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Asymptomatic Infection of Adult Volunteers with a Temperature Sensitive Mutant of *Mycoplasma pneumoniae*

(*Mycoplasma* genetics/respiratory disease immunoprophylaxis)

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ABSTRACT **Temperature sensitive mutants of *Mycoplasma pneumoniae* were developed with the expectation that their temperature sensitive defects would restrict replication *in vivo* at the temperature of the lower respiratory tract, whereas such defects would not seriously impair replication in the cooler environment of the upper respiratory passages. One such *ts* mutant, *ts-H43*, which does not replicate at a temperature of 37° or above, although noninfectious for hamsters, infected each of 16 seronegative adult volunteers when given by the intranasal route. The mutant remained genetically stable throughout the course of infection and stimulated a moderate systemic and local antibody response. The mutant was entirely avirulent for the volunteers but appeared to stimulate resistance to subsequent challenge with partially attenuated wild-type (*ts*$^+$) *Mycoplasma pneumoniae*.**

Mycoplasma pneumoniae, the etiologic agent of primary atypical pneumonia associated with cold agglutinins, is an important cause of respiratory disease in older children and young adults (1, 2). In some closed populations of military recruits, the organism can assume major importance since it causes pneumonia with a frequency 20 times that seen in the general civilian population (3). Although *M. pneumoniae* pneumonia responds satisfactorily to treatment with the appropriate antibiotic, the organism is not eradicated from the respiratory tract (4). Furthermore, the onset of disease is often insidious and considerable debility can occur before the illness is diagnosed correctly. For these reasons, an effective vaccine would be desirable for prevention of *M. pneumoniae* disease.

Critical to the development of effective immunoprophylaxis is an understanding of the pathogenesis of the disease and the host defense mechanisms which prevent the illness. Although there are many gaps in our knowledge in this area, several relevant findings have emerged from recent studies. Infection with the organism under experimental conditions appears to be quite superficial, involving only the respiratory epithelium (5). This suggests that local immune mechanisms in the respiratory tract should be of greater importance than systemic immunity in protecting against disease. This inference can also be drawn from the finding that resistance to experimental challenge in the hamster was stimulated more effectively by infection or intranasal instillation of inactivated organisms than by parenteral inoculation of inactivated *M. pneumoniae*, although the latter method stimulated higher levels of serum antibody (ref. 6 and unpublished data). In a recent study, we detected the development of local respiratory IgA antibody for *M. pneumoniae* in man after experimental infection with the organism. Preexisting local antibody appeared to be more closely correlated with resistance to disease than was serum antibody (7). These findings further support the view that local immune mechanisms (secretory immunoglobulin and/or cell mediated immunity) in the respiratory tract may be of prime importance in protection from disease. These observations also provide a basis for pursuing the development of a live attenuated mutant which could be administered locally into the respiratory tract and which would stimulate local immune processes in this area.

In previous work we have described the production and characterization of a series of temperature sensitive (*ts*) mutants of *M. pneumoniae* (8, 9). Mutants were selected which were restricted in growth on agar at 36°, 37°, or 38°. At these temperatures, the wild-type organism grew without restriction. Missense mutants of the *ts* class were sought with the expectation that their temperature sensitve defects would restrict growth *in vivo* at the temperature of the lower respiratory tract (37°), whereas replication would not be seriously impaired in the cooler environment of the upper respiratory passage (32°–34°). Five *ts* mutants of *M. pneumoniae* with restrictive temperatures between 36° and 38° were selected for *in vivo* studies because of their high degree of *in vitro* genetic stability. It was shown that these mutants were attenuated for the Syrian hamster, and prior infection with those mutants able to replicate in the hamster induced prolonged resistance to subsequent challenge with wild type organism (9). Importantly, the mutants were completely stable genetically *in vivo* as well as *in vitro*.

The mutant with intermediate temperature sensitivity (*ts-640*) was the first to be evaluated in man (9). In the laboratory *ts-640* was found to be partially restricted in growth on agar at 37° and completely restricted at 38°. The mutant was studied in 11 volunteers who lacked preexisting serum metabolism-inhibiting (MI) antibody. Each volunteer was infected and nine underwent a silent infection. However, two of the volunteers developed bronchitis which indicated that the mutant retained sufficient residual virulence to disqualify it as a candidate vaccine strain. Nonetheless, the results of the study were encouraging since *ts-640* induced a significant antibody response in the volunteers and the mutant was genetically stable in man.

The next mutant after *ts-640* in the temperature sensitivity gradient was *ts-H43*. This organism was completely restricted in growth on agar at 37° and was highly attenuated, being

Abbreviations: MI, metabolism-inhibiting, CF, complement fixation; MCT, complement dependent mycoplasmacidal; RIP radioimmunoprecipitation; CFU, colony forming unit; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; ts, temperature sensitive.

noninfectious for hamsters and guinea pigs. In contrast, *ts* mutants less defective than *ts-H43*, i.e., mutants with a higher temperature shutoff, produced an extensive, prolonged infection in these rodents. Although mutant *ts-H43* lacked infectivity for hamsters and guinea pigs, we were prompted to evaluate this organism in man because a mutant which was slightly less temperature sensitive (*ts-640*) was infectious for man and retained some virulence. In this report we will describe the response of adult volunteers to the *ts-H43* mutant.

MATERIALS AND METHODS

Organisms. The PI 1428 strain of *M. pneumoniae* was exposed to 100 μg/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and mutants were selected from clonal populations produced on agar by a suspension of treated organisms which were filtered through a 220-nm Millipore filter (9). The *ts-H43* mutant was subjected to three colony-to-colony passages and then studied for the effect of temperature on growth on agar and for its ability to infect hamsters and guinea pigs. The suspension of *ts-H43* administered to volunteers was prepared after the 14th passage of the PI 1428 strain in artificial medium. The mutant was grown in a broth medium consisting of seven parts PPLO broth (Difco), two parts swine serum, and one part 25% yeast extract without thallium or penicillin. The 14th passage suspension was tested for evidence of adventitious microbial agents and none were found. In addition, the *ts-H43* mutant was shown to be completely inhibited by 5 μg/ml of erythromycin or tetracycline.

The wild-type suspension of strain PI 1428 was prepared after the 3rd passage in artificial medium. It was grown in broth medium identical to that used for preparation of the *ts-H43* mutant suspension described above. In studies of human volunteers, this wild-type suspension proved to be only marginally virulent and was, therefore, classified as partially attenuated.

Recovery of Organisms and Test for Genetic Stability of Isolates. Volunteers infected with either wild type or temperature sensitive organisms were tested for the presence of *M. pneumoniae* in their throat and sputum at 2- to 5-day intervals over a 28- to 60-day period. Throat swab fluid and sputum were inoculated in duplicate at the bedside into diphasic mycoplasma medium which was incubated at 32° and observed for color change and spherule formation over a 2-month period (10). Fluid from presumptively positive cultures was inoculated onto agar medium and the agar plates were incubated at 32° or 38° in an air incubator. *M. pneumoniae* isolates were identified by (*1*) their ability to grow as spherules in diphasic media, (*2*) their colonial morphology on agar, and (*3*) the ability of colonies on agar to hemadsorb guinea pig erythrocytes. Temperature sensitivity of the isolates was evaluated in a preliminary fashion by comparing the efficiency of colony formation at 32° (a permissive temperature) and at 38° (a restrictive temperature). Definitive characterization of the ts phenotype was performed subsequently in tests in which incubation was carried out in water baths which were maintained at 37° or 38° ± 0.05°. The last isolate, from each volunteer following infection with *ts-H43*, was titrated and the inoculated agar cultures were incubated at 32° in an air incubator and at 37° or 38° in a water bath. Failure of the isolate to produce colonies on agar medium at the restrictive temperatures of 37° and 38° indicated that it possessed the ts phenotype of the *ts-H43* mutant.

Immunologic Procedures. Serum antibody was measured by complement fixation (CF), metabolism inhibition (MI), and complement dependent mycoplasmacidal (MCT) tests as described previously (10, 11). The radioimmunoprecipitation (RIP) test for secretory antibody was performed as described previously with two modifications (12): [^{3}H]oleic and [^{3}H]-palmitic acids instead of ^{14}C-labeled fatty acids were used to label the mycoplasma. The anti-IgA serum used to precipitate *M. pneumoniae*–antibody complexes was produced in rabbits by Behringwerke Ag., Germany. Titer of IgA antibody was adjusted to a level of 20 mg/100 ml of IgA.

Collection and Processing of Respiratory Secretions. Nasal wash fluids and sputum samples were sonicated at 10 kHz for 2 min and centrifuged at 2000 rpm for 10 min. The supernatant obtained from the nasal washes was concentrated 10-fold by exposure to Aquacide (Calbiochem, San Diego, Calif.) whereas the supernatant from sputum was tested for antibody without further concentration. IgA content in the secretions was determined by use of the radial immunodiffusion method of Mancini *et al.* (13). The diffusion plates and standards were obtained from Meloy Laboratories, Springfield, Va.

Volunteers. Volunteers were male residents of the Lorton Reformatory, Lorton, Va. and the Maryland House of Corrections at Jessup, Md. Men were selected on the basis of general good health, age (21–50 years), absence of cardiorespiratory or allergic disease, normal chest x-ray and absence of detectable serum MI antibody for *M. pneumoniae* (serum titer <1:2). Informed consent was obtained from each volunteer prior to participation in the study.

Clinical Studies. The *ts-H43* mutant of *M. pneumoniae* was administered to 16 men who lacked serum MI antibody. Six of these men were challenged 3 months later with a suspension of partially attentuated wild-type organisms; for purposes of comparison, the partially attenuated wild-type suspension was also administered to a group of six men who lacked serum MI antibody. Two other comparison groups were also challenged with the partially attenuated, wild-type suspension; one group included four men who were initially free of serum MI antibody and who were given a formalin inactivated *M. pneumoniae* vaccine by the intranasal route; the other group included three men who were initially free of serum MI antibody and who received the inactivated vaccine by the intramuscular route. Inactivated vaccine, lot OSU 1A, was prepared by Drs. Somerson and Hamparian of Ohio State University and was found to be highly effective in inducing the development of serum MI antibody when given parenterally (14). The vaccine was given 4 months prior to challenge with wild-type organisms.

The volunteers were housed in an isolation area for 3 days before and for 28 days after the administration of live mycoplasma organisms. Two milliliters of *M. pneumoniae* suspension were given intranasally to each volunteer by DeVilbiss no. 15 atomizer. $10^{6.7}$ colony-forming units (CFU) of the *ts-H43* mutant were given, while the wild-type inoculum contained $10^{7.5}$ CFU. The volunteers were examined each day by two physicians. Chest x-rays were obtained at the beginning and end of each study. Nasal wash, sputum, and serum specimens were obtained at approximately weekly intervals.

RESULTS

Properties of the ts-H43 Mutant. As described previously, of the five genetically stable *ts* mutants of *M. pneumoniae* investigated extensively *in vitro* and *in vivo*, the *ts-H43* mutant exhibited the next to the most temperature sensitivity (9).

TABLE 1. *Response of volunteers to intranasal administration of ts-H43 mutant of M. pneumoniae*

Vol. no.	Reciprocal of pre-inoculation serum antibody titer			Maximal fold increase in post-infection serum antibody			Duration of shedding of *ts-H43* (initial day)	No. of isolates/ No. of specimens tested*
	CF	MI	MCT	CF	MI	MCT		
1	<4	<2	20	16	2	>64	≥42 (6)	16/52
2	16	<2	910	4	2	N.I.	10 (8)	9/56
3	<4	<2	40	8	2	8	27 (21)	6/56
4	<4	<2	80	16	8	5	1 (1)	1/56
5	<4	<2	80	8	2	3	12 (1)	6/56
6	<4	<2	64	16	4	7	12 (6)	6/56
7	<4	<2	240	8	2	4	7 (6)	6/52
8	<4	<2	216	32	2	2	2 (7)	2/44
9	<4	2	159	8	N.I.	2	12 (2)	7/44
10	<4	<2	136	16	4	2	0	0/44
11	<4	<2	33	2	4	5	0	0/44
12	<4	<2	<32	64	16	32	0	0/44
13	<4	<2	<32	N.I.	2	9	5 (4)	2/44
14	8	<2	66	8	2	8	1 (9)	1/44
15	4	<2	104	16	N.I.	5	≥26 (2)	14/44
16	<4	<2	101	N.I.	2	4	6 (9)	2/44

Vol., volunteer; N.I., no increase.

None of the volunteers shed wild-type revertant organisms or developed any signs or symptoms of respiratory illness.

* Throat swab fluid and sputum were each inoculated into two diphasic *Mycoplasma* cultures. Men who had a total of 44 isolation attempts performed were sampled on 11 occasions over a 28-day period; men with 52 isolation attempts were sampled on 13 occasions over a 48-day period; and men with 56 isolation attempts were sampled on 14 occasions over a 60-day period.

Unlike the wild-type organism which formed colonies on agar medium without restriction between 32° and 38°, the *ts-H43* mutant was unable to produce colonies at 37° or 38°. The shutoff temperature of *ts-H43* was rather sharp in that colony formation was efficient at 36°. The mutant was noninfectious for hamsters and guinea pigs which have body temperatures of 37° and 38.4°, respectively.

Infection of Volunteers with ts-H43. Each of the 16 men given the mutant by the intranasal route became infected. Thirteen of the volunteers shed *M. pneumoniae*, while all 16 developed a significant rise in serum antibody as measured by CF, MI, and/or MCT (Table 1). The initial day of shedding varied considerably, i.e., from the first to the 21st day after administration of the mutant. The duration of shedding was also variable in that it ranged from 1 day to 42 days or greater.

Serologic responses were detected most often by CF (13 men) and least often by MI (5 men). The mycoplasmacidal test (MCT) assay was reasonably efficient in detecting a seroresponse in that 11 of the men developed a 4-fold or greater increase in this type of antibody 4 weeks after administration of the mutant.

Genetic Stability of ts-H43 in Man. None of the 78 isolates recovered from the volunteers produced colonies on agar at 38°, whereas colony formation was efficient at 32°. A more stringent test for genetic alteration of the ts lesion(s) was performed with the last isolate recovered from each infected volunteer. The last isolates were tested for efficiency of colony formation at both 37° and 38° in water baths which did not vary more than 0.05° in temperature. None of the last isolates produced colonies at 37° or 38°. These findings suggest that the *ts-H43* mutant did not undergo a change in its ts phenotype during growth in man.

Secretory Antibody Response to ts-H43. Because of the apparent importance of local immune mechanisms in resistance to *M. pneumoniae* disease, we studied the secretory IgA antibody response of volunteers infected with the *ts-H43* mutant (Table 2). Thirteen of the 16 men were able to produce serial sputum samples. Eleven of these men (84%) developed a significant (3-fold or greater) increase in *M. pneumoniae* IgA antibody as measured by radioimmunoprecipitation using organisms labeled with [^{3}H]oleic and [^{3}H]palmitic acids. Eight of the responses were 6-fold or greater. In each instance, the maximum secretory response occurred at the end of the second week or later. An increase in secretory antibody in nasal secre-

TABLE 2. *Secretory IgA antibody response of 16 volunteers who received the ts-H43 mutant of M. pneumoniae*

Vol. no.	Development of 4-fold or greater rise in serum antibody in the indicated tests	*M. pneumoniae* IgA antibody in secretions as measured by radioimmunoprecipitation			
		Reciprocal of titer* prior to infection		Maximum fold rise during first month postinfection (day of maximum rise)	
		Nasal wash	Sputum	Nasal wash	Sputum
1	CF, MCT	71	1	N.I.	30 (20)
2	CF	13	6	13 (18)	6 (14)
3	CF, MCT	44	48	5 (11)	N.I.
4	CF, MI, MCT	71	9	13 (11)	18 (20)
5	CF	33	20	7 (18)	3 (28)
6	CF, MI, MCT	44	3	2 (18)	7 (28)
7	CF, MCT	53	4	3 (25)	9 (20)
8	CF	80	64	3 (21)	4 (14)†
9	CF	27	128	2 (21)	N.I.
10	CF, MI	20	N.A.	2 (14)	—
11	MI, MCT	34	N.A.	N.I.	—
12	CF, MI, MCT	82	10	2 (14)	8 (14)
13	MCT	7	16	8 (14)	4 (14)
14	CF, MCT	74	37	3 (14)	9 (14)
15	CF, MCT	40	N.A.	2 (14)	—
16	MCT	17	21	N.I.	9 (14)

N.A., not available; N.I., no increase.

* Adjusted to 20 mg/100 ml of IgA.

† Sputum was collected from volunteers 8-16 only through day 14.

TABLE 3. *Response to intranasal administration of partially attenuated wild-type M. pneumoniae of volunteers who were previously infected with mutant ts-H43 or given inactivated vaccine*

Previous treatment of men who were given $10^{7.5}$ CFU of wild-type *M. pneumoniae*	No. of men	No. who developed a 4-fold or greater rise in serum antibody			Maximum fold increase in IgA antibody in secretions as measured by radioimmunoprecipitation		No. who shed *M. pneum.*	No. of isolates recovered from 56 sputa and throat swab fluids tested
		CF	MI	MCT	Nasal secretions	Sputum		
Infection 3 months before with *M. pneumoniae* mutant *ts-H43*	6	0	0	0	N.I., 2, 2, 4, 6, 13	N.I., N.I., N.I., 2, 21*	2	3, 2, 0, 0, 0, 0
Inactivated *M. pneumoniae* vaccine IN 4 months before	4	4	4	2	2, 2, 4, 9	4, 8, 12, 23	4	7, 8, 16, 25
Inactivated *M. pneumoniae* vaccine IM 4 months before	3	3	3	3	4, 4, 12	5†	3	1, 1, 11
No treatment	6	5	5	4	2, 3, 6, 7, 12, 18	N.I., 2 3, 6, 8, 16	6	2, 9, 11, 12, 14, 24

Three men in the 1st group, one in the 2nd group, and two in the 3rd group possessed serum MI antibody at the time of challenge, whereas men in the 4th group (controls) were seronegative.

* Sputum not available from one volunteer.

† Sputum not available from two volunteers.

N.I., no increase; IN, intranasally; iM, intramuscularly.

tions was seen less often than in sputum. Eight of the volunteers developed a 3-fold or greater increase in *M. pneumoniae* IgA antibody in nasal secretions.

The secretory antibody responses to *ts-H43* appeared to occur as frequently and to be of the same magnitude as the responses of volunteers who were infected with a partially attenuated wild-type suspension of *M. pneumoniae* (Tables 2 and 3). In addition, the secretory antibody responses of the *ts-H43* infected volunteers were similar in frequency and magnitude to those described for a larger group of men infected with wild-type *M. pneumoniae* (7).

Clinical Response to Infection. None of the men infected with the *ts-H43* mutant developed any signs or symptoms of illness during the 28-day period of intensive medical surveillance following administration of the inoculum. There were no elevations of temperature nor did any of the men develop any signs or symptoms involving the respiratory tract. Hematocrit, white cell count and cold agglutinins were investigated weekly and there were no abnormalities noted. Similarly, SGPT (serum glutamic pyruvic transaminase) and serum alkaline phosphatase were not elevated on day 14 or day 28. Finally, chest x-rays were normal in each instance on day 28.

Response of Men Infected with ts-H43 to Later Challenge with Partially Attenuated Wild-Type M. pneumoniae. A third passage suspension of the PI 1428 strain of *M. pneumoniae*, which was wild type (ts^+) and partially attenuated, was used as a challenge inoculum in an attempt to evaluate the extent of resistance induced by the *ts-H43* mutant. In order to aid in this evaluation, a group of six men who did not have serum MI antibody was also challenged, as were seven men who were initially seronegative and who received an inactivated *M. pneumoniae* vaccine either intranasally or intramuscularly (Table 3). The 3rd passage suspension of the PI 1428 strain produced illness in only two of the volunteers. Both of these men had received inactivated vaccine by the nasal route previously; they developed bronchitis with low grade fever and these symptoms responded rapidly to treatment with erythromycin.

It was not possible to assess the capacity of *ts-H43* to induce resistance to disease since the challenge inoculum did not produce objective signs of illness with sufficient frequency to permit such an analysis. However, there was a suggestion that infection with the ts^+ suspension of *M. pneumoniae* was suppressed in men who had received the *ts-H43* mutant previously (Table 3). The *ts-H43* vaccinees failed to develop a serum antibody response, whereas 12 of the 13 men in the comparison groups developed a seroresponse. Further, *ts-H43* vaccinees appeared to shed fewer organisms from the respiratory tract than the seronegative controls or the men who had received inactivated *M. pneumoniae* vaccine previously. *ts-H43* vaccinees also appeared to have local respiratory secretory antibody responses which were less extensive than those observed in men in the seronegative control and inactivated vaccine groups.

DISCUSSION

An organism, to be used in a live vaccine, must achieve a very delicate balance between attenuation and the ability to replicate sufficiently well so that the defense mechanisms of the host are stimulated. In a previous study of volunteers, we observed that the *ts-640* mutant had not achieved this desired balance. Two of the 11 volunteers infected with this mutant developed symptoms of mild, lower respiratory tract disease. This result created a dilemma in that the mutant (*ts-H43*) which was slightly more temperature sensitive than *ts-640* was not infectious for hamsters or guinea pigs. Both of these rodents are easily infected by wild-type *M. pneumoniae*, mutant *ts-640* and mutants less temperature sensitive than *ts-640* (9). Nevertheless, *ts-H43* was evaluated in volunteers who lacked serum MI antibody and to our surprise the mutant was found to be infectious for each of the 16 men studied. One suspension of *ts-H43* was employed in all the studies in man and laboratory animals so that the difference in infectivity appears to be

an intrinsic property of the organism and cannot be ascribed to differences in mycoplasma populations used. Although much important information concerning pathogenesis of disease and immunological response has been obtained from the study of infected hamsters and guinea pigs, it appears that these animals are not as sensitive to *M. pneumoniae* as man.

In the present study, each of 16 volunteers underwent a completely silent infection with mutant *ts-H43*. Infection of the respiratory tract was extensive enough, however, to stimulate a moderate local secretory antibody response as well as a systemic antibody response. These observations suggest that the *ts-H43* organism had achieved the desired balance between attenuation (defectiveness) and antigenicity. The development of a respectable local antibody response was particularly encouraging because of the mounting evidence that local immunological factors in the respiratory tract play a major role in protecting the host against *M. pneumoniae* disease. The 16 men challenged with *ts-H43* would be considered seronegative if only standard serologic techniques such as metabolism-inhibition were used. However, when highly sensitive immunologic procedures were employed (MCT or RIP) they possessed, in most instances, what appeared to be antibody to *M. pneumoniae* in their serum and respiratory tract secretions. In other studies we had found that almost all adults possess *M. pneumoniae* antibody when tested by the MCT and RIP techniques (7, 11). While this antibody is specific in that it is capable of lysing the organism in the presence of complement and can be blocked by *M. pneumoniae* organisms, its origin remains unclear. Whether this pre-existing antibody represents prior silent infection with *M. pneumoniae* or whether it is induced by exposure to common naturally occurring cross-reactive glycolipid antigens remains to be established.

Preliminary observations from a challenge of *ts-H43* vaccinees with partially attenuated wild type (ts^+) *M. pneumoniae* suggested that the mutant had induced resistance to infection. In the challenge study, resistance to illness could not be evaluated; however, infection with wild type (ts^+) organisms appeared to be suppressed in men previously given the *ts-H43* mutant. Previous studies in animals have shown that resistance to infection is conferred on a host only by prior infection of the respiratory tract with *M. pneumoniae* (ref. 6 and unpublished data). It appears that inactivated vaccines given either systemically or locally do not induce resistance to infection of the respiratory tract although the host may be protected against the development of lung lesions.

Another property desirable in a live vaccine is genetic stability. Initially, the *ts-H43* mutant was chosen for further study *in vitro* and in animals because of its failure to produce wild-type revertants during growth to high titer in broth medium (9). In the present study, the mutant also appeared to be stable genetically in man in that none of the isolates recovered from infected volunteers exhibited evidence of reversion of the ts phenotype. *ts-H43* was induced by exposure of wild-type *M. pneumoniae* to 100 μg/ml of NTG (9). In this circumstance it is likely that the mutant sustained more than one *ts* point mutation. Further, the presence of more than one ts lesion probably accounts for the genetic stability of *ts-H43*. Adelberg, Mandel, and Chen have commented upon the tendency for NTG to produce multiple genetic lesions, some of which are not temperature sensitive, in bacteria (15). As in bacteria mutagenized by NTG, *non-ts* genetic lesions may also be present in *ts-H43*.

In practical terms, the results of the first volunteer studies with *ts-H43* are sufficiently encouraging to warrant cautious expansion of evaluation of the mutant in larger numbers of volunteers. Furthermore, while several attempts to use temperature sensitive mutants of respiratory viruses for vaccine purposes have yielded encouraging results (16, 17), the present effort represents the first application of this technique to free-living organisms. The promising results presented here may offer a new approach toward the control of other bacterial respiratory pathogens.

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