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Biological Evaluation of *Mycoplasma pulmonis* Temperature-Sensitive Mutants for Use as Possible Rodent Vaccines

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Temperature-sensitive mutants (TSMs) of *Mycoplasma pulmonis* were produced by treating the wild-type strain with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Three TSMs were selected at 38°C, as a restrictive temperature, and at 34°C, as a permissive temperature. Two TSMs, UTCMI and UTCMII, were proven to be nonpathogenic but immunogenic. In addition, they did not induce pneumonia, tracheitis, or tympanitis but did induce mild rhinitis. They were stable after 10 passages in vitro and in vivo. They elicited excellent antibody production and cell-mediated immunity in vaccinated rats. They also were not mitogenic to rat lymphocytes. Rats immunized intranasally with these TSMs were significantly protected against challenge with wild-type organisms. These mutants were morphologically and serologically indistinguishable from the wild-type organisms. The growth characteristics and antibiotic sensitivities were similar to those of wild-type organisms, except that they grew only at 34°C. In contrast to wild-type organisms, they did not bind to or lyse sheep erythrocytes. Thus, these TSMs may qualify as a vaccine to prevent *M. pulmonis* infection in rats.

Mycoplasma pulmonis is an important pathogen of rats involving primarily the respiratory and urogenital systems. Many approaches have been used to control murine mycoplasmosis, including antibiotic therapy, establishing mycoplasma-free colonies by caesarian derivation, and vaccination. The use of effective vaccines is an attractive approach because establishment and maintenance of mycoplasma-free rats is difficult in many biomedical institutions because of economic or space limitations.

Rats vaccinated with killed *M. pulmonis* organisms were only partially protected from challenge. Viable wild-type *M. pulmonis* organisms were more protective than killed organisms. Polyarthritis and genital tract colonization (4) are possible complications. Therefore, these methods will not prevent mycoplasmosis in rat colonies.

Temperature-sensitive mutants (TSMs) of *M. pulmonis* were developed as a vaccine. The use of TSM vaccines has been investigated and tested for controlling viral (21) and bacterial (14, 28) diseases and mycoplasmosis in humans (12) and animals (19, 20, 27), with promising results. The rationale for the effective use of TSM vaccines is based on the decreased nasal cavity temperature compared with the higher temperature of the lungs and other internal organs. It is expected that TSMs will colonize only the upper respiratory tract. The higher temperatures of the lungs, uterus, and other organs are nonpermissive for the mutant. Thus, it is expected that no lesions will be produced in the lower respiratory tract and other internal organs. A series of experiments has been conducted with two TSM vaccines to determine pathogenicity and protective efficacy. Here, we report that TSM vaccines provide significant protection in rats against subsequent challenge with *M. pulmonis*.

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 the start of the experiments. The pathogen-free status of animals was determined before and at the end of experiments. Sera were tested for murine viral *M. pulmonis* and *Mycoplasma 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.

***M. pulmonis* strain.** The wild-type *M. 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°C. These organisms were thawed and diluted on the day used.

Propagation and isolation of *M. pulmonis*. PPLO broth or Chalquest's agar medium (29) was used to propagate and isolate *M. pulmonis*. Specimens from the nasal cavities, tympanic bullae, and tracheolung lavages were inoculated in the agar media and incubated in a humid atmosphere at 37°C for 7 to 10 days. Growth of organisms was determined by colony counts on agar medium or color change of phenol red-glucose broth.

Titration of organisms. Serial 10-fold dilutions of the specimens to be tested were made in PPLO broth medium. Triplicates of 1 μl of each dilution were inoculated onto a Chalquest's agar medium plate (15 by 100 mm) which can accommodate four to five serial dilutions. After incubation at 34 or at 38°C for 7 to 10 days, the number of colonies was counted and expressed as CFU per milliliter.

Production and selection of TSMs. The procedure of TSM production and selection described by Steinberg et al. (32) was followed with a modification. *M. pulmonis* UAB6510 was grown in PPLO broth to the log phase, treated with 100 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and then grown on a Chalquest's agar plate at 34°C for 10 to 14 days.

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Each plate, containing well-separated colonies, was replicated onto two Chalquest's agar plates (24), one incubated at 34 and the other at 38°C. Only colonies that grew at 34 but not at 38°C were selected for TSM candidates. The TSM candidates were further tested for temperature dependency by 10 consecutive single-colony isolations. Only genetically stable isolates were tested in rats to determine in vivo stability.

Experimental design. Each experiment was repeated three times, and results were combined. Rats were divided into three groups and treated as follows: group 1 (total, 43 rats) was nonvaccinated and challenged intranasally (IN) with 10^7 CFU of wild-type *M. pulmonis*; group 2 (total, 51 rats) was vaccinated IN three times with 10^8 CFU of TSM each time at 2-week intervals, and then challenged with 1×10^7 CFU of wild-type *M. pulmonis* 4 weeks after the last vaccination; group 3 (total, 44 rats), 32 rats were vaccinated IN with UTCMI, and 12 rats were vaccinated IN with UTCMII but not challenged. All animals were humanely killed with an intraperitoneal overdose of sodium pentobarbital 4 weeks after challenge. The nasopharyngeal duct, tympanic bullae, and tracheolung lavages were collected for culture, and sera and tracheolung lavages were diluted and tested for anti-*M. pulmonis* antibodies. The lungs and heads were fixed for histopathological examination.

Criteria for evaluation of TSM vaccine efficacy. (i) Microbiological evaluation. Nasal cavities of groups 2 and 3 were cultured every day for the first week and once for the second and third weeks after the third vaccination to determine the prevalence, time of onset, and duration of nasal colonization of the TSM organisms. At necropsy, samples were collected from nasal cavities, tympanic bullae, tracheolung lavages, and uterine washes for mycoplasmal cultures. Each sample was titrated onto two Chalquest's agar plates; one plate was incubated at 34°C (mutant and wild-type organisms will grow) and the other at 38°C (only wild-type organisms should grow).

(ii) Pathological evaluation. Rats were necropsied and examined for gross lesions. The nasal turbinates, trachea, lungs, tympanic bullae, joints, and uteri were fixed in 10% neutral buffered Formalin, decalcified, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin-eosin, and examined for microscopic lesions. The criterion for determining protection was the ability of the vaccine 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 (− to ++++) according to the severity of the lesions. This represents the individual animal lesion index. For comparison of group scores, the sum of scores for each organ from individual animals was divided by the sum of maximal scores possible, given the lesion index for that organ. A lesion index of 1.0 is the most severe change possible for an experimental group. Criteria previously described (30) were used for lesion scores and the summary is as follows. Criteria for lungs were: −, no lesions recognized; +, mild lesions—neutrophilic exudate, lymphoid hyperplasia in bronchus-associated lymphoid tissue, and peribronchiolar and perivascular lymphocytic cuffing; ++, moderate lesions—bronchiectasis with neutrophilic exudate, congestion, marked bronchus-associated lymphoid tissue, and marked peribronchiolar and perivascular cuffing; +++, severe lesions—mononuclear lymphoid cell infiltration in perivascular, peribronchiolar, alveolar space, and interstitium and marked bronchiectasis; ++++, severe lesions—atelectasis, bronchiectasis, consolidation, necrosis, severe infiltration, and fibrin in the lumen or alveolar spaces. Selected pulmonary sections were also stained by the Warthin-Starry method and examined for presence of the cilium-associated respiratory bacillus (9). For the nasal cavity; two cross sections were incised, one near the tip of the nose and the other at the midpoint of the eyes, and criteria were as follows: −, no lesions recognized; +, mild lesions characterized by hyperplasia and metaplasia at nasal septum epithelium and wall of dorsal meatus, thickening and disorganization of the nuclear pattern, and mild lymphocytic infiltration of the submucosal area; ++, moderate lesions characterized by a squamous-appearing nasal turbinate epithelium and the beginning of pseudoglandular hyperplasia in the nasal septum, neutrophils present in the lumen, and marked lymphoid cell infiltration of the submucosa; +++, severe lesions characterized by intensive lymphoid cell infiltration of the submucosa and a large amount of neutrophils in the lumen, with edema and exudate; ++++, highly severe lesions characterized by mucosal necrosis and accumulation of large numbers of inflammatory cells. For tympanic bullae, the criteria were: −, no lesions recognized; +, mild lesions characterized by lymphoid cell infiltration in the subepithelium; ++, severe lesions characterized by massive quantities of purulent exudate in the tympanic cavity, scar tissue, and neutrophilic cells filling the central part of the tympanic cavity.

(iii) Serological evaluation. Serum was collected from each rat and analyzed for anti-*M. pulmonis* immunoglobulin M (IgM) and IgG antibodies by ELISA. Tracheolung lavages and nasal pharyngeal duct washes were analyzed for anti-*M. pulmonis* IgG and IgA antibodies by a modified ELISA (15).

(iv) Cell-mediated immune response assay. Lymphocyte transformation of spleen cells of control rats and rats vaccinated with TSMs was conducted as described by Naot (25). Stimulation indices were calculated by dividing the mean counts per minute of antigen- or mitogen-stimulated cultures by the mean counts per minute of control unstimulated cultures.

Biologic characterization of TSMs. (i) Growth characteristic of TSMs and parent strain in broth and agar medium. TSMs and wild-type *M. pulmonis* organisms were inoculated into 5 ml of PPLO broth and were incubated in a 38°C water bath for 0, 2, 4, 8, 12, and 24 h. The number of organisms in the broth medium was determined on agar plates incubated at 34°C. An alternate method of testing the stability of the TSMs is to inoculate the TSMs or wild-type *M. pulmonis* into agar medium and incubate at 34°C. At an appropriate time, one plate was shifted from 34 to 38°C and the number of organisms counted after 7 to 10 days of incubation.

(ii) Hemoadsorption to sheep or rat erythrocytes. TSMs and wild-type *M. pulmonis* were inoculated on agar medium. Colonies formed after 7 days at 34°C were overlaid with 0.5 ml of a 0.5% suspension of sheep or rat erythrocytes in saline. After 30 min of incubation at room temperature, the cultures were washed three times with phosphate-buffered saline and observed for evidence of hemoadsorption (10).

(iii) Sensitivity to antibody or antibiotic. TSMs and wild-type *M. pulmonis* were inoculated in 5 ml of broth medium and incubated at 34°C overnight. Plates were seeded for the test by dipping a sterile cotton swab into the mycoplasma broth culture, pressing it against the wall of the tube to remove excess broth, and swabbing it thoroughly over the agar surface to produce a lawn of growth. When the surface of the plate was dry, a sterile filter paper disk was pressed onto the medium to ensure contact, 25 μl of rabbit anti-*M. pulmonis* antiserum or erythromycin (1.25 mg) or tetracy-

TABLE 1. Average body weight gain of rats inoculated with TSMs or wild-type *M. pulmonis*[a]

Weeks after inoculation	Mean body weight gain (grams) in rats[b]		
	Control, inoculated	TSMs, inoculated	Wild type
1	33.3 ± 27.9	23.5 ± 15.3	8.7 ± 5.0[c]
2	40.8 ± 35.8	34.0 ± 19.8	12.0 ± 5.7[c]
3	50.8 ± 32.0	51.5 ± 19.4	21.8 ± 17.3[c]
4	59.3 ± 28.8	52.6 ± 23.6	31.5 ± 29.9[c]

[a] The rats were inoculated intranasally with 4×10^8 CFU of TSMs or wild-type *M. pulmonis*. The rats were weighed before and at weekly intervals after infection.
[b] Mean ± standard deviation values presented, 10 rats in each group.
[c] Mean value significantly less, $P < 0.05$, than TSMs and control groups.

cline (125 μg) was applied to the disk, and the plates were incubated at 34°C for 7 to 10 days. At the end of the incubation period, the presence or absence of growth on the plate and around the disk was determined (6).

(iv) Peroxide hemolysin production. TSMs and wild-type *M. pulmonis* were inoculated onto agar plates and incubated at 34°C for 7 to 10 days. When the colonies were well formed, they were overlaid with a 4% sheep erythrocyte-agar mixture and incubated at 34°C for 3 to 5 days. The plates were observed daily for hemolysis in the region of the mycoplasma colonies (31).

(v) Lymphocyte proliferation. This test is similar to the cell-mediated immune response assay. *M. pulmonis* organisms were lysed with distilled water at 37°C for 30 min. The membranes were washed three times with phosphate-buffered saline after centrifugation at 26,900 × *g* for 15 min. The membranes were sonicated for 3 min and filter-sterilized by using a 0.45-μm-pore-size membrane filter (Millipore Corp.). Protein concentration was measured by the Lowry method (22). Spleen cells were obtained from the control rats and rats infected with *M. pulmonis*. The spleen suspensions were plated in triplicate in flat-bottom microtiter plates. Thirty micrograms of soluble UTCMI or UTCMII antigen was added in a volume of 0.2 ml, and the solution was incubated at 37°C for 72 h. The suspensions were then pulsed with 0.5 μCi of [^{3}H]-thymidine per well for 18 h, and the cells were harvested with an automatic device. Stimulation indices were calculated as previously described (25).

Statistical analysis. Data were evaluated by analysis of variance to determine any statistical difference ($P < 0.05$) in the number of organisms in the lungs and nasal cavities among the groups of rats. The chi-square nonparametric analysis was used to determine significant differences in prevalence of tissue lesions and prevalence of *M. pulmonis* colonization in lungs among the various groups (35).

RESULTS

Production and selection of TSMs. We performed mutagenesis in over 20 experiments and selected more than 100 clones. The TSM candidates were further tested for temperature dependency by 10 consecutive single-colony isolations. Most of the clones reverted to the wild type after two or three passages in agar medium, whereas three TSMs were genetically stable after 10 passages in agar medium and stable in vivo in rats or mice. We named them UTCMI, UTCMII, and UTCMIII.

Selection of nonpathogenic but immunogenic clones from the TSMs. Of the three TSMs examined, UTCMI and UTCMII were nonpathogenic. They did not induce lower respiratory tract lesions in IN vaccinated rats and were not recovered from the lower respiratory tract. The UTCMIII produced pneumonia and tracheitis in IN vaccinated rats, although the organism was not recovered from the lower respiratory tract. Therefore, no further experiments were conducted with UTCMIII. It is possible that enough organisms reached the lungs to cause disease. It would be interesting if *M. pulmonis* antigens could be detected in the lungs of rats infected with UTCMIII.

Evaluation of vaccine efficacy. (i) Clinical observations. No clinical signs of disease were observed in vaccinated rats when challenged or not challenged with wild-type *M. pulmonis*. In contrast, most of the nonvaccinated, challenged rats developed clinical signs such as rales, weight loss (Table 1), ruffled hair, head tilt, or "twirler" activity.

(ii) Microbiology. One criterion used to evaluate the efficacy of a vaccine is whether the TSM vaccine can prevent colonization by the wild-type organism and reduce the frequency or the number of wild-type organisms in the respiratory tract after challenge. The results of this study are presented in Table 2. The samples were taken via the nasopharyngeal duct, tympanic bullae, tracheolung lavages and uterus lavages. Duplicate Chalquest's agar plates, one incubated at 34 and the other at 38°C, were used for culture. The organisms which grew at 34°C could be TSMs plus the wild-type *M. pulmonis* challenge; however, the organism that grew at 38°C would be wild type and mutant revertant organisms. The data presented in Table 2 represent the organisms that grew at 38°C. Virulent *M. pulmonis* organisms were recovered at 38°C from the nasopharyngeal duct, tympanic bullae, and tracheolung lavage with 2.1×10^7, 7.1×10^7, and 7.1×10^7 CFU/ml organisms, respectively, in the group 1 (nonvaccinated, challenged) rats. Conversely, challenge organisms were isolated from group 2 (vaccinated, challenged) rats with 2×10^1 CFU/ml in the tracheolung lavage and none from the tympanic bullae. The differences are significant ($P < 0.001$). There was also a significant

TABLE 2. Isolation of *M. pulmonis* from respiratory tracts of rats vaccinated with TSMs and challenged with wild-type *M. pulmonis*

Group	Treatment	Tracheolung lavage		Tympanic bullae		Nasopharyngeal duct	
		Prevalence	Mean ± SEM[a]	Prevalence	Mean ± SEM	Prevalence	Mean ± SEM
1	Nonvaccinated, challenged	43/43	7.32 ± 0.05	43/43	7.32 ± 0.05	43/43	7.32 ± 0.05
2	Vaccinated, challenged						
	UTCMI, challenged	13/20	1.71 ± 0.02[b]	0/20	0.00[b]	20/20	5.60 ± 0.01[b]
	UTCMII, challenged	7/31	1.30 ± 0.01[b]	4/31	1.30 ± 0.01[b]	24/31	5.60 ± 0.03[b]
3	Vaccinated, nonchallenged						
	UTCMI	0/32	0.00[b]	5/32	0.05 ± 0.001[b,c]	10/32	0.00[b]
	UTCMII	0/12	0.00[b]	3/12	0.01 ± 0.001[b,c]	0/12	0.00[b]

[a] Values are means ± standard errors of the $\log_{10}$ CFU per milliliter. CFU of *M. pulmonis* only grew at 38°C.
[b] Mean values significantly less, $P < 0.01$, than group 1.
[c] The organisms found in the vaccinated, nonchallenged group were TSMs because they only grew at 34°C.

TABLE 3. Histopathologic lesions in rats vaccinated with TSMs and challenged with wild-type *M. pulmonis* (UAB 6510)

Group	Treatment	Tympanitis		Rhinitis		Pneumonia	
		Prevalence[a]	Mean[b] ± SD	Prevalence	Mean ± SD	Prevalence	Mean ± SD
1	Nonvaccinated, challenged	43/43	0.75 ± 0.1	43/43	0.65 ± 0.1	37/43	0.70 ± 0.1
2	Vaccinated, challenged						
	UTCMI challenged	10/20	0.09 ± 0.03[c]	20/20	0.20 ± 0.08[c]	6/20	0.06 ± 0.01[c]
	UTCMII challenged	9/31	0.08 ± 0.03[c]	21/31	0.10 ± 0.03[c]	1/31	0.00[c]
3	Vaccinated, nonchallenged						
	UTCMI	0/32	0.00[c]	25/32	0.10 ± 0.01[c]	0/32	0.00[c]
	UTCMII	0/12	0.00[c]	3/12	0.01 ± 0.01[c]	0/12	0.00[c]

[a] Number positive/total number animals.
[b] Represents the severity of the lesions of the group. A lesion index of 1.0 is the most severe change possible for a group.
[c] Mean score significantly less, $P < 0.01$, than group 1.

reduction ($P < 0.05$) in the number of organisms isolated from nasal cavities (1×10^5 CFU/ml) as compared with nonvaccinated, challenged group 1 rats. Generally, rats vaccinated with TSMs showed reductions of 6 $\log_{10}$ in organisms in the tracheolung lavage and tympanic bullae and 2 $\log_{10}$ in the nasal cavities, as compared with the corresponding group 1 rats. Rats from group 3, which had been vaccinated with TSMs but were nonchallenged, had no wild-type organisms isolated even though these animals were housed in the same room as group 1 (nonvaccinated, challenged) and group 2 (vaccinated, challenged) for more than 3 months. Another criterion used to evaluate the efficacy of a vaccine is to determine whether the vaccine can reduce the frequency of colonization by challenge organisms in the respiratory tract. There was a significant reduction in the prevalence of the challenge organism in tracheolung lavage and tympanic bullae but no significant reduction in the nasal cavity. These data suggest that the TSM vaccine does not prevent the colonization by virulent *M. pulmonis* in the nasal cavities of vaccinated rats. However, the numbers of organisms recovered from group 2 vaccinated, challenged rats showed significantly lower numbers of *M. pulmonis* in the nasal cavities, as compared to the nonvaccinated, challenged group 1 rats. The TSM vaccine, however, almost completely prevented the colonization by virulent organisms in the tympanic bullae, trachea, and lungs. The wild-type challenge organisms were not recovered from joints or uteri in group 2 and 3 rats.

To test in vivo reversion and evaluate TSMs, we determined whether the TSM *M. pulmonis* can colonize and multiply in nasal cavities. Ninety sequential nasal cultures were taken from the 10 vaccinated rats (groups 2 and 3) at 1 to 7 day intervals, beginning on day 1 after the third vaccination, every day for the first week, and once for the second and third weeks after the last vaccination. In the first week, 100% (70 of 70) of TSMs can be recovered from the nasal cavity; 50% (5 of 10) can be recovered in the second week and 40% (4 of 10) can be recovered in the third week. TSMs were not isolated from tracheolung and tympanic bulla samples (0 of 10; 0 of 10; 0 of 10) at 7, 14, and 21 days after the last vaccination. In vitro testing of the isolated TSM showed retention of temperature dependency of the mutant in Chalquest's agar because the mutant grew only at 34°C. This indicates that the TSM had not reverted to the wild-type organism. It appears that TSM vaccines do colonize the nasal cavities of vaccinated rats, a circumstance which may cause a mild rhinitis.

(iii) Histopathologic changes. No significant differences were noted between experimental groups; therefore, the data were combined in Table 3. No gross lesions were observed in vaccinated rats. Gross lung lesions such as atelectasis, consolidation, bronchiectasis, and hemorrhage were noted in most nonvaccinated, challenged animals. Microscopically, 100% of the rats in group 1 (nonvaccinated, challenged) developed tympanitis and rhinitis and 86% developed pneumonia. Of the rats in group 2 (vaccinated, challenged) 29.6 or 50% developed very mild tympanitis, 68% of one group and 100% of a second group developed rhinitis, but only 3 or 30% developed a very mild pneumonia. None of the rats in group 3 (vaccinated, nonchallenged) had tympanitis or pneumonia. However, 25% of one group and 78% of a second group of rats in this group had a mild rhinitis. This clearly showed that the TSM would colonize the nasal cavities of vaccinated rats but in all probability should not be able to invade the lower respiratory tract because of higher temperature in those areas; consequently, no lesions develop. The mean score of the lesion index in Table 3 was used to measure the severity of the lesions in a group. The group lesion index of pneumonia in group 1 (0.70 ± 0.1) was 12 times higher than that of group 2 (0.06 ± 0.01), and the difference is highly significant ($P < 0.001$). Similarly, the group mean scores of tympanitis were 0.75 ± 0.1 in group 1, 0.09 ± 0.03 (vaccinated UTCMI), and 0.08 ± 0.03 (vaccinated UTCMII) in group 2, and the difference is significant ($P < 0.01$). The mean scores of rhinitis were 0.65 ± 0.1 in group 1 and 0.20 ± 0.08 (vaccinated UTCMI) and 0.10 ± 0.03 (vaccinated UTCMII) in group 2, and the difference is also significant ($P < 0.05$). No tympanitis or pneumonia and only a very mild rhinitis were observed in group 3 (vaccinated, nonchallenged). No histopathologic changes were observed in uteri and joints of all groups; also no cilium-associated respiratory bacilluslike organisms were identified in lung tissues of all groups.

(iv) Immune response. The results of humoral immune response are shown in Table 4. Rats vaccinated IN with TSMs developed low titers of IgM antibody (1:10), while a very high IgG antibody (1:1,000) was observed in the serum of group 3 rats (vaccinated only) 8 weeks after the last vaccination. However, both IgM and IgG in the serum increased 10-fold when the vaccinated rats were challenged with virulent organisms. High IgM and IgG antibody titers were always observed in the serum of group 1 (nonvaccinated, challenged) rats. The low titer of IgM antibody observed in the serum collected from necropsied animals (group 3) may be due to the prolonged time period after the last vaccination.

The results of the secretory immune response in tracheolung lavage are shown in Table 4. A total of 100% of the

TABLE 4. Humoral immune response (ELISA) in rats vaccinated with TSMs and challenged with wild-type *M. pulmonis*

Group	Treatment	Tracheolung lavage[a]				Serum[a]			
		IgA		IgG		IgM		IgG	
		Prevalence	Mean titer[b]	Prevalence	Mean titer[b]	Prevalence	Mean titer[b]	Prevalence	Mean titer[b]
1	Challenge only	43/43	500 ± 105	40/43	100 ± 23	22/43	100 ± 24	43/43	$5.3 \pm 0.3 \times 10^4$
2	Vaccinated, challenged								
	UTCMI, challenged	20/20	550 ± 120	19/20	100 ± 43	10/20	100 ± 35	20/20	$1.2 \pm 0.1 \times 10^4$
	UTCMII, challenged	31/31	550 ± 125	30/31	100 ± 45	17/31	100 ± 40	31/31	$4.8 \pm 0.1 \times 10^4$
3	Vaccinated, nonchallenged								
	UTCMI	32/32	100 ± 26	10/32	10 ± 0	3/32	10 ± 0	32/32	$1.4 \pm 0.1 \times 10^3$
	UTCMII	31/31	100 ± 25	5/12	50 ± 10	2/12	10 ± 0	12/12	$1.0 \pm 0.1 \times 10^3$

[a] Samples were collected at necropsy.
[b] Mean ± standard deviation value presented. Titer is expressed as the reciprocal of the highest dilution giving a positive reaction in the ELISA.

group 3 rats immunized IN with TSM developed a good titer of IgA antibody, while only 30 to 40% of the rats had IgG antibody with a low titer (1:10 or 1:50). However, when the vaccinated rats were challenged with virulent *M. pulmonis* (group 2), the IgA antibody increased significantly (from 1:100 to 1:550), while 100% of the rats had a moderate increase in IgG antibody. Similarly, 100% of the group 1 rats (nonvaccinated, challenged) developed a high IgA antibody titer (1:500) and a moderate IgG antibody titer (1:100) in tracheolung lavage (Table 4).

The results of the cell-mediated immune response are shown in Table 5. Lymphocyte transformation was used to examine the cell-mediated immune response. The spleen cell suspensions taken from group 3 rats were mixed with sonicated antigen (30 μg/ml) prepared from wild type *M. pulmonis* or concanavalin A (5 μg/ml) in quadruplicate on a microtiter plate. The results shown in Table 5 demonstrate that the TSM vaccinated rats had significantly higher ($P < 0.05$) stimulated indexes of 32 and 26 for UTCMI and UTCMII, respectively. This indicates that the cell-mediated immune response was activated in rats vaccinated with TSMs alone.

Biologic characterization of TSMs. (i) Temperature sensitivity of the mutants. A permissive temperature of 34°C and a nonpermissive temperature of 38°C were used. TSMs were inoculated onto Chalquest's agar plates and incubated at 34°C, and an agar plate was shifted from 34 to 38°C at appropriate time periods. TSMs can grow well at 34 but not at all at 38°C, irrespective of the time the agar plate was incubated at 34°C (Table 6).

(ii) Thermal inactivation of TSMs. TSMs and wild-type *M. pulmonis* were grown in a water bath at 38°C. At appropriate time intervals, the heated cultures were removed and titrated on Chalquest's plates at 34°C. The titer of wild-type *M. pulmonis* increased by approximately 15-fold in a 24-h period at 38°C; however, the TSMs decreased drastically; in 12 h at 38°C, UTCMI was completely nonviable and UTCMII decreased by 2 $\log_{10}$ in that period. Both mutants were completely nonviable by 24 h at 38°C.

(iii) Hemoadsorption to sheep or rat erythrocytes. Both TSMs completely lost their ability to bind to sheep or rat erythrocytes, while wild-type *M. pulmonis* had strong binding activity to sheep or rat erythrocytes (Table 6).

(iv) Antigenic relatedness. A filter paper disk soaked with rabbit anti-*M. pulmonis* antibody inhibited the growth of the *M. pulmonis* UAB6510, as was demonstrated by a clear zone of inhibition around the disk. The disk also inhibited the growth of UTCMI and UTCMII (Table 6). Therefore,

TABLE 5. Cell-mediated immune response in rats vaccinated with TSMs and challenged with wild-type *M. pulmonis*

Lymphocyte donor	[^{3}H]thymidine uptake in counts per minute (stimulation index)[a]		
	Control	*M. pulmonis* antigen[b]	ConA[c]
Rats vaccinated with UTCMI			
1	150	4,780 (38.9)	20,374 (135.8)
2	247	10,800 (43.5)	31,210 (126.4)
3	248	4,470 (18.0)	37,422 (150.0)
4	185	4,920 (26.6)	22,297 (120.5)
Mean stimulation index		31.8 ± 11.6[d]	133 ± 12.9[d]
Rats vaccinated with UTCMII			
1	358	11,459 (32.0)	53,790 (150.2)
2	399	10,800 (27.1)	35,833 (89.7)
3	325	6,834 (21.0)	55,352 (170.1)
4	494	11,362 (23.0)	32,498 (65.8)
Mean stimulation index		25.8 ± 4.8[d]	119.9 ± 49.2[d]
Normal rats, control	490	1,540 (3.2)	17,836 (36.4)

[a] Stimulation indices were calculated by dividing the mean counts per minute of antigen- or mitogen-stimulated cultures by the mean counts per minute of control cultures.
[b] *M. pulmonis* wild-type sonicated antigen, 30 μg/ml. Note the stimulation index of 3.2 for the normal rat spleen cells; with the TSMs, the stimulation indexes were less than 1.0.
[c] ConA, Concanavalin A, 5 μg/ml.
[d] Significant difference ($P < 0.05$) between control and UTCMI and between control and UTCMII.

TABLE 6. Biologic characterization of TSMs

Experimental variable	UTCMI	UTCMII	*M. pulmonis* UAB6510
Temperature			
34°C	Growth	Growth	Growth
38°C	No growth	No growth	Growth
Aerobic growth	+	+	+
Anaerobic growth	+	+	+
Growth inhibition by rabbit anti-*M. pulmonis* antibodies	+	+	+
Hemoadsorption to sheep or rat erythrocytes	−	−	+
Peroxide hemolysin production	−	−	+
Antibiotic sensitivity			
Tetracycline	+	+	+
Erythromycin	+	+	+

UTCMI and UTCMII contain common antigens with wild-type *M. pulmonis*.

(v) Sensitivity to antibiotics. Both TSMs and wild-type *M. pulmonis* remain sensitive to erythromycin and tetracycline (Table 6).

(vi) Peroxide hemolysin production. Wild-type *M. pulmonis* produced a toxic hemolysin which lysed sheep erythrocytes, while both TSMs completely lost this ability (Table 6).

(vii) Mitogenicity studies. In two experiments, stimulation indices for UTCMI in spleen cells of infected (wild-type *M. pulmonis*) and normal rats were 1.5 ± 0.2 and 0.7 ± 0.1, respectively, which suggests that UTCMI was not mitogenic for rat spleen cells. Similar results were obtained with UTCMII.

DISCUSSION

This study demonstrates that the TSM vaccine is effective in preventing murine respiratory mycoplasmosis in rats after IN challenge with highly virulent wild-type *M. pulmonis* UAB6510 (Tables 2, 3, and 4). Both TSMs elicited strong local (IgA) and systemic (IgG in sera) humoral antibody (Table 4) and cell-mediated immune responses (Table 5), consequently preventing the development of lesions in the lung and tympanic bullae, while almost completely preventing *M. pulmonis* colonization in these organs (Table 3). The enhanced lymphocyte response to concanavalin A in rats vaccinated with the TSMs could have been due to a systemic stimulation of the immune system by the mild rhinitis in otherwise specific-pathogen-free animals. An important characteristic of the vaccines is that they do not cause clinical disease or microscopic lesions in rats when repeatedly introduced IN. In addition, no reversion has been observed in vitro or in vivo (Table 2), which makes them well qualified as a safe vaccine.

We chose *M. pulmonis* UAB6510 to produce the TSM vaccine and to challenge the animals because this strain was isolated originally from a rat's lung and causes severe lung lesions in infected rats (4, 8). LEW rats were chosen because they are susceptible to this disease (8) and provide a good model for vaccine studies.

The temperature of the rat nasal cavity ranges from 30°C at the tip of the nose to 34°C at a 16-mm depth inside the nasal passage, with a normal body temperature of 38.5°C (17). It is reasonable, therefore, to select 34°C for the permissive temperature of the TSMs and 38°C for the nonpermissive temperature.

The IN route of vaccination and challenge was selected in this study to mimic natural exposure to *M. pulmonis*. In naturally occurring murine mycoplasmosis, there is a high nasal carrier rate and these animals developed severe lower respiratory tract lesions (7). This may be due to the lack of localized and systemic humoral or cellular immunity, or both, in the naturally occurring disease (2, 5, 11, 16). There could be similar problems as sequelae to IN inoculation of live vaccines. In our study, protection against challenge with a highly virulent *M. pulmonis* strain was attained. The vaccine did prevent colonization by the virulent *M. pulmonis* in the lungs and other organs, excluding the nasal cavities. Although the precise protective mechanism(s) is still unclear, the local IgA and IgG antibodies in tracheolung lavages, the systemic IgG antibodies in the sera, and the induction of the cell-mediated immune responses by the TSM vaccine could be responsible. It has been reported that rabbits vaccinated IN with a potassium thiocyanate extract from *Pasteurella multocida* exhibit persisting serum IgG and nasal IgA antibodies. The persisting nasal IgA antibodies correlate with the better protection observed in IN vaccinated rabbits (23). Similar results have been reported in humans (13), in whom a TSM of *Mycoplasma pneumoniae* produces a nasal IgA response which protects against wild-type challenge. It is undetermined whether or not the humoral or cell-mediated immune response plays a significant role in protecting the rat against *M. pulmonis* infection. It has been said that cell-mediated immunity may be more important than the humoral immune response in protecting the rat against infection (3), but the other mechanisms involved in this protection, such as natural killer cells, can not be ruled out (18).

Live vaccines are superior to killed vaccines. Live vaccines usually provide both quantitatively and qualitatively better protective immunogens because chemical and physical procedures used to prepare killed vaccine may alter immunogens. Live vaccines may also stimulate long-lasting immunity because the organism multiplies in the host and thus continuously stimulates the immune system both locally and systemically (12).

One would argue that TSMs inoculated IN into rats should be able to reproduce in the nasal cavity of the host continuously so as to stimulate an immune response. However, this is not the case. Recovery of the TSMs from the nasal cavities decreased over time (100% in the first week, 50% in the second week, and 40% in the third week). The longer the organism survives, the greater the magnitude of the developing immune response would be anticipated. Therefore, we attempted to get maximal immune response by vaccination of rats with three doses of TSMs. It would be interesting to determine whether one IN inoculation of TSMs would suffice. We have never been able to recover TSMs from the joints, lungs, spleens, kidneys, or uteri. If the temperatures in these organs are nonpermissive for TSMs, the amount of even nonviable mitogenic membranes may be too small to cause lesions. In contrast, it has been reported that 40% of the rats challenged with viable wild-type *M. pulmonis* showed polyarthritis and 100% carried organisms in the genital tract (4). In our study, there were no histopathological changes noted in joints and uteri in all groups. This may be due to short time periods after challenge studies.

It has been suggested that a relatively small dose of IN antigen elicits a local immune response of the respiratory tract (33); however, if the antigen is applied with a large

single dose, multiple administrations, or both, then the response may be amplified and may become systemic (34). It also has been reported that a high level of systemic antibody titers decreases the local pulmonary response to antigen (13). This may explain the fact that when all of the rats are studied, they produce very high IgG antibody titers in serum but relatively low IgA antibody titers in the tracheolung lavage (Table 4). Some of the differences in IgA and IgG titers could be explained by sensitivity of the assay.

Temperature-shift experiments demonstrated the temperature sensitivity of the TSMs. In shift-up experiments, TSMs grew at 34°C for up to 24 h but could not overcome the 38°C temperature shock. These data indicate that TSMs are really sensitive to temperature changes. In shift-down experiments, there was little or no decrease in the titer for the first 4 h of incubation, indicating that the events in the first 4 h were not critical temperature-sensitive steps, but titers dropped dramatically when shift-down experiments were done at 8 h for UTCMI and 12 h for UTCMII. These results indicated that incubation at 38°C for 8 to 12 h was detrimental to the growth of TSMs.

Growth inhibition tests demonstrated that the TSMs were serologically related to the wild-type *M. pulmonis* (Table 6), so that the immunity elicited by TSMs would protect the animal against wild-type *M. pulmonis* infection.

TSMs were tested for antibiotic sensitivity to allow the termination of nasal infection (vaccination) if required or desired in future. Furthermore, TSMs recovered from vaccinated rats which still retain sensitivity to the antibiotic suggest that the recovered TSMs were the original organisms used for vaccination. With the development of new molecular biology techniques, it will be desirable to have a marker in the genome of our organism for future studies.

Another property desirable in a live vaccine is genetic stability. Our UTCMI and UTCMII were chosen for in vitro and in vivo studies because of their failure to revert to the wild type in temperature-shift experiments (Table 6). In the in vivo study, the TSMs also appears to be stable genetically in rats in that none of the isolates recovered from vaccinated rats exhibited evidence of reversion of the TSM phenotype (Table 2).

It has also been shown that *M. pulmonis* cell membranes, which are mitogenic, can cause lung lesions similar to those caused by viable organisms (26). Our two TSMs have been tested for mitogenicity at 37°C, and both were negative for this characteristic (Results). This indicated that the *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced mutant has two characteristics, temperature sensitivity and loss of mitogenicity of *M. pulmonis*. We do not know whether these are related. Numerous publications indicate there is a high probability that every cell had mutated at more than one site. Some organisms even had three different properties from their parent strain (1, 32), suggesting that two or more cistrons had been affected by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

TSMs display many advantageous characteristics, including temperature sensitivity, lack of hemolysin, inability to attach to cells, retention of antigenicity, lack of pathogenicity and mitogenicity, and retention of antibiotic sensitivity. All of these characteristics qualified our TSMs as candidates for a vaccine to prevent *M. pulmonis* infection in rats.

Vaccinated animals may not be acceptable as specific-pathogen-free animals for biomedical research because of persistent mild rhinitis. However, the research community has a tremendous problem with *M. pulmonis* infection. TSM vaccine could be offered as a solution to the current *M. pulmonis* infection until such time in the future that only specific-pathogen-free animals are used and *M. pulmonis* infections are no longer a problem. Until that date we must develop alternatives such as vaccines to battle the disease.

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