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Respiratory Syncytial Virus Infection in Owl Monkeys: Viral Shedding, Immunological Response, and Associated Illness Caused by Wild-Type Virus and Two Temperature-Sensitive Mutants

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Intranasal inoculation of owl monkeys with wild-type respiratory syncytial virus induced upper respiratory tract disease in each of seven animals. The response of owl monkeys to two highly defective, temperature-sensitive, multiple-lesion mutants was then compared to the pattern seen with wild-type respiratory syncytial virus. These mutants, *ts*-1 NG-1 and *ts*-1 NG-16, were derived from the *ts*-1 mutant that had been remutagenized with nitrosoguanidine (NG). Previously the *ts*-1 NG-1 and *ts*-1 NG-16 mutants had been shown to be more temperature sensitive and more stable genetically than their *ts*-1 parent. Both *ts*-1 NG-1 and *ts*-1 NG-16 produced infection that was delayed in onset compared to wild-type virus infection. However, the mutants were shed from the upper respiratory tract for the same period of time and at the same titer as wild-type virus. The serum neutralizing antibody response to infection with the mutants was nearly equivalent to that elicited by wild-type virus. However, the extent of disease induced by the mutants was significantly less than that seen with wild-type virus. These observations suggest that the mutants are potential vaccine condidates and should be subjected to additional in vivo testing in primates and, ultimately, humans.

In the past 3 years several animal models of respiratory syncytial (RS) virus infection have been described, including the ferret (10), cotton rat (9), cebus monkey (12), owl monkey (11), and chimpanzee (1). Of these, the chimpanzee most closely resembles humans in its response to RS virus infection since this primate develops clinical signs of illness (profuse rhinorrhea, mild cough, and sneezing) similar to those seen during upper respiratory illness of children and adults (1). However, the extreme scarcity of chimpanzees lacking antibody to RS virus severely limits their use as an experimental model of infection.

The only other nonhuman species in which RS virus has been shown to produce clinically evident disease (i.e., rhinorrhea) is the owl monkey (11). In contrast to chimpanzees, owl monkeys lacking antibody to RS virus are available in sufficient numbers to permit their use in large-scale experiments.

This paper describes the pattern of wild-type RS virus infection in owl monkeys, and compares it to the effect of two highly defective, temperature-sensitive, multiple-lesion mutants derived from the wild-type virus.

MATERIALS AND METHODS

Viruses. The wild-type A-2 strain of RS virus (F-059; Flow Laboratories) was isolated in human embryonic kidney culture and subsequently passaged only in primary bovine kidney cells. It was previously shown to produce upper respiratory tract illness in adult volunteers (8).

The *ts*-1 mutant was initially isolated from the progeny of wild-type virus (strain A-2) grown in the presence of 10^{-4} M 5-fluorouridine (4, 15). Remutagenization of the *ts*-1 mutant with nitrosoguanidine (NG) yielded the two clones in which one or more additional temperature-sensitive (*ts*) mutations were introduced. These multiple mutants were designated *ts*-1 NG-1 and *ts*-1 NG-16 (13). Suspensions of *ts*-1 NG-1 (F-481; Flow Laboratories) and *ts*-1 NG-16 (F-486; Flow Laboratories) were prepared in bovine kidney cells.

Animals. Wild-caught adult owl monkeys (*Aotus trivirgatus*), which lacked RS virus neutralizing antibodies detectable by the plaque-reduction technique (8), were selected from a colony maintained by Meloy Laboratories, Inc., Rockville, Md. The animals were maintained in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care and were housed as previously described (1).

Experimental protocol. Animals were anesthetized lightly with ketamine hydrochloride and then

inoculated intranasally with 1.0 ml of virus suspension. Nasal and throat swabs, taken daily for the first 2 weeks postinoculation and every other day for an additional week, were combined into 1.5 ml of veal infusion broth on each day of collection. Blood specimens were taken immediately before virus inoculation, and 7, 14, 28, and 56 days thereafter. Animals were evaluated daily for signs of upper respiratory disease. Rhinorrhea was scored from 0 to +3 on the basis of criteria outlined in Table 1.

Virus isolation. Swab specimens were promptly inoculated into RS virus-sensitive HEp-2 tissue culture tubes, for detection of virus, and onto HEp-2 monolayers for quantitation of infectivity by plaque assay (14).

Antibody assay. Serum specimens were tested for the presence of neutralizing antibody to RS virus by the plaque-reduction technique previously described (8).

Statistical testing. Mean values were compared by Student's *t* test. Differences were considered significant when the probability (P) was <0.05.

RESULTS

Wild-type virus. Seven animals were inoculated intranasally with wild-type RS virus; each animal received $10^{4.4}$ plaque-forming units (PFU) of virus. Virus was first recovered from six of the animals on the second day postinoculation; the remaining animal began shedding virus the next day (Table 1). Peak titers ranged from $10^{4.7}$ to $10^{5.3}$ PFU/ml of nasopharyngeal swab specimen, with a geometric mean value of $10^{5.0} \pm 10^{0.1}$ PFU/ml (standard error). The time at which peak viral titer was attained varied from the day 3 to the day 6 postinoculation (mean 4.9 ± 0.5 days). Duration of infection, defined as the interval between the first and last virus isolation, ranged from 8 to 17 days, with a mean of 11.1 ± 1.1 days.

All seven animals developed clinical signs of disease, which began as early as day 4 and persisted as late as day 19 postinoculation. For purposes of comparing the severity of disease, a combined disease score was calculated for each animal. This score was a summation of the daily disease scores: a +1 daily score was given one point, a +2 was given two points, etc. On this basis, the combined disease scores for the seven animals ranged from 10 to 21, with a mean of 15.6 ± 1.7. None of the animals succumbed to RS virus infection.

The serum neutralizing antibody response of the five animals tested was similar (Table 2). Antibody did not appear until 14 days after inoculation, reached peak titer in most cases by 28 days, and remained at a high level throughout the experiment (day 56).

***ts*-1 NG-1 mutant.** Six animals were inoculated intranasally with the *ts*-1 NG-1 mutant; each animal received $10^{4.5}$ PFU of virus. Virus was first recovered from two animals on day 2 after inoculation, from two others on day 3, and from the remaining animals on day 4 or 5 (Table 1). This represented a significant delay in the onset of infection compared with wild-type virus ($P < 0.05$).

The mean peak titer of *ts*-1 NG-1-inoculated animals, $10^{4.7} \pm 10^{0.4}$ PFU/ml of nasopharyngeal swab specimen, did not vary significantly from wild-type infection. However, the attainment of peak titer was delayed in the *ts*-1 NG-1 animals, with a mean of 8.7 ± 1.2 days. In comparison with wild-type infection, this delay was significant ($P < 0.01$). The duration of infection (13.5 ± 1.6 days) did not differ from wild-type infection.

The *ts*-1 NG-1 mutant produced a milder clinical disease than its wild-type virus parent. Although the duration of clinical signs induced by

TABLE 1. *Infection of owl monkeys with wild-type RS virus and ts-1 NG-1 and ts-1 NG-16*[a]

Virus inoculum	No. of animals	Mean time to first viral isolate (days)	Geometric mean peak viral titer (log_{10} PFU/ml of swab specimen)	Mean time of peak viral titer (days)	Mean duration of viral shedding (days)	Mean combined disease score[b]
Wild type, $10^{4.4}$ PFU, i.n.[c]	7	2.1 ± 0.1 + 3.2 ± 0.5 □	5.0 ± 0.1	4.9 ± 0.5 □ 8.7 ± 1.2 △	11.1 ± 1.1	15.6 ± 1.7 □ 5.3 ± 2.5 △
ts-1 NG-1, $10^{4.5}$ PFU, i.n.	6		4.7 ± 0.4		13.5 ± 1.6	
ts-1 NG-16, $10^{5.3}$ PFU, i.n.	6	2.8 ± 0.2	5.2 ± 0.3	7.0 ± 0.5	10.7 ± 0.5	9.3 ± 1.2

[a] Symbols: ±, standard error; +, $P < 0.05$; △, $P < 0.02$; □, $P < 0.01$.

[b] Clinical disease was evaluated daily in each animal on a scale of 0 to 3, based on the following criteria: 0, no nasal discharge; 1, nasal discharge not visible until dislodged during swabbing; 2, nasal discharge visible, but did not extend beyond nares; 3, nasal discharge extending beyond nares.

[c] i.n., Intranasal route of inoculation.

TABLE 2. *Serological response of owl monkeys to infection with ts mutant or wild-type RS virus*

Virus administered	Owl monkey no.	Reciprocal serum neutralizing antibody titers on indicated day after inoculation				
		0	7	14	28	56
Wild type	233	<20	<20	689	>1,280	544
	242	<20	<20	439	432	>1,280
	263	<20	NT[a]	241	>1,280	>1,280
	265	<20	NT	265	>1,280	>1,280
	266	<20	NT	142	>1,280	>1,280
ts-1 NG-1	231	<20	<20	22	>1,280	226
	235	<20	<20	<20	470	189
	236	<20	<20	148	>1,280	>1,280
	254	<20	<20	82	711	1,115
	307	<20	<20	<20	66	249
	308	<20	<20	<20	350	568
ts-1 NG-16	208	<20	<20	85	754	388
	209	<20	<20	<20	620	702
	246	<20	<20	25	279	1,028
	248	<20	<20	244	1,164	>1,280
	249	<20	27	240	>1,280	>1,280
	276	<20	<20	<20	271	101

[a] NT, Not tested.

ts-1 NG-1 was similar to that observed for wild-type virus infection, the severity of disease was reduced. Furthermore, two of the six animals that received *ts*-1 NG-1 showed no signs of disease throughout the experiment, although they shed substantial amounts of virus (up to $10^{5.6}$ PFU/ml). The combined disease scores for the six *ts*-1 NG-1 animals, therefore, ranged from 0 to 14, with a mean of 5.3 ± 2.5. None of the animals died. The difference in combined disease scores between animals receiving wild-type virus or *ts*-1 NG-1 virus was significant ($P < 0.01$).

The serum antibody response of animals that received *ts*-1 NG-1 was similar to that seen with wild-type virus (Table 2). Antibody was not detected before day 14 postinoculation, and peak titers were generally reached by about day 28 and remained high through day 56. Although some *ts*-1 NG-1 animals did not produce antibody by day 14 (unlike wild-type virus), and the mean peak titer of the *ts*-1 NG-1 animals was slightly lower, these differences were not statistically significant.

ts-1 NG-16 mutant. Six animals were inoculated intranasally with the *ts*-1 NG-16 mutant; each animal received $10^{5.3}$ PFU of virus. Virus was first recovered on day 2 after inoculation from a single animal. The other five began to shed virus on day 3 (Table 1). This delay, in comparison with wild-type virus, was significant ($P < 0.01$); however, the pattern of the *ts*-1 NG-16 mutant did not differ from that of the other mutant.

The mean peak titer of *ts*-1 NG-16–inoculated animals, $10^{5.2} \pm 10^{0.3}$ PFU/ml of nasopharyngeal swab specimen, did not vary significantly from either wild-type virus or the *ts*-1 NG-1 mutant. However, as with *ts*-1 NG-1, *ts*-1 NG-16–inoculated animals shed peak quantity of virus later in infection (mean 7.0 ± 0.5 days) than did wild-type virus-inoculated animals ($P < 0.02$). The difference between *ts*-1 NG-16 and *ts*-1 NG-1 in this regard was not significant. Mean duration of virus shedding (10.7 ± 0.5 days) did not differ either from wild-type or *ts*-1 NG-1 infection.

Like *ts*-1 NG-1, *ts*-1 NG-16 produced a milder form of disease than wild-type virus. Although none of the six *ts*-1 NG-16–inoculated animals was free of upper respiratory tract signs, the severity of disease was reduced. The combined disease scores for the six animals ranged from 6 to 13, with a mean of 9.3 ± 1.2. None of the animals died. The difference in combined disease scores between these animals and those receiving wild-type virus was significant ($P < 0.02$); however, there was no significant difference between animals receiving *ts*-1 NG-16 and those receiving *ts*-1 NG-1.

The serum antibody response of animals receiving *ts*-1 NG-16 was essentially the same as that seen with wild-type or *ts*-1 NG-1 viruses (Table 2), with no significant difference either in time of onset or peak titer of serum neutralizing antibody.

DISCUSSION

Efforts to develop an effective vaccine against respiratory syncytial virus have met with only

limited success. An early vaccine, prepared by Formalin inactivation of the virus and administered parenterally, produced a substantial immunological response, as measured by serum neutralizing antibody. However, vaccinees were not protected against subsequent natural infection, and in fact developed a more severe form of disease than unvaccinated controls (2, 3, 5, 7).

This experience with inactivated vaccine led to the development of candidate live, attenuated vaccine strains. Treatment of RS virus with chemical mutagens induced a series of *ts* mutants (4). The first of these to be tested in humans, *ts*-1 (16), appeared promising when evaluated in adult volunteers. However, tests in seronegative infants showed the virus to possess a low level of residual virulence and to exhibit some genetic instability (6, 17).

Subsequent work has focused on a second mutant, *ts*-2, which has been shown to be completely avirulent in primates (11) and adult volunteers (unpublished data). Field trials of this vaccine candidate are soon to begin. However, the genetic defect in *ts*-2 is restrictive for both virulence and infectivity. Initial studies in primates (11) raised the question of whether this virus may be insufficiently infective to consistently induce immunity.

In an attempt to further attenuate *ts*-1, which appears to be nearly as infective as wild-type virus, NG was used to remutagenize the mutant (13). The two mutants evaluated in this study, *ts*-1 NG-1 and *ts*-1 NG-16, were recovered from the progeny of NG-treated *ts*-1 and were shown to exhibit greater temperature sensitivity and genetic stability than *ts*-1. We used the owl monkey to test these two mutants in vivo because it is the only experimental animal which develops clinically evident disease when infected with wild-type RS virus and which is also available in sufficient numbers to permit statistically valid comparison with wild-type virus.

In evaluating *ts*-1 NG-1 and *ts*-1 NG-16 in vivo, we examined three phenomena. Pattern of infection was assessed by examining the time of onset of shedding of infectious virus, duration of shedding, peak viral titer, and time of peak titer. Virulence was evaluated by a composite disease score, consisting of the sum of daily disease scores for each animal. Finally, antigenicity was evaluated in terms of the time of onset and the peak titer of serum neutralizing antibody.

Neither *ts*-1 NG-1 nor *ts*-1 NG-16 differed significantly from wild-type virus in either duration of infection or peak virus titer. This would suggest that both mutants produced an infection that was comparable in extent to that of wild-type virus, a desirable property for a vaccine strain. However, the time of onset of virus shedding and the time of peak titer of both mutants differed significantly from wild-type virus, suggesting that both mutants were, nonetheless, functionally defective compared to wild-type virus.

Although capable of producing extensive infection, both mutants were significantly attenuated compared to wild-type virus. That is, the composite disease scores were significantly lower for both *ts*-1 NG-1- and *ts*-1 NG-16–infected animals. Reduced virulence, like high infectivity, is another property desirable for a potential vaccine strain.

Finally, both mutants were nearly as antigenic as wild-type virus when various aspects of the humoral antibody response were measured. Again, antigenicity equivalent to that produced by wild-type virus is desirable for a vaccine candidate.

In vitro testing of *ts*-1 NG-1 and *ts*-1 NG-16 (13) previously showed them to be more temperature sensitive and genetically stable than their *ts*-1 parent virus. Subsequent in vivo study in chimpanzees (11) confirmed the greater stability of both mutants. However, in vivo analysis of infectivity, virulence, and antigenicity was limited to observations on two chimpanzees for each mutant, a number inadequate for statistical analysis.

In the present study the *ts*-1 NG-1 and *ts*-1 NG-16 mutants were each evaluated in six owl monkeys. The resulting observations permit the following conclusions. (i) Both mutants produced an infection as extensive as wild-type virus, although the onset of infection appeared to be delayed. (ii) Both mutants were significantly attenuated compared to wild-type virus, in terms of the severity of clinical disease produced. (iii) Both mutants were nearly as antigenic as wild-type virus.

These observations, in conjunction with previous studies showing *ts*-1 NG-1 and *ts*-1 NG-16 to be more defective than the *ts*-1, parental mutant, and more genetically stable both in vitro and in vivo, suggest that they are potential candidates for use in a live vaccine. The fact that the two mutants did not differ significantly from each other in any of the observed parameters suggests that both be subjected to additional in vivo testing in primates and, ultimately, humans.

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