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Genetic Alteration in a Temperature-Sensitive Mutant of Respiratory Syncytial Virus After Replication *in Vivo* (37972)

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Genetic stability *in vivo* is of considerable interest to those concerned with the development of live attenuated virus vaccines. We report here on studies of the genetic stability *in vivo* of a chemically induced temperature-sensitive mutant of respiratory syncytial virus (RSV) which is currently being evaluated as a vaccine strain (1).

Materials and Methods. Virus. The ts-1 temperature-sensitive mutant of RSV was produced by 5-fluorouridine treatment of the A2 strain (2). The mutant employed in these studies was grown in bovine kidney (BK) tissue culture and was characterized by an inability to form characteristic RSV syncytial plaques in HEp-2 or HeLa cells at temperatures of 37° or above (restrictive temperatures). In contrast, the wild-type parent A2 virus formed plaques without restriction at 39°. The ts-1 mutant also demonstrated diminished viral replication at restrictive temperatures. Viral plaque formation and replication at 32° (permissive temperature) approximated those of the parent strain. No evidence of genotypic lability of this mutant had been noted prior to these trials despite extensive studies in animals and in adult volunteers (3, 4).

Evaluation of mutant in children. After written, informed consent from their parents or guardians had been obtained, 32 children, aged 6 months to 6 years, were admitted in pairs to the Clinical Research Center of the Children's Hospital of the District of Columbia (1). They were kept in isolation, observed for 5 days, and then approximately 10^4 $TCID_{50}$ of the ts-1, candidate, vaccine virus was administered into the nose by pipet and into the pharynx as a coarse spray. Subsequently, daily pharyngeal swabs were obtained from all children for 3 weeks in an attempt to recover the mutant and describe its temporal pattern of replication. The swab fluid was inoculated into HEp-2 cell roller tube cultures which were incubated at 32°. Virus was harvested from tube cultures showing cytopathic effects (CPE) characteristic of RSV, and 1–2 further passages in HEp-2 cells at permissive temperature were carried out. The progeny virus from these passages was tested for temperature sensitivity of plaque formation.

Efficiency of plaque formation at different temperatures. The techniques employed in these investigations have been described elsewhere (3). Briefly, 0.1-ml aliquots of virus were adsorbed for 90 min onto 3-day-old HEp-2 cell monolayers in 35-mm wells of plastic petri dishes (Flow Laboratories, Rockville, MD). The cells were then overlaid with a medium consisting of equal parts of (a) twice-concentrated Leibowitz 15 medium containing 20% inactivated agamma calf serum (BBL, Cockeysville, MD), glutamine (0.002 *M*), penicillin (250 U/ml), and amphotericin B (50 U/ml) and (b) 2% agarose in 0.05% L-tyrosine. Parallel cultures were then incubated at 32° in a forced air incubator and at 37, 38, and 39° in water baths maintained with Braun constant-temperature water circu-

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lators. The temperature in these water baths was controlled within ±0.05°. Cells incubated at restrictive temperatures were fixed with 10% formalin after 3 days, stained with hematoxylin and eosin, and examined for RSV plaques. Cells incubated at 32° were fixed and stained after 5 days.

Results. Virus shedding. As reported in an earlier publication, virus was recovered from the throat swabs of 27 of the 32 children who were given the ts-1 candidate vaccine strain (1). Virus was first isolated as early as the 2nd day after administration and as late as the 20th day; the mean time to initial excretion was 6.1 days. The mean duration of virus excretion was 5.2 days. Those children with lower preinfection serum anti-RSV neutralizing antibody titers (principally the younger individuals) tended to shed virus earlier, in greater amounts, and for more prolonged periods of time.

Genetic stability of the virus. When the viruses isolated from throat-swab material were passaged two times in HEp-2 cells at permissive temperature and the progeny virus examined for efficiency of plaque formation at both permissive and restrictive temperatures, evidence of genetic alteration of the ts phenotype was observed in some instances.

In all, 135 throat-swab specimens from 27 vaccinees yielded virus when tested in HEp-2 cells. After two passages in HEp-2 cells, the progeny of 34 of these isolates recovered from 16 vaccinees showed evidence of genetic alteration. Altered virus tended first to appear late in the course of infection (average 11 days postinfection), but there was no correlation between a child's shedding altered virus and his age or serologic status. Two types of genetic alteration were noted. The progeny of 8 isolates formed plaques with equal efficiency at permissive temperature (32°) and at all 3 restrictive temperatures (37, 38, and 39°). This total loss of temperature sensitivity suggested reversion to wild type. The other 26 isolates showed a pattern of temperature sensitivity intermediate between that of wild type RSV and the ts-1 vaccine virus, i.e., some degree of plaque formation at restrictive temperatures could be demonstrated, but the efficiency of such plaque formation was less than that shown by wild type and tended to decrease with increasing temperature.

Table I compares the efficiency of plaque formation at permissive and restrictive temperatures of wild type RSV, ts-1 vaccine, and representative isolates recovered from the infected children. As can be seen, some of the isolates consisted of virus populations which were intermediate in their temperature sensitivities between wild type RSV and the ts-1 vaccine strain. In contrast, isolate 9151 exhibited no real loss of efficiency of plaque formation with increasing temperature and was thus indistinguishable from wild type. It should be emphasized that these isolates represent progeny derived from virus which had undergone two pas-

TABLE I. Efficiency of Plaque Formation at Permissive and Restrictive Temperatures of RSV Strains Recovered from Infants and Young Children Infected with ts-1 Mutant Virus—Representative Findings from a Single Test.

RSV	Titer in PFU/ml at indicated temperature (log_{10})			
	32°C	37°C	38°C	39°C
Wild type	5.2	5.2	5.3	5.3
ts-1 vaccine[a]	4.2	<0.7	<0.7	<0.7
Isolate 9151[b]	3.9	3.9	3.7	3.5
Isolate 9625[b]	4.3	3.5	3.3	2.5
Isolate 8519[b]	4.7	3.8	3.8	1.2
Isolate 9127[b]	4.8	3.4	<0.7	<0.7
Isolate 8989[b]	5.5	3.2	<0.7	<0.7

[a] Suspension of ts-1 mutant which was administered to infants and young children.

[b] These isolates were tested after 2 passages in HEp-2 cells at 32°.

sages in HEp-2 cells at the permissive temperature of 32°. As described previously, when the daily pharyngeal swabs of 20 of the vaccinees were inoculated directly onto HEp-2 cells which were incubated at both permissive (32°) and restrictive (39°) temperatures, the proportion of virus with wild phenotype was less then 0.1% (1). Thus, the genetically altered wild type-like virus did not become the predominant species *in vivo*. The presence of any form of genetically altered virus was detected only after the isolates had been subjected to passage in cell culture. Presumably altered virus had a growth advantage over ts virus and this was responsible for the emergence of an appreciable quantity of altered virus on passage *in vitro*.

Characterization of genetically altered virus. The results presented in Table I represent tests performed upon virus present in tissue culture passage material. The intermediate type of temperature sensitivity exhibited by four of the isolates in Table I might represent a composite of patterns produced by a mixture of virus populations. Thus, these virus suspensions might consist of a heterogeneous population of virions, the majority being temperature sensitive with a minority being wild-type revertants or virions of an intermediate temperature sensitivity. On the other hand, these four virus populations might be homogeneous with all the virions exhibiting intermediate temperature sensitivity. To discriminate between these possibilities, it was necessary to determine the temperature-sensitivity phenotypes of individual virions within these populations. To this end, 3 of the isolates, 9127, 9625, and 8519, were serially diluted and various dilutions inoculated in 0.1-ml amounts onto monolayers of HeLa cells. Adsorption and overlay were carried out as described above. Cultures were then incubated at 32, 37, 38, and 39°. At the end of 4 days, the overlay medium was supplemented by the addition to each well of 1 ml of medium containing 1:10,000 neutral red. The cultures were then incubated for an additional 24 hr, care being taken to avoid exposing them to light. At the end of the 5th day of incubation, RSV plaques were

TABLE II. Titer (PFU/ml) of Clones Derived from Plaques Produced by Isolate 9127 at Various Temperatures.

Temperature of plaque production	Clone No.	Titer (log_{10})			
		32°	37°	38°	39°
32°	1	6.1	<0.7	<0.7	<0.7
	2	5.4	<0.7	<0.7	<0.7
	3	5.8	<0.7	<0.7	<0.7
	4	6.0	<0.7	<0.7	<0.7
	5	6.0	<0.7	<0.7	<0.7
	6	6.3	<0.7	<0.7	<0.7
37°	1	5.2	4.9	3.6	1.3
	2	4.2	4.1	3.5	3.6
	3	5.2	4.8	4.5	3.9
	4	5.2	4.6	4.0	3.5
	5	4.8	4.4	3.6	1.3
	6	5.7	5.0	4.7	2.5
38°	1	3.7	3.4	3.3	3.0
	2	3.6	3.2	3.4	3.1
	3	5.4	4.6	4.5	3.6
	4	6.1	5.5	5.3	4.3
	5	5.2	4.4	4.0	1.9
	6	5.0	4.4	4.4	3.6
	7	4.2	4.0	3.8	3.0

easily identifiable as they concentrated neutral red. Clones were isolated from wells containing no more than 3 plaques. To generate stocks of plaque-purified virus, the cloned material was then inoculated into tube cultures of HeLa cells that were incubated at permissive temperature. When CPE were noted, the tube cultures were harvested by scraping the cells, sonicating the resulting suspension in a Raytheon Model DF 101 sonic oscillator (1.0 A for 20 sec), and clarifying by low-speed centrifugation. The resulting virus clones were then quick-frozen in an alcohol–dry ice bath and stored at −70° until temperature-sensitivity testing could be carried out.

Six viable clones were obtained from monolayers of HeLa cells inoculated with isolate 9127 and incubated at 32°. The temperature-sensitivity profiles of these clones (presented in Table II) were indistinguishable from that of the ts-1 vaccine strain. These results suggest that the makeup of this isolate was heterogeneous with the majority of the viruses in the population retaining the temperature-sensitive phenotype. Five of the six clones isolated at 37° had a temperature sensitivity intermediate between the wild type A2 strain and the ts-1 vaccine strain. The remaining clone did not show a significant (i.e., ten-fold or greater) difference in titer between permissive and restrictive temperatures and could therefore not be positively distinguished from wild type. This would suggest that complete reversion of phenotype had occurred in this instance. Of the 7 clones isolated at 38°, 5 showed intermediate-type temperature sensitivity, while 2 appeared to be wild-type revertants. Viable clones were not isolated from the 9127-infected HeLa monolayers incubated at 39°.

The ability of clones from isolate 9127 to show some degree of plaque formation at 38° may seem surprising in light of the failure to demonstrate such plaque formation by the original isolate. Incubation at this temperature was carried out for 5 days during the cloning procedure, however, and only for 3 days during the initial characterization of the original isolate. Variation from week to week in the sensitivity of HeLa cells to virus plaque formation at restrictive temperature might also provide an explanation.

TABLE III. Titers (PFU/ml) of Clones Derived from Plaques Produced by Isolate 9625 at Various Temperatures.

Temperature of plaque production	Clone No.	Titer (log_{10})			
		32°	37°	38°	39°
32°	1	5.1	<0.7	<0.7	<0.7
	2	5.2	<0.7	<0.7	<0.7
	3	6.0	<0.7	<0.7	<0.7
	4	6.0	<0.7	<0.7	<0.7
37°	1	5.7	3.9	3.7	2.3
	2	5.9	5.3	5.6	5.2
	3	5.9	5.3	5.3	5.0
	4	5.8	5.0	5.0	4.9
38°	1	5.1	4.8	4.4	4.2
	2	5.9	4.9	5.0	4.8
	3	5.4	5.0	4.9	4.7
	4	5.1	4.5	5.0	4.4
39°	1	5.8	5.4	4.9	5.2
	2	5.9	5.4	4.4	5.0
	3	5.8	5.4	5.4	4.9
	4	5.8	5.1	5.1	4.7

The temperature-sensitivity profiles of clones obtained from isolate 9625 are presented in Table III. As in the case of the clones obtained from isolate 9127, all clones derived from plaques produced at 32° retained complete temperature sensitivity, indicating that this isolate also consisted of a heterogeneous populations of virions the majority of which were temperature sensitive. Of 4 clones isolated at 37°, 1 showed intermediate temperature sensitivity, while 3 others did not exhibit a significant difference in titer at the permissive and restrictive temperatures and were thus not distinguishable from wild type. Wild type-like clones were obtained at 38 and 39°.

Table IV shows the temperature-sensitivity profiles of clones derived from isolate 8519. In contrast to the results seen with the other two isolates, 3 of the 4 clones derived at 32° showed intermediate patterns of temperature sensitivity, while the 4th showed a phenotype indistinguishable from wild type. These results suggest that, unlike the other two isolates, isolate 8519 contained many virions whose temperature-sensitivity pattern was altered from that of the ts-1 vaccine virus. Four clones were isolated at 37°, 4 at 38°, and 2 at 39°. None of these clones showed a significant difference in titer between restrictive and permissive temperatures and thus could not be distinguished from wild type.

The above results indicated that the ts-1 vaccine virus had undergone genetic alteration. It was not immediately obvious, however, whether this alteration had occurred *in vivo* in the vaccinees or *in vitro* during the passage of the isolates in HEp-2 cells at permissive temperature. To discriminate between these two possibilities, 20 separate tube cultures of HEp-2 cells were inoculated with 10^4 $TCID_{50}$ of the ts-1 vaccine virus and incubated at permissive temperature until CPE were noted. The tubes were then harvested and a second passage in HEp-2 cells was performed. When the progeny populations of the second passage were tested for efficiency of plaque formation at restrictive temperatures, none of the 20 populations was different from the ts-1 vaccine. These results suggest that the genetic alterations previously observed probably occurred *in vivo* in the vaccinees.

Discussion. The data indicate that a temperature-sensitive mutant of RSV underwent genetic alteration during replication in

TABLE IV. Titers (PFU/ml) of Clones Derived from Plaques Produced by Isolate 8519 at Various Temperatures.

Temperature of plaque production	Clone No.	Titer (log_{10})			
		32°	37°	38°	39°
32°	1	4.8	4.3	<0.7	<0.7
	2	5.4	4.9	4.6	4.4
	3	5.1	4.3	4.1	3.7
	4	5.1	4.7	4.7	4.3
37°	1	5.1	4.9	4.6	4.5
	2	4.6	4.8	4.4	4.3
	3	5.1	5.2	4.9	4.6
	4	4.9	4.7	4.4	4.3
38°	1	4.8	4.7	4.4	4.0
	2	4.9	4.6	4.4	4.1
	3	5.2	5.0	4.4	4.6
	4	5.1	5.1	4.8	4.6
39°	1	5.3	5.2	4.9	4.7
	2	4.9	4.7	4.2	4.9

man. Clones of virus from the tissue-culture-grown progeny of isolates recovered from individuals infected with the mutant exhibited a loss of temperature sensitivity which in most instances was not complete. Three sorts of events may have brought about these results. First, the temperature-sensitive phenotype of ts-1 might have been due to several different mutations. Reversion ("back mutation") of one or more of these mutations might have resulted in a decrease, but not a complete loss of temperature sensitivity. Analysis of the data does not provide much support for this explanation. Clones with a phenotype similar to wild type, i.e., putative revertants, were isolated not infrequently at restrictive temperature. Were the temperature sensitivity of ts-1 dependent on several reversion mutations, we would expect reversion to wild-type phenotype to occur far less frequently than alteration to an intermediate sensitivity since true reversion would require back mutation to occur simultaneously at 2 or more loci. On the contrary, 3 of the 13 phenotypically altered clones of isolate 9127 produced at 37 or 38° were wild type-like in temperature sensitivity (Table II). Further, 9 of 12 clones of isolate 9625 which were picked at 37, 38, or 39° had a wild-type phenotype. Finally, 1 of the 4 clones of isolate 8519 produced at the nonselective temperature of 32° was like wild type in temperature sensitivity.

A second possible mechanism which might explain the results is a second mutation at the same locus as the original mutation of ts-1. Such a mutation might produce a gene product less temperature sensitive than the ts-1 gene product, but more temperature sensitive than the wild-type gene product. From the data, this explanation cannot be ruled out. However, as the intermediate patterns of temperature sensitivity differed among themselves, several different sorts of mutation at the same locus would have had to occur. This would require of the locus a rather strong tendency toward mutation.

A third possibility is the occurrence of a second (suppressor) mutation which was distinct from the original mutation and which left it intact. Such a mutation might partially correct the temperature-sensitive lesion brought about by the original mutation (5). Suppressor mutations have been noted in *Drosophila* (6), and their significance in bacterial and phage genetics has been reviewed recently (7). In classical genetics, the presence of a suppressor mutation is demonstrated by recovering the original mutant type from the progeny of a back-cross between a putative suppressor mutant and the wild type. This can occur because, as noted, the suppressor mutation leaves the original mutation intact. Unfortunately, such a demonstration requires the exchange of genetic material. This molecular mechanism has not yet been demonstrated with RSV (8) and, save for poliovirus, has not been demonstrated to occur with any of the RNA animal viruses whose genome is not segmented.

The means by which a suppressor mutation might alter an existing mutation are many (7). Briefly, such a mutation might (a) produce a gene product capable of substituting for the product of the original mutation, (b) open alternate pathways for the synthesis of viral products, (c) alter cellular conditions in such a way as to circumvent the defectiveness of the product of the original mutation, or (d) make a second alteration in the same product (intracistronic suppression) which would serve partially to normalize the function of this product. Alterations in the way genetic information is transformed into structural proteins might be a further possibility. In the current state of knowledge of the molecular biology of RSV, it is not possible to decide amongst these possibilities.

Possible suppressor mutations in cloned animal virus mutants have not, to our knowledge, been formally commented upon. It would appear, however, that such mutations might have occurred in the case of the ts mutants of vesicular stomatitis virus reported by Cairns *et al.* (9). These workers found that the virion-associated RNA polymerases of certain ts mutants were ineffective at restrictive temperatures. A number of revertant clones, which had reacquired the ability to replicate at restrictive tem-

perature, had a polymerase whose activity at restrictive temperature was intermediate between that of the stable mutants and that of wild type.

Summary. In the course of a clinical trial of a temperature-sensitive mutant RSV vaccine, a proportion of the virus recovered exhibited genetic alteration. Some of the altered virus showed a temperature-sensitivity pattern intermediate between that of the temperature-sensitive vaccine virus and the temperature-insensitive wild type. Possible genetic mechanisms which might have produced these findings are discussed.

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1. Kim, H. W., Arrobio, J. O., Brandt, C. D., Wright, P. F., Hodes, D. S., Chanock, R. M., and Parrott, R. H., Pediatrics **52**, 56 (1973).

2. Gharpure, M. A., Wright, P. F., and Chanock, R. M., J. Virol. **3**, 414 (1969).

3. Wright, P. F., Woodend, W. G., and Chanock, R. M., J. Infect. Dis. **122**, 501 (1970).

4. Wright, P. F., Mills, J. V., and Chanock, R. M., J. Infect. Dis. **124**, 505 (1971).

5. Sturtevant, A. H., Proc. Soc. Exp. Biol. Med. **17**, 70 (1920).

6. Beadle, G. W., and Ephrussi, B., Proc. Nat. Acad. Sci. USA **22**, 536 (1936).

7. Gorini, L., and Beckwith, J. R., Ann. Rev. Microbiol. **20**, 401 (1966).

8. Wright, P. F., Gharpure, M. A., Hodes, D. S., and Chanock, R. M., Arch. Ges. Virusforsch. **41**, 238 (1973).

9. Cairns, J. R., Hollaway, A. F., and Cormack, D. V., J. Virol. **10**, 1130 (1972).

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