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A Hamster-Attenuated, Temperature-Sensitive Mutant of Venezuelan Encephalitis Virus

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Pathogenicities of 10 temperature-sensitive mutants of Venezuelan encephalitis virus were studied using the hamster model of human virulence. The parental strain and nine of the temperature-sensitive mutants produced lethal infections in hamsters. Strain ts 126 showed reduced hamster virulence. Deaths with the lethal mutants usually occurred 1 to 3 days later than with parental virus. Nine mutants produced lower levels of viremia than parental virus. Attenuation of ts 126 was related to restriction of viral growth in spleen and probably bone marrow and to absence of the usual pathological lesions in hemopoietic tissues and brain, but was functionally unrelated to temperature sensitivity since temperatures of both normal and infected hamsters remained within the permissive range of the mutant. Deaths did not correlate with titers of the 10 mutants in blood at permissive temperatures or with reversions of four temperature-sensitive mutants to non-temperature-sensitive virus in hamsters.

Virulence and pathogenicity of Venezuelan encephalitis (VE) virus strains have been studied in hamsters as a model system (1, 6, 9, 10, 22). Numerous naturally occurring strains are virulent for hamsters inoculated subcutaneously (s.c.), but only three benign strains have been available for study, two isolated from nature and one selected by repeated passages in cell cultures (2, 16). In an attempt to find benign strains closely related genetically to virulent strains, 10 temperature-sensitive (ts) mutants were isolated after treatment of a non-ts strain of VE virus with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (B. A. Pancake, Z. P. Harsanyi, M. E. Wiebe, E. Emini, and W. F. Scherer, manuscript in preparation). These ts mutants were selected to produce plaques at permissive temperatures (PT) of 30 to 32°C but not at nonpermissive temperatures (NPT) of 39 to 40°C. To study pathogenicities of these mutants in vivo, hamsters were inoculated subcutaneously, intracardially, or intracranially and observed for illness, death, viremia, virus in tissues, histopathology, antibody development, and reversion of mutants to parental non-ts virus.

MATERIALS AND METHODS

VE viruses. Parental strain 68U201 was isolated from a sentinel hamster exposed at La Avellana on the Pacific coast of Guatemala in August 1968 (13). It was used in these experiments after three passages in suckling mice and four passages in primary cultures of chicken embryonic cells (CEC). Treatment of this virus with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and isolation of 10 ts mutants are described elsewhere (Pancake et al., manuscript in preparation). Mutants 3, 85, 121, and 126 were classified ribonucleic acid negative RNA^- (1), and 4, 40, 73, 127, 151, and 238 were classified RNA^+. Each ts mutant was used after two sequential clonings in CEC at PT and one or two further passages in fluid cultures of CEC at PT for amplification. Virus suspensions were fluids from CEC prepared as previously described (12). They were mixed with equal volumes of 1% bovine albumin in Hanks solution at pH 8.0 before storage at −60°C. Virus was assayed by counting plaques in 8-cm^2 sheets of CEC maintained under agar medium for 3 days (14). Cultures held at 39 to 40°C were placed in a water-jacketed incubator; for 30 to 32°C incubation a conventional incubator was used.

Inoculation, observation, and autopsy of hamsters. Male Syrian hamsters (*Mesocricetus auratus*) were inoculated at 6 to 10 weeks of age with 0.2 ml s.c., 0.4 ml intracardially, or 0.05 ml intracranially. Doses of virus were determined by concurrent titration of inocula in CEC at PT. Hamsters were caged individually or in pairs receiving equal virus doses in an air-conditioned room with a 12-h light cycle, and they were observed for 3 weeks for illness or death. Blood samples were taken by intracardiac puncture using syringes wetted with heparin (200 U/ml). For tissue specimens, hamsters were killed by ether or chloroform anesthesia if not dead from virus infection; tissues were removed aseptically, and weight/volume suspensions were prepared in bovine albumin-Hanks solution containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Blood (undiluted or diluted 1:10 in bovine albumin-Hanks solution) and supernatant fluids removed from tissue suspensions after centrifugation at 10,000 × *g* for 10 min at 5°C were stored at −60°C; plasma samples were stored at −20°C.

For histopathological examination, tissues were fixed in 10% or 20% Formalin, decalcified in 7% nitric acid if necessary, embedded in paraffin, and stained with hematoxylin and eosin.

Body temperatures of hamsters were taken rectally using one clinical thermometer calibrated daily in a water bath. Hamsters were held as gently as possible to minimize movement that might affect body temperatures. Temperatures were taken usually in the morning, but sometimes during the afternoon.

VE antibody tests. Plasmas from surviving hamsters were tested for hemagglutination inhibition or plaque-reduction neutralizing antibodies by methods previously recorded (4, 15). VE strain 63U2 was employed for hemagglutination inhibition tests, and strain 68U201 was used for neutralization tests.

Determination of the maximal PT of VE ts mutant 126 in cultured hamster cells. Primary cultures of hamster embryonic cells were prepared as previously recorded (14). Cells attached to the flat bottoms of screw-capped glass vials (15 by 48 mm) were infected with VE ts mutant 126 5 or 6 days after initiation of cultures by adsorbing 7,000 plaque-forming units (PFU) in CEC to approximately 70,000 cells for 1 h at 30, 37, 38, or 40°C. Cells were then covered with 0.8 ml of 1% calf serum in maintenance solution (14). Vials were incubated with the bottom half in a water bath regulated to 37 or 38 ± <0.1°C by use of a constant temperature circulator (model E52; Haake Inc., Saddle Brook, N.J.). Other vials were also incubated in a water-jacketed incubator at 40°C and in a solid-wall incubator at 30°C. Vials were withdrawn at time 0 and after incubation for 8 or 24 h; cultural fluids were pooled from three vials, mixed with equal volumes of bovine albumin-Hanks solution, and stored at −60°C until virus was assayed.

RESULTS

Lethalities of 10 ts mutants of VE strain 68U201 for hamsters. Male hamsters 6 to 8 weeks of age died after s.c. inoculation of 100 to 6,300 PFU of 9 out of 10 different ts mutants or of parental virus, strain 68U201 (Table 1). Nine of nine hamsters died 2 days after receiving parental virus, and all of two or three hamsters died 3 to 5 days after inoculation of seven mutants (ts 3, 40, 73, 85, 121, 127, and 238) (except for one hamster that died on day 10 with mutant 73). With one mutant (ts 4), six of seven hamsters died (five on day 5 and one on day 12), and with another mutant (ts 151), five of eight hamsters died (four on day 5 and one on day 15). Additional hamsters died after inoculation of 0.8, 8, 80, and 800 PFU of ts 151 (1/1, 2/3, 1/4, and 3/3, respectively). The hamster that survived with ts 4 and three survivors with ts 151 were infected, because they had detectable virus in blood on days 1 and 2 after inoculation. In the tables and text below, these nine ts mutants will be referred to as lethal, recognizing that, in some instances, revertant virus (discussed subsequently in the text) may contribute to the lethal process.

TABLE 1. *Viremia levels in hamsters inoculated s.c. with parental VE virus or ts mutants*

VE virus	No. of hamsters tested[a]		Log_{10} PFU[b] per ml of blood			
	Dead	Surviving	Mean		Range	
			day 1	day 2	day 1	day 2
Parent	6		8.8		8.1–9.4	
68U201	3			7.8		7.1–8.7
ts mutants						
3	3		3.8	6.1	3.7–4.0	5.6–6.4
4	6		3.5	4.5	<1.4–4.9	2.6–6.7
4		1	2.0	4.5		
40	3		6.0	7.2	5.6–6.3	7.0–7.5
73	3		4.4	6.5	3.7–4.8	6.0–6.7
85	2		4.2	5.4		5.3–5.5
121	3		4.9	7.3	3.6–5.9	6.2–8.4
127	2		4.8	6.9	4.5–5.0	5.9–7.8
151	5		4.2	5.8	3.2–5.4	5.5–6.1
151		3	4.2	4.9	2.4–5.5	2.4–6.2
238	3		>6.7	7.9		7.6–8.4
126	1		3.1	6.5		
126		9	4.2	5.4	3.1–5.3	4.0–6.0

[a] Doses of viruses inoculated = 100 to 6,300 PFU per hamster, s.c. Surviving animals developed detectable hemagglutination inhibition or plaque-reduction neutralizing antibodies in plasma by 22 to 23 days after inoculation.

[b] PFU were measured at PT.

The tenth mutant (ts 126) was of reduced virulence for hamsters since only 1 of 10 hamsters died (on day 8 after s.c. inoculation). The nine surviving hamsters were infected, because they had detectable viremias on days 1 and 2 after inoculation. Doses of ts 126 as large as 40,000 and 2,000,000 PFU each infected six of six hamsters inoculated s.c., but only one died (with 2,000,000 PFU). In contrast, 6 and 63 PFU of parental strain 68U201 killed three of three and four of four hamsters, respectively.

When ts 126 was inoculated intracardially in a large dose (8,000,000 PFU) into hamsters weighing 130 to 140 g, only one of six died (on day 2), and another became ill on day 3 and was sacrificed on day 5. Virus recovered from heart and brain tissues of both hamsters was ts. Rapid clearance of ts 126 virus did not occur in blood of the sick hamster or of two survivors; concentrations of virus in blood of each of these three hamsters at 70 min after intracardial inoculation were only $10^{-0.2}$, $10^{-0.7}$, and $10^{-0.1}$ of the 10-min values of $10^{5.4}$, $10^{5.5}$, and $10^{5.3}$ PFU/ml. The four surviving hamsters had detectable VE hemagglutination inhibition antibody (to strain 68U201) in plasmas 29 days after inoculation.

After intracranial inoculation of ts 126 into hamsters 8 weeks of age, three of seven died on days 4, 9, and 12 at a dose of 20,000,000 PFU, three of eight on days 4, 12, and 13 at a dose of 160,000 PFU, and zero of eight with a dose of 200 PFU. However, there were no histopathological lesions in hemopoietic or brain tissues of two hamsters examined after deaths on days 4 and 9 with a dose of 20,000,000 PFU; spleens, bone marrows, and cerebrums were normal, and Peyer's patch and thymus available from one hamster, and lung from the other, were normal. Revertants to non-ts virus were ≤2.5% of the total virus in brains of these two hamsters and of the one dying 4 days after 160,000 PFU. Thus, deaths of these hamsters remain unexplained. The hamsters that survived inoculation with 200 PFU were not infected, since plasmas obtained 19 days after inoculation contained no detectable plaque-reduction neutralizing antibody at 1:5 dilution using parental strain 68U201, and the hamsters subsequently died within 2 to 4 days of s.c. challenge with 100 PFU of parental strain 68U201. In contrast, nine hamsters surviving the larger intracranial doses of ts mutant 126 survived a similar challenge with strain 68U201 on days 19 to 21.

Growth of ts mutants of VE virus in hamsters inoculated s.c. Concentrations of virus in blood were measured 1 and 2 days after inoculation as a manifestation of viral growth in hamsters. Means of virus titers in bloods measured at PT were usually 10^3 to 10^6 PFU/ml on day 1 and 10^5 to 10^8 on day 2 (Table 1). In contrast, titers of parental strain 68U201 were 10^7 to 10^9 on these days. Viremia titers of attenuated ts mutant 126 were in the same range as some of the lethal ts mutants (Table 1).

In previous studies of other strains of VE virus in hamsters, viremia titers were usually as high or higher than virus titers in tissues until day 3 or 4 after inoculation, after which viremia titers usually began to decrease and titers of virus in host tissues were sometimes higher than in blood (10). Therefore, to distinguish between replication of ts 126 in tissues and mere contamination of tissues with virus in blood, virus concentrations were measured in tissues and blood on days 3 to 5 after inoculation, i.e., after the period of initial viremia produced by this mutant on days 1 and 2 (Table 1). Infectious ts 126 virus was detectable in blood of only one of eight hamsters on day 3 and in none of three on days 4 and 5 (Fig. 1). Titers of virus in Peyer's patches and adjacent ileum were significantly higher than in blood on days 3 to 5, as were titers in brain and kidney on days 3 and 4 (Fig. 1). In contrast, virus was undetectable in spleen, bone marrow, liver, and skeletal muscle; for one hamster the titer of virus in spleen on day 3 was similar to that in blood. Because only small quantities of marrow could be obtained, virus tests of marrow could not be performed at dilutions as low as other tissues. Only ts virus was detectable in single suspensions of Peyer's patches, brain, or kidney from day 3 or 4. Four hamsters that had no detected viremia on day 3 were kept until day 24 and bled; their plasmas contained VE plaque-reduction neutralizing antibody at 1:4 dilution ($\log_{10}$ neutralization indexes, >2.7 versus parental VE strain 68U201).

Lethal ts 4 was tested as a positive control in case no replication of ts 126 in tissues had been found. Viremia titers with ts 4 were too high on day 3 to permit evaluations of virus titers in tissues. However, on day 4 after inoculation, virus titers were higher in Peyer's patches and adjacent ileum, spleen, bone marrow, brain, and kidney than in blood and were similar to or below blood levels in liver and skeletal muscle (Fig. 1). These patterns of replication in tissues were like those seen with other hamster-lethal strains of VE virus (1, 6, 10). In this experiment, reversions of ts mutant 4 occurred; virus titers of bloods from each of six hamsters on day 3 and two on day 4 were as high at NPT as at PT.

Reversion of VE ts mutants to non-ts virus in hamsters. To assess further the frequency of reversion of VE ts mutants during growth in hamsters, blood samples 2 days after inoculation of each of the 10 ts mutants were titrated at PT and NPT. Reversions to non-ts virus did not occur uniformly, and deaths of hamsters did not always correlate with reversions (Table 2). For example, in this experiment with ts mutant 4, only one of six hamsters that died had revertant, non-ts virus in blood on day 2; similarly, with ts mutants 121, 151, and 126 and possibly 40 and 238, some hamsters that died had no detectable revertant virus in blood (Table 2). The reverse also occurred, i.e., hamsters survived but had revertant virus in blood (one hamster with ts mutant 4 and one with ts mutant 126) (Table 2). Thus in these experiments there were 41 deaths, but only 23 deaths with reversions. There were also two reversions among 27 survivors.

Revertant, non-ts virus was detected also in hamster tissues, though less frequently than in blood (Table 3). It was found more commonly and in more tissues of hamsters infected with lethal ts 121 than with attenuated ts 126.

Histopathological lesions in hemopoietic and brain tissues of hamsters inoculated with VE ts mutants 126 and 151. Lesions produced by virulent non-ts strains of VE virus

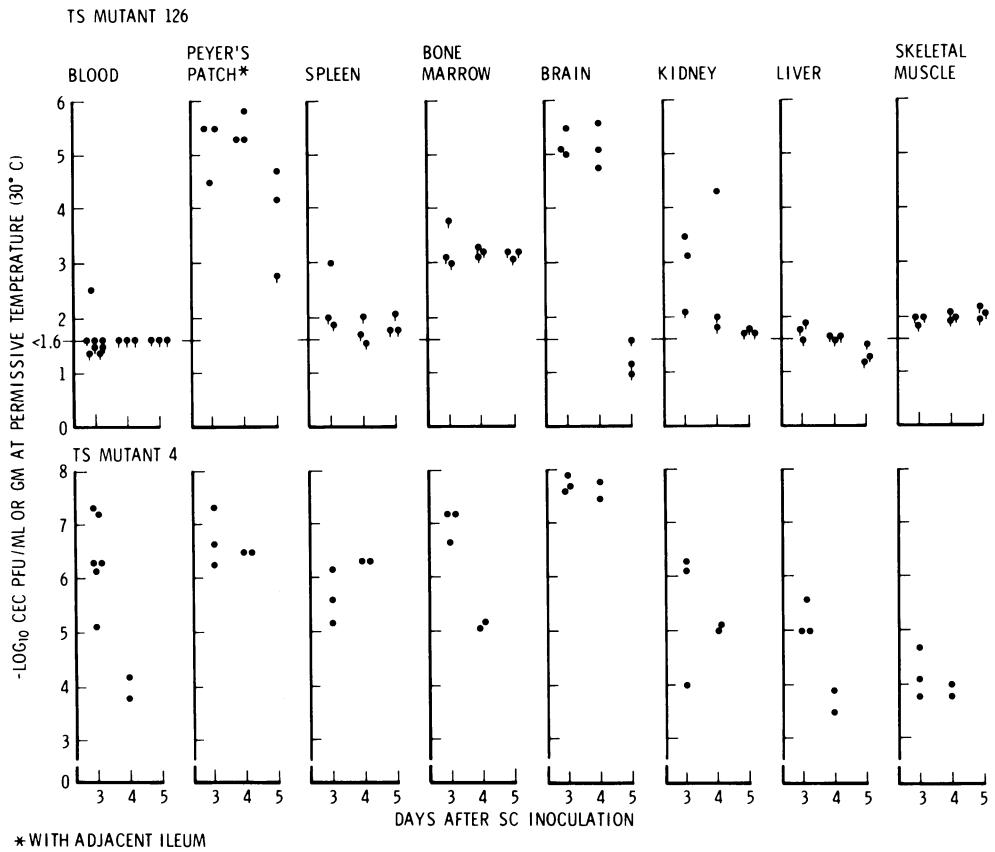

FIG. 1. *Concentrations of infectious VE virus in blood and tissues of hamsters inoculated s.c. with ts 126 or ts 4. Each dot represents one hamster, and a dot with a line descending from it indicates a value equal to or less than the value on the ordinate.*

in hamsters are located in hemopoietic tissues and brain and have been extensively described (1, 9). Therefore, histopathological studies of VE ts mutants were limited to the attenuated and one lethal ts virus.

Two hamsters sacrificed at 3 days and two sacrificed at 7 days after s.c. inoculation of 80 PFU of attenuated ts 126 were shown to be infected; concentrations of virus in blood on day 2 were 10^4 to $10^{6.4}$ PFU/ml. Yet bone marrow, spleen, brain, kidney, liver, jejunum, ileum, and skeletal muscle tissues were normal microscopically. One Peyer's patch examined from one hamster sacrificed on day 7 was also normal.

Two hamsters sacrificed 3 days after s.c. inoculation of 120 PFU of lethal ts 151 showed no lesions in marrow, spleen, and kidney; liver, skeletal muscle, and pancreas were normal in one animal. Brains had slight increases in nuclei associated with vessel walls, but no hemorrhages or neuronal damage. These two hamsters had viremias of $10^{5.2}$ PFU/ml on day 2 after s.c. inoculation.

Previous studies have shown that lethal strains of VE virus produce necrosis of hemopoietic and brain tissues (1, 9). One hamster was examined after death from infection with parental virus (i.e., 2 days after s.c. inoculation of 320 PFU, when a viremia of $10^{8.1}$ PFU/ml was present). There was marked necrosis of cells in bone marrow and white pulp of spleen and slight accumulations of mononuclear cells around a small vessel in brain; kidney, liver, and skeletal muscle were normal.

Body temperatures of hamsters in relation to the NPT of VE ts mutant 126 in primary cultures of hamster embryonic cells. Hamsters are nocturnal, and temperatures vary during the diurnal cycle. However, since we could find no practical method of measuring temperatures of hamsters sleeping during daytime, we had to take rectal temperatures of aroused, though not overly active or stimulated, hamsters. Temperatures of normal and infected hamsters were measured using a single clinical thermometer. Means of morning and afternoon

rectal temperatures of hamsters 6 to 10 weeks of age were as follows: 11 males—36.6°C (range, 34.8 to 37.8°C) during 6 days; 4 males—36.1°C (range, 35.2 to 37.0°C) during 6 days; and 2 females—36.7°C (range, 36.0 to 37.5°C) during 4 or 6 days. Twenty-eight morning and/or afternoon temperatures of two males averaged 36.5°C (range, 35.5 to 37.0°C) during 16 days, and those of two females averaged 36.4°C (range, 36.0 to 37.0°C). Thus the average rectal temperature of aroused normal male or female hamsters was about 36.5°C when measured by the method we employed.

Experiments with ts 126 grown in primary hamster embryonic cells in vitro revealed that 37.0°C was the PT and 38.0°C was probably the NPT, since virus concentrations in cultural fluid increased between 0 and 24 h at 37.0°C but decreased at 38.0°C to a level compatible with that of residual input virus (Table 4). Thus the usual body temperatures of aroused normal hamsters were within the permissive range for ts 126 grown in hamster cells in vitro. Since body temperatures of hamsters are probably lower while sleeping than when aroused, there would seem to be no restriction placed by body temperature on replication of VE ts mutant 126 in hamsters.

The question then arose of whether ts 126 induced sufficient fever during infection that body temperature reached the NPT range and inhibited further replication of ts virus. Five hamsters infected s.c. with 110 PFU of virulent parental strain 68U201 developed fever to an average of 37.5°C on day 1 after inoculation; thereafter temperatures of some fell below normal as terminal illness developed (Fig. 2). Average rectal temperatues of four hamsters infected s.c. with 200 PFU of ts 126 remained below 37°C and thus were within the in vitro PT range during 5 days after infection (Fig. 2).

TABLE 2. *Occurrence of revertants in blood of hamsters 2 days after s.c. inoculation of VE ts mutants*

Mutant	Hamsters			
	Dead		Surviving	
	No.	No. with revertants[a]	No.	No. with revertants
3	3	3	0	
4	6	1	1	1
40	3	2	0	
73	3	3	0	
85	2	2	0	
121	3	1	0	
127	2	2	0	
151	12	6	3	0
238	3	2	0	
126	4	1	23	1

[a] Revertants were indicated by PFU titers at 40°C of $>10^{2.0}$/ml of blood; absence of revertants, titers of $<10^{1.4}$/ml. Titers of virus in blood of these hamsters at PT are summarized in Table 1.

DISCUSSION

The mechanisms of virulence of VE virus

TABLE 4. *PT and NPT for growth of VE ts 126 in cultures of primary hamster embryonic cells*

Temp of incubation for virus growth (°C)	Expt no.	Concn of virus[a] in cultural fluid at h of incubation:		
		0	8	24
30–32	A	3.5	<1.7	5.8
	B	3.2		5.7
37.0	A	3.5	<1.7	5.3
38.0	B	3.3		2.6
39–40	A	3.5	<1.7	<1.7
	B	3.3		<1.7

[a] Log_{10} CEC PFU/ml when assayed at 30°C. All values were <1.7 when assayed at 40°C.

TABLE 3. *Occurrence of revertants in blood and tissues of hamsters inoculated s.c. with VE ts 121 or ts 126*

Mutant	Days after inoculation	No. of hamsters					
		Inoculated	With revertants in[a]:				
			Blood	Spleen	Brain	Liver	Kidney
121	1	5	5	1	0	1	1
	2	5	5	5	5	5	5
126	1	2	0	0	0	0	0
	2	6	2	1	0	0	0

[a] Revertants were determined by PFU titers at 40°C of $>10^{2.0}$/ml of blood or g of tissue; absence of revertants, titers of $<10^{1.4}$/ml or g. All inoculated hamsters were infected, because virus was detected in blood on day 1 or 2.

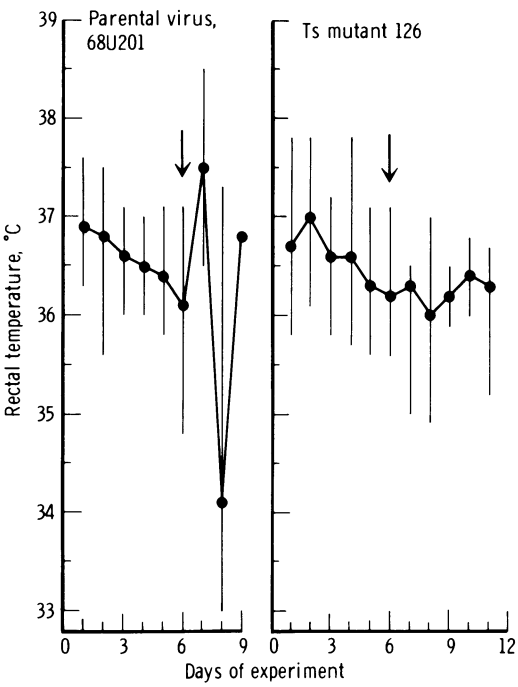

FIG. 2. *Rectal temperatures of hamsters infected s.c. with parental VE strain 68U201 or ts 126. Dots indicate means of five and four hamsters, respectively, and bars are ranges of morning and afternoon temperatures before and after virus inoculation on day 6 (indicated by arrow). Only one 68U201-infected hamster was alive on day 9.*

strains in hamsters are not fully understood, but the in vivo data presented here offer interesting insights. Attenuation of VE mutant ts 126 for hamsters was related to restriction of viral growth in spleen and probably bone marrow, although not in Peyer's patches or brain, and to lack of pathological lesions in the usual target hemopoietic and brain tissues (1, 9). The restriction of ts 126 growth in spleen and probably bone marrow was shown by comparison with a virulent mutant, ts 4. On day 4 after inoculation, ts 4 titers were 10- to 100-fold higher in spleen and bone marrow than in blood, whereas ts 126 titers were less than 10-fold higher in spleen and less than 100-fold higher in marrow than in blood. Unfortunately the small quantities of marrow that could be obtained prevented virus testing at lower dilutions. Tissues without evidence of virus growth (i.e., ts 4 concentrations less than blood on days 3 and 4 after inoculation) were liver and skeletal muscle.

Attenuation was functionally separate from temperature sensitivity since body temperatures of normal or infected hamsters remained within the PT range of the mutant. Virulence was not related in a simple manner to titers of VE virus in blood. Some mutants, such as ts 151, killed hamsters even though blood titers of virus at PT were low (2.4 $\log_{10}$ PFU/ml on day 1 or 2). Other mutants, such as ts 126, did not kill hamsters even though blood titers were high (5.3 and 6.0 $\log_{10}$ PFU/ml on days 1 and 2). Lethality in hamsters also did not always correlate with reversion to non-ts. Some hamsters died with no revertant virus in blood on day 2 after inoculation, and some that survived had revertants in blood.

Selection of ts mutants of other viruses has also resulted in attenuated strains. For some viruses, such as polio (5) and influenza (17), decreased virulence of ts mutants appears to be related to temperature-induced inhibition of viral replication. A similar mechanism may exist with ts mutants of eastern encephalitis virus in mice, although the evidence is not conclusive (3). The factor thought to be responsible for low virulence of ts mutants of western encephalitis virus for mice was slow viral growth in brain (18). With dengue 2 virus, the relationship between temperature sensitivity and virulence in vivo is complicated and has not been completely defined (19, 20). Attenuation of the encephalitogenic potential of ts mutants of measles virus seems related to partial defectiveness under permissive conditions rather than a temperature-induced inhibition of virus replication (8).

Attenuated VE mutant ts 126 could be either a single or a double mutant. Since ts mutants are generally base pair substitutions leading to changes in amino acids of proteins, it is conceivable that one genetic lesion and a resultant protein change could make virus lose its ability to bind to cells of a tissue such as bone marrow whose destruction is an integral part of the lethal process of parental virus. Alternatively, since *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine often induces multiple mutation in closely linked genes (7), it is possible that temperature sensitivity and attenuation were generated in ts 126 as separate genetic lesions.

Attenuated ts 126 VE virus and its parental, lethal virus or its sibling, lethal ts mutants now provide genetically related pairs of viruses with which to study mechanisms of virulence in the hamster model of human VE viral disease. VE ts 126, however, is not a mutant that currently warrants much consideration as a vaccine candidate, since it has passage histories in multiple hosts (vector mosquito to hamster to baby mice to CEC in culture) that would probably make it unsuitable, by today's standards, for human inoculation. Moreover, the attenuated TC83 strain of VE virus is an effective, safe, live virus vaccine for equine animals, although it is not completely

satisfactory for human use since it produces some febrile reactions (11). However, chemical mutagenesis of virus with subsequent selection of ts viruses, as employed in these studies, might be utilized to develop a more suitable human VE virus vaccine or attenuated vaccines for other virus diseases.

The growth patterns in hamster tissues of lethal VE ts mutant 4 were as expected from previous studies of other strains of VE virus (1, 6, 10). Hemopoietic tissues and brain were major sites of viral replication. However, for the first time kidney was shown to be a site of VE viral replication, whereas liver and skeletal muscle clearly did not show evidence of virus growth.

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LITERATURE CITED

1. **Austin, F. J., and W. F. Scherer.** 1971. Studies of viral virulence. I. Growth and histopathology of virulent and attenuated strains of Venezuelan encephalitis in hamsters. Am. J. Pathol. **62**:195–210.
2. **Berge, T. O., I. S. Banks, and W. D. Tigertt.** 1961. Attenuation of Venezuelan equine encephalomyelitis virus by *in vitro* cultivation in guinea pig heart cells. Am. J. Hyg. **73**:209–218.
3. **Brown, A., R. Vosdingh, and E. Zebovitz.** 1975. Attenuation and immunogenicity of ts mutants of eastern encephalitis virus for mice. J. Gen. Virol. **27**:111–116.
4. **Clarke, D. H., and J. Casals.** 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am. J. Trop. Med. Hyg. **7**: 561–573.
5. **Fiszman, M., M. Reynier, D. Buczherini, and M. Girard.** 1972. Thermosensitive block of the Sabin strain of poliovirus type I. J. Virol. **10**:1143–1151.
6. **Gorelkin, L., and P. B. Jahrling.** 1975. Virus initiated septic shock: acute death of Venezuelan encephalitis virus infected hamsters. Lab. Invest. **32**:78–85.
7. **Guerola, N., J. L. Ingram, and E. Ceroda-Olmedo.** 1971. Induction of closely-linked multiple mutations by nitrosoguanidine. Nature (London) New Biol. **230**:122–125.
8. **Haspel, M. V., R. Duff, and F. Rapp.** 1975. Experimental measles encephalitis: a genetic analysis. Infect. Immun. **12**:785–790.
9. **Jahrling, P. B., and W. F. Scherer.** 1973. Histopathology and distribution of viral antigens in hamsters infected with virulent and benign Venezuelan encephalitis viruses. Am. J. Pathol. **72**:25–38.
10. **Jahrling, P. B., and W. F. Scherer.** 1973. Growth curves and clearance rates of virulent and benign Venezuelan encephalitis viruses in hamsters. Infect. Immun. **8**:456–462.
11. **McKinney, R. W.** 1972. Inactivated and live VEE vaccines—a review, p. 369–376. *In* Venezuelan encephalitis. Proceedings of the Workshop-Symposium on Venezuelan Encephalitis Virus. Scientific Publication no. 243. Pan American Health Organization, Washington, D.C.
12. **Scherer, W. F.** 1964. Inapparent viral infection of cells *in vitro*. I. Conversion of inapparent to apparent infection by environmental alteration of chicken embryonic cells in cultures inoculated with Japanese encephalitis virus. Am. J. Pathol. **45**:393–411.
13. **Scherer, W. F., and J. Chin.** 1977. Responses of guinea pigs to infections of Venezuelan encephalitis virus and correlations with equine virulence. Am. J. Trop. Med. Hyg. **26**:307–312.
14. **Scherer, W. F., C. A. Ellsworth, and A. K. Ventura.** 1971. Studies of viral virulence II. Growth and adsorption curves of virulent and attenuated strains of Venezuelan encephalitis in cultured cells. Am. J. Pathol. **62**: 211–219.
15. **Scherer, W. F., and B. A. Pancake.** 1977. Comparisons of Venezuelan encephalitis virus strains by hemagglutination-inhibition tests with chicken antibodies. J. Clin. Microbiol. **6**:578–585.
16. **Shope, R. E., O. R. Causey, A. H. Paes de Andrade, and M. Theiler.** 1964. The Venezuelan equine encephalomyelitis complex of group A arthropod-borne viruses, including Mucambo and Pixuna from the Amazon region of Brazil. Am. J. Trop. Med. Hyg. **13**:723–727.
17. **Svobodová, J., E. Tučková, and V. Vonka.** 1974. Linkage between reproductive capacity at increased temperature and neurotropic activity in A/NWS HONi (H_0N_1) influenza virus. Infect. Immun. **10**:400–401.
18. **Takayama, N., and M. Nakano.** 1975. Pathogenicity of an attenuated temperature-sensitive mutant of western equine encephalitis virus induced by a chemical mutagen. Infect. Immun. **12**:858–865.
19. **Tarr, G. C., and A. S. Lubiniecki.** 1976. Chemically induced mutants of dengue virus type 2. I. Isolation and partial characterization. Arch. Virol. **50**:223–235.
20. **Tarr, G. C., and A. S. Lubiniecki.** 1976. 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. Infect. Immun. **13**:688–695.
21. **Wagner, R. R.** 1974. Pathogenicity and immunogenicity for mice of temperature-sensitive mutants of vesicular stomatitis virus. Infect. Immun. **10**:309–315.
22. **Zarate, M. L., and W. F. Scherer.** 1969. A comparative study of virulences, plaque morphologies and antigenic characteristics of Venezuelan encephalitis virus strains. Am. J. Epidemiol. **89**:489–502.