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Chemically Induced Temperature-Sensitive Mutants of Dengue Virus Type 2: Comparison of Temperature Sensitivity In Vitro with Infectivity in Suckling Mice, Hamsters, and Rhesus Monkeys

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A series of temperature-sensitive (*ts*) mutants of dengue virus type 2 (DEN-2, TH-36 isolate) were induced by treatment with 5-azacytidine. These mutants and parental viruses were compared for the *ts* trait and/or attenuation in four systems: primary hamster kidney cells, suckling mice, golden Syrian hamsters, and rhesus monkeys. Seven clones judged to possess the *ts* trait in vitro demonstrated a variety of patterns in vivo. On initial isolation, five of seven *ts* mutants exhibited reduced mouse lethality. The remaining two mutants possessed parental levels of mouse lethality. In hamsters, neither *ts* mutant nor parental viruses replicated very well, and then only when inoculated intracerebrally. Studies in rhesus monkeys indicated that all seven *ts* clones and parental viruses were capable of inducing antibody responses; however, *ts*-1 and *ts*-2 failed to produce detectable viremia. After challenge with parental virus, all vaccinated monkeys demonstrated rapid secondary-type antibody responses. Reversion from *ts* to ts^+ was confirmed for *ts*-1 in mice and *ts*-3 in monkeys, and was strongly suspected in several other instances.

The four known serotypes of dengue (DEN) viruses represent significant human health problems in numerous tropical and semitropical areas (for recent reviews, see 8, 14). Vector eradication has been the only available method of control (11, 16), but it has been difficult to maintain, except in relatively isolated areas (11, 16, 24). An alternative control measure might be active immunization.

It has been clearly shown that inactivated DEN virus vaccines are ineffective (3, 18, 31, 43). Live, attenuated type 1 and type 2 DEN virus vaccines have been derived from virus passaged extensively in mouse brain and/or cell culture (17, 28, 33, 35, 43). In man, inoculation of vaccine virus produced significant neutralizing antibody (18, 35, 43) and, where examined, resistance to homologous (18, 33) and possibly heterologous (2) virulent virus challenge. By present standards, however, virus strains that have been extensively mouse passaged are probably unacceptable as human vaccines.

We have attempted to develop alternative methods of developing candidate vaccine strains. We chose to approach the problem through the isolation of temperature-sensitive (*ts*) mutants (40a). This was prompted by: (i) the frequent association of temperature sensitivity and reduced virulence observed with several licensed and experimental live, attenuated vaccine viruses (1, 6, 7, 9, 19-21, 25, 27, 32) and several experimental host-virus systems (4, 36-39, 41); and (ii) the virtual lack of animal models of DEN virus diseases. DEN virus infection of laboratory animals does not produce disease similar to that in humans, although several primate species have been shown to develop viremia and antibodies after peripheral inoculation of any of the DEN serotypes (11, 12, 34, 42). Since temperature sensitivity represents, at least theoretically, a host-independent marker of attenuation, behavior of *ts* mutants in vivo could give indications of degree of attenuation and/or stability of the *ts* phenotype, and of their potential efficiency in active immunization in the absence of totally relevant models of the human DEN diseases.

MATERIALS AND METHODS

Cells. Primary hamster kidney (HK) cells were prepared as described (30) or purchased as a suspension of 10^6 cells/ml (GIBCO, Grand Island, N.Y.). Cultures were grown in LAH medium consisting of 5 mg of lactalbumin hydrolysate per ml (GIBCO), 50

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μg of penicillin per ml, 50 μg of streptomycin per ml, 100 μg of neomycin per ml, 25 U of mycostatin per ml, 30 mg of glutamine per ml, and BME vitamins (Microbiological Associates, Bethesda, Md.) in Hanks balanced salt solution, supplemented with 5% (vol/vol) newborn bovine serum (GIBCO) and $NaHCO_3$ (0.8 or 2 mg/ml for closed systems or CO_2 incubator, respectively).

Viruses. The origin of DEN-2, TH-36 isolate, and the *ts* mutants derived from it has been described (40a). Briefly, P_4 represents parental virus in its fourth serial mouse brain passage; P_3T_{11} represents virus after receiving three mouse brain passages and eleven passages in HK cells at 28 C. All mutants and wild-type (ts^+) clones were obtained from the P_3T_{10} stock grown in the presence of the mutagen 5-azacytidine (25 or 100 μg/ml).

Cloning and infectivity titrations were performed by direct immunofluorescent techniques as previously described, using fluorescein-conjugated globulin from mouse anti-DEN-2 immune ascites fluids (23). Fluorescent focus-forming units (FFU) assays of infectivity were performed at 33.5 C (permissive temperature) and 40 C (nonpermissive temperature). The difference between the titers at these two temperatures was taken as an indication of the degree of the *ts* trait exhibited by a given virus clone. These differences were confirmed virus yield experiments performed at 33.5 and 40 C (40a).

Rodents. Pregnant HaM/ICR outbred Swiss mice were obtained from Charles River Breeding Laboratories, Wilmington, Mass. Litters were utilized for mouse lethality studies at 1 to 2 days of age. Suckling mouse intracerebral mean lethal dose ($SMICLD_{50}$) was determined as described by Reed and Muench (29). Male 40- to 50-g weanling Syrian golden hamsters (Lakeview Hamster Colony, Newfield, N.J.) were utilized for cell culture preparation and for experimental infection studies.

Rhesus monkeys. Before their selection and shipment, sera from juvenile female rhesus monkeys (4 to 6 lb [about 1.8 to 2.7 kg], Primate Imports, Port Washington, N.Y.) were screened for the presence of hemagglutination-inhibiting (HI) and complement-fixing antibody against DEN-2 (TH-36), JBE, West Nile, Murray Valley encephalitis, and Langat viruses, and for neutralizing antibody against DEN-2 (TH-36) virus (15). Monkeys selected for subsequent studies were free of detectable antibody in the above tests. All monkeys were initially free of tuberculin reactivity and remained free during the experimental period.

Viremia. Pairs of monkeys were inoculated subcutaneously in the forearm with 0.1 ml of a given mutant or parental virus stock. Second HK passage stocks of the mutants were used. As discussed below, the passage levels of *ts*-4 and *ts*-6 used for monkey studies behaved as if both viruses had reverted to ts^+. The monkeys were bled at intervals by femoral venipuncture. Sera collected on days 3 through 7 after inoculation were assayed for infectious DEN virus. Sera were diluted 1:5 and 1:50 in sucrose phosphate-gluconate (15), and 0.01-ml quantities of the respective dilutions were inoculated intracerebrally into suckling mice (eight littermate suckling mice per dilution). The mice were observed for 21 days for death. Selected sera were also inoculated at 1:5 or 1:10 dilution (in LAH medium) onto HK monolayers.

HI tests. Sera collected 7, 13, 20, and 32 days after infection were acetone extracted and assayed for HI antibody by a microtechnique test as described by Hammon and Sather (15). Twofold dilutions of test sera were made in 0.4% bovine albumin in borate-buffered saline and mixed with sucrose acetone-extracted DEN-2 (TH-36) antigen. A goose erythrocyte suspension in dextrose-gelatin-veronal buffer was added as the indicator of hemagglutination. The HI titer was the highest dilution of serum that caused partial inhibition of hemagglutination.

RESULTS

Relationship between mouse lethality and the ts trait. This problem was examined at several levels. Figure 1 shows a comparison of the *ts* trait ($\log_{10} FFU_{40\ C}/FFC_{33.5\ C}$, mean of one to five determinations) with the mouse lethality trait ($\log_{10} SMICLD_{50}/FFU_{33.5\ C}$) for each of 112 clones in first HK cell passage and two parental viruses. The process of adaptation of mouse brain virus to cell culture during 11 serial passages at 28 C may have caused small but reproducible changes in the mouse lethality trait, although P_4 does not appear to be significantly less temperature sensitive than P_3T_{11}. The P_3T_{11} virus pool is more or less representative of the 105 ts^+ clones shown in Fig. 1.

When the data for 112 clones were examined by linear regression analysis, a statistically significant degree of correlation was observed between the two traits ($r = 0.359$, $P < 0.001$). However, similar analysis performed on the 105 ts^+ clones showed no correlation between the two traits ($r = 0.0003$). This finding is further explored in Table 1, in which the distributions of the mouse lethality and *ts* traits are compared. The values of the *ts* trait for the 105 ts^+ clones are normally distributed ($P > 0.05$), as might be expected of randomly sampled members of a population homogeneous for the ts^+ trait. These 105 ts^+ clones manifested a mean value of -1.0 ± 0.3 for the ts^+ trait, whereas the standard deviation for multiple determinations on single clones varied from 0.1 to 0.4. The mean value for the 7 *ts* mutants was -2.2 ± 0.4; the standard deviation for multiple determinations or single clones ranged from 0.2 to 0.5. These *ts* mutants also demonstrated reduced capacity to replicate at 40 C (40a).

In Fig. 1, seven clones were considered ts^+ and yet had $\log_{10} (FFU_{40\ C}/FFU_{33.5\ C})$ values <-1.5. Results of repeated titrations of these clones varied, sometimes indicating temperature sensitivity, sometimes indicating a ts^+ clas-

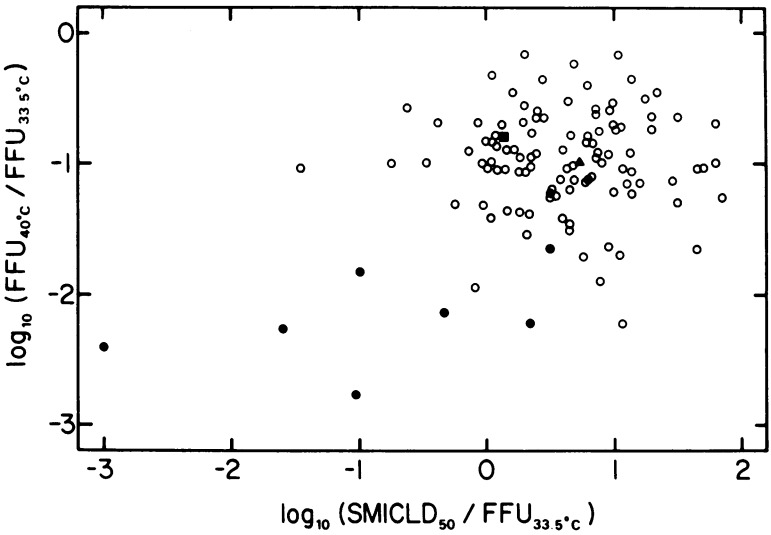

FIG. 1. *Mouse lethality and ts trait values for 112 clones of DEN-2 virus. Open circles designate clone considered ts⁺; closed circles indicate ts mutant clones. The square identifies the P_4 virus, and the triangle identifies the P_3T_{11} virus.*

TABLE 1. *Distribution of ts and mouse lethality trait values among 105 wild-type clones of DEN-2 virus*

Log_{10} ($FFU_{40°C}$/ $FFU_{33.5°C}$) value[b]	Log_{10} ($SMICLD_{50}/FFU_{33.5°C}$) value[a]						Total	ϵ_N[c]
	$<-2\sigma$	$<-1\sigma$	<0	>0	$>1\sigma$	$>2\sigma$		
$<-2\sigma$	0	0	0	0	0	0	0	2.5
$<-1\sigma$	1	1	1	3	1	2	9	15.0
<0	9	5	6	6	3	10	39	32.5
>0	10	7	3	4	8	8	40	32.5
$>1\sigma$	1	3	1	3	2	3	13	15.0
$>2\sigma$	2	0	0	0	1	1	4	2.5
Total	23	16	11	16	15	24	105	(>0.05)[d]
ϵ_N	2.5	15.0	32.5	32.5	15	2.5	(<0.0001)[d]	
ϵ_R[c]	18.45	18.45	18.45	18.45	18.45	18.45	(>0.10)[d]	

[a] Used as measure of mouse lethality trait.
[b] Used as measure of *ts* trait.
[c] Expected value based on assumption of normal distribution.
[d] Probability that the trait actually possesses the distribution predicted by the expectation function ϵ_x, employing the χ^2 frequency test.
[e] Expected value based on random distribution.

sification. However, in subsequent experiments, these clones showed no significant yield reduction yield when grown at 40 C relative to 33.5 C (data not shown). We have therefore conservatively classified these seven clones as *ts*⁺.

In contrast to the normally distributed values for the *ts* trait, the values of the mouse lethality trait for the 105 *ts*⁺ clones are randomly ($P > 0.10$) rather than normally ($P < 0.0001$) distributed. This strongly implies that the two traits are independent of each other in *ts*⁺ clones. This was confirmed by χ^2 contingency analysis ($P > 0.975$). Yet the *ts* trait appears to be accompanied initially by reduced value of the mouse lethality trait (Fig. 1).

This suggested relationship between the *ts* trait and reduced mouse lethality was investigated further. Table 2 compares the values of the two traits for the first and second passages of the *ts* mutants in HK cells. Interestingly, *ts*-1 appeared to completely regain its mouse lethality during T_2 but remained *ts* by titration (Table 2) and yield reduction methods. However,

TABLE 2. *Effect of passage upon values of the ts trait and the mouse lethality trait for ts, ts⁺, and parental viruses*

Virus	Log_{10} ($SMICLD_{50}$/$FFU_{33.5\ C}$)		Log_{10} ($FFU_{40\ C}$/$FFU_{33.5\ C}$)	
	t_1[a]	t_2	t_1	t_2
ts-1	−3.0	+0.1	−1.6	−1.7
ts-2	−0.4	−0.1	−2.2	−2.0
ts-3	−1.6	−1.6	−2.3	−2.2
ts-4	+0.5	+0.4	−1.6	−0.7
ts-5	−1.0	−1.0	−2.9	−0.7
ts-6	+0.4	+0.3	−2.3	−0.4
ts-7	−1.0	−0.6	<−1.6	<−1.1
wt-1(*ts*⁺)	0	ND[b]	−1.1	ND
wt-2(*ts*⁺)	+0.3	ND	−0.4	ND
P_3T_{11}	+0.7		−1.0	
P_4	+0.1		−0.8	

[a] t_1 and t_2 refer to first and second HK cell passage levels, respectively.
[b] ND, Not done.

ts-5 and *ts*-7 are probably still *ts* by yield reduction experiments (40a). It is also interesting that *ts*-4 and *ts*-6, the only clones which reverted to all examined parental traits, alone possessed positive (parental type) values of the mouse lethality trait at initial isolation (t_1). This potential relationship is of unknown validity or importance.

Other facets examined included the fate of the *ts* trait during virus replication in mouse brain (Table 3). The infected brain materials demonstrated values of the *ts* trait that were intermediate between those of the inoculum (last row of Table 3) and *ts*⁺ viruses (Table 2). Therefore, virus was cloned by terminal dilution from a number of these harvested, infected brains. The results shown in Table 4 indicate that only one of the 11 clones isolated was actually *ts*. Nine of the 10 remaining clones were clearly *ts*⁺. This suggests that *ts*-1, and probably other *ts* mutants, tended to revert upon replication in mice. Additionally, the relationship between survival time index and log_{10} $SMICLD_{50}$ (23) was similar for both *ts* mutants and *ts*⁺ viruses (unpublished observations). This implies that the intrinsic properties (but not necessarily the relative abundance) of the lethal virus particles in *ts* mutant- and *ts*⁺-infected mouse brains were similar.

Infection of weanling hamsters. P_4 and P_3T_{11} viruses replicated to a limited extent ($10^{2.5}$ $SMICLD_{50}$/mg of brain) in weanling hamster brains, even if cyclophosphamide immunosuppression was used (40; unpublished observations). No disease was observed in normal or immunosuppressed animals. Small amounts of DEN-2 neutralizing antibody were detected 14 days after infection. Inoculation of either virus by subcutaneous or intraperitoneal route into normal or immunosuppressed animals failed to cause detectable disease, viremia, intracerebral virus replication, or antibody production.

Hamsters were inoculated intracerebrally with the *ts*-1 (t_1 or t_5) or *wt*-3 (*ts*⁺) mutant

TABLE 3. *Effect of growth in suckling mouse brain on the ts phenotype of ts-1 virus*

Day harvested[a]	Log_{10} FFU/mg of brain assayed at temp (C):		
	33.5	37	40
1	<1.0	<1.0	<1.0
2	<1.0	<1.0	<1.0
3	2.0	1.7	<1.0
4	2.4	2.2	1.6
5	3.0	2.8	1.7
6	4.6	4.0	3.5
7	5.0	4.6	3.8
8	4.2	3.8	3.4
9	5.0	4.4	3.8
10	3.5	3.5	2.2
11	4.5	4.1	3.1
ts-1[b]	3.6	2.5	1.1

[a] Pairs of brains were harvested at time shown, triturated, and made 10% (vol/vol) in buffered saline. Dilutions of this material were made in LAH medium.
[b] Same stock as the inoculum.

TABLE 4. *Infectivity, measured at two temperatures, of virus clones isolated from brains of mice infected with ts-1 virus*[a]

Day[b]	Clone no.	Log_{10} FFU/0.1 ml assayed at temp (C):		Log_{10} $FFU_{40\ C}$/$FFU_{33.5\ C}$	Phenotype[c]
		33.5	40		
3	1	2.7	2.3	−0.4	*ts*⁺
	2	2.8	1.9	−0.9	*ts*⁺
	3	3.3	1.5	−1.8	*ts*⁻
	4	3.6	2.2	−1.4	*ts*⁺
4	1	3.4	2.4	−1.0	*ts*⁺
6	1	3.4	3.1	−0.3	*ts*⁺
	2	3.2	2.6	−0.6	*ts*⁺
	3	3.4	3.0	−0.4	*ts*⁺
9	1	2.1	1.5	−0.6	*ts*⁺
	2	3.0	2.7	−0.3	*ts*⁺
	3	1.3	1.3	0	*ts*⁺

[a] Infected brain material was from pools described in Table 3.
[b] Days postinoculation on which brain material was harvested.
[c] Determined by using the 95% confidence limits about the respective $D_{40/33.5}$ values for *ts*-1 (Table 1) and average wild-type clones. The approximate division between *ts*⁺ and *ts*⁻ is a value of −1.60, which lies at a point slightly greater than two standard deviations above the mean for *ts*⁺ wild-type clones.

clones. Although all three viruses appeared to replicate intracerebrally, neither disease nor detectable serum antibodies were observed. None of the mutant, wild-type, or parental viruses produced detectable disease, viremia, or antibody after intraperitoneal inoculation. Thus, the *ts*, *ts*$^+$, and parental viruses tested did not acquire hamster neurovirulence by repeated passage in HK cell culture.

Infection of rhesus monkeys. Pairs of seronegative monkeys were inoculated with various DEN-2 *ts* mutants or parental viruses as described above. The pattern of viremia observed after virus inoculation is shown in Table 5. Mutant clones *ts*-1 and *ts*-2 failed to produce detectable viremia. The remaining *ts* mutants and parental viruses all produced at least some viremia detectable in suckling mice. Attempts to isolate virus in HK cells from numerous aliquots of all sera collected were negative except for the day-6 serum from a *ts*-3-infected monkey. The three clones isolated from this sample were titrated at 33.5 and 40 C and found to have values for the *ts* trait similar to those of *ts*$^+$ virus (−1.0 to −0.3). Repeated attempts with other sera (positive or negative in mouse assay) were uniformly negative in HK cells. Our pretest screening for flavivirus antibody in sera from these monkeys did not include a search for heterologous dengue antibody. Thus, viremia may have been suppressed by low levels of antibody to DEN-1, -3, or -4 that were not detectable in the screening tests used. However, the HI antibody response of the monkeys, after immunization with either mutant or parental virus, appeared to be of the primary type. Further, viremia was detected even in monkeys inoculated with the low-titered *ts*-7 virus. If all four monkeys inoculated with *ts*-1 or *ts*-2 possessed sufficient preexisting antibody to neutralize over 500 times the inoculum which produced viremia in *ts*-7-infected animals, then this antibody level would presumably have been detectable in preinfection bleedings. These data strengthen our conclusion that *ts*-1 and *ts*-2 viruses are likely attenuated in their ability to produce viremia in monkeys.

TABLE 5. *Viremia detected in monkeys inoculated with DEN-2 virus ts mutant or parental strains*

Virus	Inoculum (log_{10} $FFU_{33.5\ C}$/0.1 ml)	Viremia on day:				
		3	4	5	6	7
ts-1	4.3	0[a]	0	0	0	0
-2	4.3	0	0	0	0	0
-3	4.6	0	0	+[b]	+	+
-4	4.9	0	+	+	+	+
-5	3.4	0	0	+	+	+
-6	4.5	0	0	+	+	+
-7	1.6	0	0	0	+	+
P_3T_{11}	4.3	0	0	+	+	+
P_4	6.3	+	0	+	+	0

[a] No detectable viremia in either monkey.

[b] Positive viremia in at least one monkey; log_{10} $SMICLD_{50}$/0.01 ml infectivity titers were usually 0.5 to 1.0.

Table 6 shows that all inoculated monkeys developed antibody to varying degrees. It is most significant that monkeys infected with *ts*-1 or *ts*-2 developed antibody without detectable viremia. Six weeks after the primary infection, the monkeys were challenged subcutaneously with 8.0×10^5 FFU of P_4 virus. Results of HI tests on postchallenge sera (i.e., sera collected on days 49 and 56) are shown also in Table 6. With the exception of one monkey that had been infected with *ts*-3 virus, all monkeys developed a significant antibody response after P_4 virus challenge. The unimmunized control monkeys responded with much lower titers, as expected in a primary response pattern.

TABLE 6. *HI antibody levels detected in sera of monkeys inoculated on day 0 with various ts mutant or parental DEN-2 viruses and challenged on day 42 with P_4 virus*

Virus inoculum	HI titer (geometric mean of 2 monkeys) on day:						
	7	13	20	32	42	49	56
ts-1	<10	640	ND[a]	320	320	1280	ND
-2	<10	40	ND	40	28	896	ND
-3	<10	320	ND	160	320	640	ND
-4	<10	225	112	ND	28	225	112
-5	<10	160	112	ND	40	225	160
-6	<10	56	40	ND	20	225	56
-7	<10	<10	28	ND	20	320	160
P_3T_{11}	<10	640	ND	ND	320	1280	ND
P_4	<10	320	ND	ND	160	2560	ND
Control	ND	ND	ND	ND	<10	80	ND

[a] ND, Not done.

Detection of viremia in sera from this P_4 virus challenge was attempted by intracerebral inoculation of suckling mice, immunofluorescent assay in HK cells at 33.5 C, and plaque assay on LLC-MK2 cells. All such attempts have been unsuccessful. It is feared that progeny virus may have been inactivated by mishandling of sera after collection, or, in the case of vaccinated monkeys, neutralized by preexisting antibody. It is unclear, therefore, whether the antibody responses in vaccinated monkeys indicate replication of the challenge virus or a booster response to the mass of viral protein injected. The latter possibility seems reasonable since Halstead et al. (13) observed two- to fourfold increases in HI antibody titers with no detectable concomitant viremia after homologous DEN virus challenge and since significant titer increases were detected even in P_3T_{11} and P_4 virus immunized monkeys after P_4 virus challenge.

DISCUSSION

The data presented here indicate that the relationship between the existence and expression of *ts* mutations and virulence in vivo is a complicated one. *ts* mutants often exhibited reduced values of the mouse lethality trait, but not always. Further, *ts*-1 at t_2 regained the parental mouse lethality trait without an apparent high frequency of reversion to ts^+. The 105 ts^+ clones examined also showed no relationship between the ts^+ trait and mouse lethality trait. It is concluded that temperature sensitivity and lack of mouse lethality may be associated under some conditions in a manner yet to be defined.

One possible explanation for this problem is that the *ts* mutants possessed distinct minimum temperatures, below which their *ts* mutations were not expressed. This critical temperature for some of the *ts* mutants might have occurred below 40 C but above animal body temperatures. Such mutant clones would have appeared *ts* in vitro but not in vivo. No evidence currently exists in this system to confirm or deny such a hypothesis.

The possibility of reversion to ts^+ in vivo complicates the problem considerably. Evidence of reversion in both the mouse (*ts*-1) and monkey (*ts*-3) have been presented. In view of the viremia evidenced by *ts*-5 and *ts*-7 viruses, these may also have reverted. However, no virus was isolated from monkey sera to evaluate this hypothesis. *ts*-4 and *ts*-6 were considered revertants based on in vitro data in Table 2, and behaved as such in monkeys. However, mutant *ts*-1 virus was also capable of replication in suckling mouse brain. Thus, no definitive conclusion can be drawn regarding the independence of the *ts* trait and replication and/or virulence in vivo for DEN-2 *ts* mutants. In other systems, data suggesting independence (4, 38) or covariation (4, 5, 10, 32, 36, 39) of the two traits have been presented.

Whereas the expression of the *ts* mutation in vitro has an obscure relationship to mouse lethality in vivo, the *ts* mutations had no discernible effect on virus replication in hamster brains. The same level and temporal pattern of virus replication was observed in *ts*-1 (t_1), of very low mouse lethality, and *wt*-3-infected hamsters. In contrast, *ts*-1 (t_2) did not produce viremia in monkeys. It is apparent that the replication of DEN-2 viruses in hamsters may differ in some as yet undefined manner, since suckling mouse, hamster, and monkey body temperatures are believed to be virtually identical (37.5 ± 0.5 C).

In spite of these dilemmas, it is possible that some *ts* mutants may have properties suitable for live attenuated vaccines. *ts*-1 and *ts*-2 clones failed to produce detectable viremia in monkeys, but did stimulate HI antibody production. The actual nature of the *ts* mutation seems to be of little importance. *ts*-1 and *ts*-2 are ribonucleic acid positive and ribonucleic acid negative at 40 C, respectively, and *ts*-7 belongs to the same complementation group as *ts*-1 (40a). Murphy et al. (26) concluded that the determining factor in the degree of attenuation appeared to be the severity of the *ts* defect. In view of these observations, the utility of a *ts* mutant clone as vaccine material must be evaluated independently and generally cannot be deduced from virological data alone. The effectiveness of a given *ts* mutant as a vaccine virus may depend heavily upon the "leakiness" of the mutation and the amount of virus-specific neutralizing antigen produced (41). It is concluded that the data presented give sufficient support to the potential usefulness of chemically derived DEN-2 *ts* mutants as vaccines to stimulate further serious feasibility studies for the other DEN virus serotypes.

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