medium, and suspended in RPMI 1640–10% fetal bovine serum to \(2 \times 10^8\) viable nucleated cells per ml. The function of the spleen cells was tested in vitro by exposure to sonicated \( M.\ \text{pulmonis} \) cell membranes or medium only for 4 days or to concanavalin A or medium only for 3 days to determine stimulation indices (ratios of [\( ^3\text{H} \)] thymidine incorporation into spleen cells [counts per minute], sonicated \( M.\ \text{pulmonis} \) membranes, or concanavalin A/medium only as measures of responsiveness [19]). The cell suspensions were also cultured in Chalquest agar medium to test for \( M.\ \text{pulmonis} \). The ratio of donor spleen cells equivalent to recipient animals was 2:1. Recipient syngeneic rats were infused intravenously with \(6 \times 10^8\) cells or 3 to 4 ml of serum and challenged 1 h later with \(10^7\) CFU of \( M.\ \text{pulmonis} \) inoculated i.n. Rats were humanely killed 28 days after i.n. challenge. Nasopharyngeal ducts, tympanic bullae, and tracheolung lavages were collected and titrated for mycoplasma, and the whole respiratory systems were collected and examined for histopathologic changes.

**Mycoplasma isolation, titration, and identification.** The procedures for mycoplasma isolation, titration, and identification were modified as described previously (20). We used a macrotitration method: 0.1-ml samples were cultured in Chalquest agar medium. Numbers of organisms are expressed as CFU per milliliter.

**Criteria for evaluation of protection.** (i) Microbiological evaluation. Samples were collected from nasal cavities, tympanic bullae, and tracheolung lavages for mycoplasma cultures.

(ii) Pathological evaluation. Rats were necropsied and examined for gross lesions. The nasal turbinates, trachea, lungs, and tympanic bullae were fixed in 10% neutral buffered Formalin, decalcified, embedded in paraffin, sectioned at 5 \( \mu \)m, stained with hematoxylin-eosin, and examined for microscopic lesions. The criterion for determining protection was the ability of the cells or sera to prevent or reduce the severity of the characteristic lesions of murine respiratory mycoplasmosis. To determine the severity of otitis, tracheitis, rhinitis, and pneumonia, histological sections were identified by code and scored as follows: \(-\), no lesions recognized; +, mild lesions or lymphatic hyperplasia in bronchial tract-associated lymphoid tissue and perivascular and peribronchial cuffing; ++, moderate lesions, bronchiectasis, and congestion; ++++, severe lesions and mononuclear cell infiltration in perivascular, peribroncholar, and alveolar space; +++++, most severe lesion, atelectasia, bronchiectasis, and necrosis.

**Statistical analysis.** Data were evaluated by analysis of variance to determine statistically significant differences \((P < 0.05)\) in the number of organisms in the lungs and nasal cavities among the groups of rats. Chi-square analysis was used to determine significant differences of tissue lesions and \( M.\ \text{pulmonis} \) colonization in lungs among the various groups.

**RESULTS**

\( M.\ \text{pulmonis} \) infection in immunized rats. The number of mycoplasmas isolated from the respiratory tracts averaged \(2 \times 10^7\) CFU/ml at \(38^\circ\text{C}\) in tracheolung lavage, tympanic bullae, and nasopharyngeal ducts in group 1 (nonvaccinated, challenged) rats. All of the rats harbored mycoplasmas in their respiratory systems, with typical murine mycoplasmosis lesions in their nasal cavities, tympanic bullae, and lungs. The pathologic lesion index averages were 0.65 to 0.75, as reported recently (15). In contrast, the numbers of organisms isolated from the respiratory tracts of group 2 rats (vaccinated, challenged) were significantly less than those isolated from group 1 animals \((P < 0.01)\). In general, rats vaccinated with a TSM showed reductions of \(6 \log_{10}\) in the number of organisms in the tracheolung lavages and tympanic bullae and \(2 \log_{10}\) in the nasal cavities compared with the corresponding group 1 rats. No gross lesions were observed in vaccinated rats (group 4, vaccinated only). Gross lung lesions, such as atelectasis, consolidation, and bronchiectasis, were noted in most group 1 nonvaccinated, challenged animals. Microscopically, all of the rats in group 1 developed tympanitis and rhinitis and 86% developed pneumonia. Of the rats in group 2, 30% developed very mild tympanitis, 100% developed rhinitis, but only 3% developed very mild pneumonia. The lesion index of pneumonia in group 2 \((0.06 \pm 0.01)\) was 12 times lower than that of group 1 \((0.70 \pm 0.1)\) \((P < 0.001)\). Similarly, the mean tympanitis score was \(0.09 \pm 0.03\) in group 2 versus \(0.75 \pm 0.1\) in group 1 \((P < 0.01)\). The mean rhinitis score was also lower in group 2 \((0.20 \pm 0.08)\) than in group 1 \((0.65 \pm 0.1)\) \((P < 0.05)\). Group 3 rats (nonvaccinated, nonchallenged) had no lesions in the lungs, tympanic bullae, or ears, while group 4 rats (vaccinated, nonchallenged) had no lesions in the lungs or tympanic bullae and only very mild rhinitis, although they were housed in the same room with rats of groups 1 and 2 infected with \( M.\ \text{pulmonis} \).
M. pulmonis infection in passively immunized rats. Since the rats vaccinated with TSMs were protected against wild-type M. pulmonis infection, it was important to determine the abilities of cells and sera obtained from these rats to protect syngeneic recipients. Therefore, pooled spleen cells or sera obtained from group 1 and 2 rats were mixed together and referred to as immune cells or sera to be examined from rats infected with wild-type M. pulmonis. The antibody titers for pooled immune sera in the ELISA was $2 \times 10^4$ for immunoglobulin G (IgG) and $5 \times 10^5$ for IgM. These were the peak titers obtained. Spleen cells and sera collected from group 3 control rats were tested in parallel. Control sera contained no IgG or IgM (titer, $<10^3$). Pooled sera and spleen cells from group 4 (vaccinated, not challenged) were considered vaccinated sera or cells. The peak titers of IgG and IgM in this group were $10^3$ and $10^2$, respectively, and were also examined. No mycoplasmas were isolated from immune, vaccinated, or normal sera. Immune and vaccinated spleen cells had stimulation indices of 30 and 25, respectively, against sonicated M. pulmonis cell membranes, which were significantly higher ($P < 0.05$) than that of normal control (group 3) cells (stimulation index, 3). These results suggested that immune and vaccinated spleen cells contained memory T cells. A small number of M. pulmonis organisms were isolated from pooled immune (group 1 and 2) cell suspensions but not from normal or vaccinated (group 3 or 4) cell suspensions.

Effects of passively transferred cells. (i) Lung lesions. Twenty-eight days after i.n. challenge, the pneumonia lesion indices were 0.21, 0.24, and 0.83 in rats that received immune, vaccinated, and control cells, respectively (Table 1). The differences between the two experimental groups and the controls were highly significant ($P < 0.01$). The prevalences of lung lesions in rats which had received immune, vaccinated, and control spleen cells were 22, 27, and 100%, respectively. These differences were also significant ($P < 0.01$). These data indicate that rats which received immune or vaccinated spleen cell suspensions could inhibit the frequency and severity of lung lesions induced by mycoplasma infection.

(ii) Isolation of M. pulmonis. Another criterion used to evaluate protection is whether passively transferred cells or sera can prevent colonization by the wild-type challenge organism and reduce the frequency or number of wild-type organisms in the respiratory tract postchallenge. The results of this study are presented in Table 2. Samples were taken from nasopharyngeal ducts, tympanic bullae, and tracheo-ung lavages and cultured on Chalquest agar medium. Rats that received immune or vaccinated cells had $10^5$-fold fewer organisms in each area than did rats that received normal cells ($P < 0.001$). The incidences of recovery of challenge organisms was 27, 32, and 100% for rats which received immune, vaccinated, and normal spleen cells respectively.

Effects of passively transferred serum. Notable gross lesions, such as atelectasis, consolidation, and bronchiectasis, were observed in rats which received immune, vaccinated, or normal serum and were challenged with M. pulmonis. Microscopically, all of the rats in these three groups developed severe pneumonia. The mean lesion index scores were 0.80, 0.75, and 0.85 for the immune, vaccinated, and normal serum groups, respectively (Table 1). The differences were not significant.

Immune or vaccinated serum (or normal serum) also failed to prevent recipient animals from colonization by M. pulmonis UAB 6510 28 days after i.n. challenge (Table 2). All of the rats from the three groups had large numbers of M. pulmonis organisms at the three sites tested, and there were no significant differences in numbers of M. pulmonis organisms detected.

DISCUSSION

Since rats vaccinated with TSMs were protected against wild-type M. pulmonis, it was important to determine the

TABLE 1. Incidence and severity of respiratory disease due to M. pulmonis in passively immunized Lewis rats

<table>
<thead>
<tr>
<th>Treatment with:</th>
<th>Incidence of pneumonia</th>
<th>Severity of pneumonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splne cells</td>
<td>Sera</td>
<td></td>
</tr>
<tr>
<td>Immune</td>
<td>No</td>
<td>8/37d</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>No</td>
<td>6/22d</td>
</tr>
<tr>
<td>Normal</td>
<td>No</td>
<td>20/20</td>
</tr>
</tbody>
</table>

| No               | Immune                 | 37/37                 |
| No               | Vaccinated             | 22/22                 |
| No               | Normal                 | 20/20                 |

a Immune donors were Lewis rats vaccinated or not with an M. pulmonis TSM and challenged with strain UAB 6510 28 days before harvest of serum and spleen cells (pool of groups 1 and 2). Vaccinated donors received an M. pulmonis TSM but were not challenged with strain UAB 6510 (group 4). Normal donors were not exposed to M. pulmonis (group 3).

b Number of animals that developed pneumonia/number given transfer of cells or sera at 28 days after i.n. infection with $10^7$ CFU of M. pulmonis UAB 6510.

c Lung lesion scores were calculated as described in Materials and Methods and references 15 and 27.

d Values obtained with immune or vaccinated versus normal spleen cells were significantly different ($P < 0.01$).

TABLE 2. Incidence and isolation of M. pulmonis from respiratory tracts of passively immunized Lewis rats

<table>
<thead>
<tr>
<th>Treatment with:</th>
<th>Incidence of M. pulmonis infection</th>
<th>Mean $\pm$ SD log$_{10}$ CFU of M. pulmonis in:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splne cells</td>
<td>Sera</td>
<td>Nasopharyngeal ducts</td>
</tr>
<tr>
<td>Immune</td>
<td>No</td>
<td>10/37</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>No</td>
<td>7/22</td>
</tr>
<tr>
<td>Normal</td>
<td>No</td>
<td>20/20</td>
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<tr>
<td>No</td>
<td>Immune</td>
<td>37/37</td>
</tr>
<tr>
<td>No</td>
<td>Vaccinated</td>
<td>22/22</td>
</tr>
<tr>
<td>No</td>
<td>Normal</td>
<td>20/20</td>
</tr>
</tbody>
</table>

a See Table 1, footnote a.

b Number of rats with detectable M. pulmonis/number given cells or sera at 28 days after i.n. infection with $10^7$ CFU of M. pulmonis UAB 6510.

c See Table 1, footnote d.
activates macrophages exposed to potential either T-tinguishable (about compare M. pulmonis with control sions) versus susceptible) rather than antibody-mediated responses have been observed (5, 24). However, both resistant F344 (2) and susceptible Lewis (this report) rats transfer passive immunity to cells with but not with sera. We therefore conclude that rats and mice differ in their mechanisms of immune protection.

T cells were no doubt present in our immune and vaccinated spleen cell suspensions and may have played a critical role in protection. A definitive confirmatory test would be to compare CD4 and/or CD8 T-cell-depleted spleen cell suspensions with control immune or vaccinated spleen cell suspensions in cell transfer studies.

Numerous cells infiltrate lungs of rats and mice following M. pulmonis infection (4, 8, 11). A significant number of cells (about 50% in tissue sections) were morphologically indistinguishable from T lymphocytes, which failed to stain with either T- or B-cell reagents, and were called null cells. One potential cell type is the NK cell. NK cells play a significant role in resistance to M. pulmonis infection (17). M. pulmonis infection augments NK cell activity in spleens and lungs of infected animals (17, 18). We have observed that NK cells exposed to viable M. pulmonis release gamma interferon, which either directly kills the mycoplasmas or indirectly activates macrophages to engulf and digest M. pulmonis (16, 17). Interleukin 2 secreted by activated T cells can activate NK cells (28), and gamma interferon release from NK cells or sensitized T lymphocytes can activate macrophages which, in turn, can engulf and digest microorganisms (1, 12, 23, 29).

However, only T and B cells with rearranged genes for T-cell and immunoglobulin receptors, respectively, are thought to be capable of memory responses, while NK cells have receptor genes in germ line configuration (28) and are probably incapable of memory responses. Therefore, we plan to test the postulate that memory T cells are the cells responsible for protection of rats vaccinated with TSMs of M. pulmonis.

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