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Temperature-Sensitive Mutants of Rabies Virus in Mice: a Mutant (ts 2) Revertant Mixture Selectively Pathogenic by the Peripheral Route of Inoculation

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Analysis of the pathogenic potential in mice of a variety of rabies and rabies serogroup viruses revealed that an apparently revertant population of virus derived from CVS mutant ts 2 had a unique capacity to selectively induce paralytic disease when given by a peripheral [intraplantar (i.pl.)] route of inoculation. Little paralytic disease was induced by high concentrations of virus administered by the intracerebral (i.c.) route, whereas paralytic disease and death were characteristically induced in mice given only a few infectious doses of virus i.c. Disease induced by i.pl. inoculation was dose dependent. Mice frequently survived paralytic disease induced by i.pl. inoculation, with clinical signs often persisting indefinitely; mice surviving i.c. inoculation of high concentrations of virus frequently exhibited chronic nonspecific signs of minor debility. Analysis of the ts 2 virus population indicated that it was composed of a mixture of ts and revertant virions, each with characteristic pathogenic (or nonpathogenic) propensities, none of which was identical to the original composite ts 2 virus populations. Despite the heterogeneity of the ts 2 virus population, the typical pathogenic pattern of selective pathogenic capacity after i.pl. inoculation at high doses was retained during 11 consecutive passages in suckling mouse brain. ts 2 virus was demonstrated to interfere with the disease-producing capacity of CVS fixed rabies virus when ts 2-CVS mixtures were inoculated i.c. However, attempts to demonstrate a particular propensity for induction in vitro of "autointerference" by ts 2 in serial passage in BHK-21 cell culture inoculated at high multiplicity were unsuccessful.

To develop an ideal model in mice for the study of rabies virus pathogenesis and the evaluation of a postexposure vaccine, a rabies virus strain is required that after a prolonged incubation period comparable to that characteristic of natural rabies virus infections in man consistently causes disease. Most standard fixed strains of rabies virus consistently cause death after a very brief (6 to 10 days) incubation period; feral "street" strains of rabies virus often kill too inconsistently to provide a useful virus challenge.

In the quest for a fixed virus with a prolonged incubation period, we have tested the behavior in mice of a variety of rabies virus mutants and variants developed in this laboratory (2, 3). A preliminary characterization of one of these viruses, ts 2, that exists in an apparently stable mixture with revertant virus and is selectively pathogenic peripherally when given in high dose to mice is described in this report.

MATERIALS AND METHODS

Cell cultures. BHK-21 cells were cultivated at 37 C in Eagle basal medium containing 2× concentrations of vitamins and amino acids and 0.0225% HCO_3^- and 10% fetal bovine serum. BHK-13S cells were grown in BHK growth medium (14) with 0.17% HCO_3^- and 10% fetal bovine serum.

Virus. Isolation of CVS virus ts mutants has been previously described (3). Plaque assays for the quantitation of infectious units and for isolation of virus clones were performed in agarose-suspended 13S cells (16). Methods for the preparation of cloned stocks of virus and for determination of temperature sensitivity at 40.5 C were as previously described (3, 5).

Mice. ICR mice were obtained from Flow Laboratories. Weanling mice were females 4 to 5 weeks old; newborn mice were less than 72 h old. Adult mice inoculated intracerebrally (i.c.) or by the intraplantar (i.pl.) route were given 0.03 ml of inoculum administered with a 26-gauge needle into the left cerebral hemisphere or the right hind footpad, respectively. Newborn mice were given a dose of 0.01

ml i.c. Animals were observed daily.

When brain tissues were harvested, the animals were sacrificed by ether inhalation or cervical dislocation. Ten percent (wt/vol) tissue suspensions were prepared by grinding the tissue in a glass grinder (Ten Broeck) in a BHK cell medium (14) containing 2% fetal bovine serum.

RESULTS

Pathogenicity for mice of rabies serogroup viruses. The results of a test for pathogenicity of various rabies serogroup viruses inoculated i.c. or i.pl. into weanling mice are presented in Table 1. Except where otherwise noted, inocula were 10% suspensions of infected suckling mouse brain. Viruses included: (i) CVS, ERA, and HEP strains of fixed rabies virus (4); (ii) ts mutants of strain CVS (3), all of which had reverted to the non-ts state during continued passage in BHK-21 cells and suckling mice; (iii) substrains of CVS virus obtained by serial passage in reptile cells (2) or in BHK-21 cells at a reduced incubation temperature of 25 C (4) or in the presence of 1.0 μg of actinomycin D per ml; and (iv) spontaneously appearing variants of rabies serogroup Lagos bat or Mokola virus (6). All inocula contained from $10^{4.0}$ to $10^{6.0}$ plaque-forming units (PFU) per dose (0.03 ml).

All CVS virus variants except ts 2 were uniformly lethal when inoculated by the i.c. or i.pl. route, with mean incubation periods observed in variant virus-infected mice commonly 1 to 2 days longer than those observed in mice infected with the parental CVS virus. ERA and HEP strain viruses were pathogenic only when inoculated i.c.; the Lagos bat and Mokola viruses tested were not pathogenic by either route.

CVS substrain ts 2 stocks of either BHK-21 cell culture or suckling mouse brain origin were selectively pathogenic after i.pl. inoculation. In initial screening experiments, only one of 25 animals inoculated i.c. with ts 2 succumbed, after a prolonged incubation period. Twenty-four of 25 mice given ts 2 by the i.pl. route developed typical rabies paralysis. The majority of mice given cell culture-orgin virus survived indefinitely with paralysis; nine of ten mice receiving suckling mouse brain-origin virus developed fatal paralysis.

Dose-response relationship in ts 2 virus infection. A typical and consistent pattern of disease was observed when serial dilutions of ts 2 virus were inoculated into mice. Results of representative experiments are shown in Table 2. Newborn (3 day old) mice experienced uniformly fatal infection at all concentrations of virus up to end point (concentration <0.01 PFU/dose) whether inoculated by the i.c. (data not shown) or i.pl. route. When ts 2 virus was titrated by i.c. inoculation into weanling mice, a characteristic and reproducible death pattern was obtained. The majority of mice receiving maximum concentrations of virus survived (al-

TABLE 1. *Central and peripheral lethality of rabies and rabies-related virus strains and substrains in weanling mice*

Virus[a]	Mouse lethality			
	Central route		Peripheral route	
	Incidence	Mean day of death	Incidence	Mean day of death
Rabies				
CVS smb_1	5/5	8.2	5/5	9.0
ts 1 smb_1 A	5/5	9.4	4/5	13.2
smb_1 B	5/5	10.0	5/5	12.2
smb_2	4/5	10.3	5/5	15.0
ts 2 BHK_8	1/5	20.0	5/5	>18.0[b]
BHK_9	0/10		10/10	>100.0[c]
smb_2 A	0/5		5/5	11.6
smb_2 B	0/5		4/5	11.5[d]
ts 3 smb_1	5/5	8.2	5/5	9.8
ts 4 smb_1	5/5	8.6	5/5	10.0
ts 5 smb_1	5/5	9.4	5/5	9.8
AD_{24} smb_1	5/5	7.6	5/5	11.0
VSW_{89} smb_1	5/5	9.0	5/5	9.0
RT_{51} smb_1 A	5/5	8.0	5/5	11.2
smb_1 B	5/5	8.8	5/5	9.0
GL_5 smb_1 A	5/5	9.4	5/5	9.8
smb_1 B	5/5	8.8	5/5	10.4
GL_{60} smb_1	5/5	9.2	5/5	9.8
ERA smb_1	5/5	11.0	0/5	
HEP smb_1	5/5	9.8	0/5	
Rabies serogroup				
Mokola 1 smb_1	0/5		0/5	
Lagos 1 smb_1	0/5		0/5	
Lagos 1 $clone_1$ smb_1	0/5		0/5	

[a] Abbreviations of virus strains and substrains are as follows: smb, passaged in suckling mouse brain (subscript indicates number of passages); "A" and "B" indicate separate replicate experiments; "ts" designations are for purposes of strain identification only; all mutants had reverted to non-ts state at time of testing in mice. CVS variants: AD_{24} passaged 24 times in BHK cells in the presence of 1 μg of actinomycin D per ml; VSW_{89} passaged 89 times in VSW (viper) cells; RT_{51} passaged 51 times in BHK-21 cells at 25 C; GL_5 and GL_{60} passaged 5 and 60 times, respectively, in GL-1 (gecko) cells.

[b] Two animals died on days 20 and 25; three paralyzed animals were sacrificed on days 18, 20, and 26.

[c] All animals were paralyzed after a mean incubation period of 17.4 days; all survived for >100 days (see text).

[d] Surviving mouse was severely paralyzed.

TABLE 2. *Dose effect on disease in weanling mice caused by rabies virus ts 2 propagated in BHK-21 cells or suckling mouse brain*

Virus dilution	BHK_8[a] (2.0 × 10^5 PFU/ml)							smb_2[b] (4.0 × 10^4 PFU/ml)				
	3 days[c] (i.pl.)		21 days[d] (i.c.)		27 days (i.pl.)			30 days (i.c.)		30 days (i.pl.)		
	Deaths	Mean day of death	Deaths	Mean day of death	Deaths	Paralysis	Mean day of onset	Deaths	Mean day of death	Deaths	Mean day of death	Nonfatal paralysis
Nondiluted	8/8	7.0	1/20	12.0	0/10	10/10	17.4	0/5		4/5	11.5	1/5
−1	8/8	6.0	2/20	12.0	0/10	3/10	18.0	1/5	12.0	3/5	11.7	2/5
−2	9/9	7.0	10/20	13.3	0/10	0/10		1/5	17.0	0/5		0/5
−3	8/8	6.4	7/20	12.5				1/5	21.0	1/5	20.0	0/5
−4	10/10	6.1	4/20	13.3				3/5	17.0	0/5		0/5
−5	7/10	7.4	0/5					4/5	18.5	0/5		0/5
−6	3/8	11.0						3/5	26.3			
−7	0/6											

[a] Virus from eighth passage in BHK cells.
[b] Virus from second passage in suckling mouse brain cells.
[c] Age of mice.
[d] Summation of two experiments.

though minor signs of encephalitis were exhibited between 4 and 10 days after infection). At a specific lower concentration of virus, a dilution characteristic for each virus stock preparation and containing ≤1 PFU/dose, ≥ 50% of infected mice died after typical encephalitic disease (primarily depression and ataxia). On the contrary, titration of ts 2 virus by i.pl. inoculation in weanling mice led to expression of disease according to a typical dose-response pattern: mice given maximum doses of virus consistently developed paralytic disease, whereas those given lesser virus doses remained free of disease.

Challenge of mice surviving ts 2 virus infection (30 to 50 mean lethal doses of CVS virus given i.c. 30 days after infection; data not shown) indicated that all mice surviving i.c. inoculation of ts 2 virus concentrations greater than that causing the maximum lethality were immune. Mice surviving inoculation of lesser concentrations of ts 2 virus given i.c., and all mice surviving i.pl. inoculation of ts 2 virus, uniformly succumbed to rabies virus challenge.

Both the substrate used for virus cultivation and the age of the weanling mice affected the severity but not the basic dose-response pattern of ts 2 virus-induced disease. Thus both a younger age of mouse and the preparation of virus in suckling mouse brain favored a higher incidence of fatal disease in mice inoculated by the i.pl. route with high concentrations of virus or by the i.c. route with terminal dilutions of virus. For this reason, suckling mouse brain-origin virus was used for all subsequent studies.

Persistence and pathology of disease induced by ts 2 virus. Rabies is traditionally considered to be uniformly lethal; there is little evidence that exceptions to this rule occur commonly in animals or man infected naturally. Nevertheless, several nonfatal disease patterns were observed in ts 2-infected mice.

Mice developing paralysis after i.pl. inoculation either died within 1 week after the onset of clinical signs or survived. Mice that survived paralytic disease either exhibited apparently complete recovery (usually within a few days after onset of disease) or, more commonly, lifelong paralysis without progression of disease. In observations taken from seven different experiments, 25 of 36 mice (69.4%) surviving paralytic disease continued to exhibit paralysis 6 months postinfection. Individual mice were observed to survive with paralysis for periods as long as 20 months. Only two of several hundred mice observed developed very late onset (i.e., longer than 90 days postinfection) of nonfatal paralysis. In all cases, mice surviving with paralysis exhibited a flaccid paralysis of one or both hind limbs, but showed no dysfunction of the central nervous system. The possibility that chronic disease induced by ts 2 virus may be accompanied by persistence of infectious virus or of virus components is under investigation.

Limited paralytic disease with survival was also observed in a few animals in two experiments involving i.pl. inoculation with ts 1 virus and in a single experiment involving i.pl. inoculation with a street strain of rabies virus (a bat isolate kindly supplied by J. F. Bell, Hamilton, Mont.). However, both of the latter viruses differ from ts 2 virus in that they kill efficiently when given by the i.c. route. In experiments in

which CVS virus, the parental strain of ts 2 and ts 1 viruses, was given by the i.pl. route, paralytic disease progressed to death within a few days in 55 of 55 mice inoculated.

Mice surviving inoculation with high concentrations of ts 2 virus usually underwent a period of transient illness within the first 10 days postinfection and then appeared to make a complete recovery; their condition, however, often deteriorated thereafter. At 1 to 2 months postinfection hyperexcitability was commonly observed, infrequently accompanied by a minor degree of ataxia. A majority of these mice, with or without obvious central nervous system symptoms, subsequently exhibited various degrees of debility and emaciation, i.e., a general "failure to thrive," which did not markedly affect their survival. In rare instances, such animals developed a nonfatal posterior paresis with very delayed onset, as late as 15 months postinfection. A detailed description of the histopathology of ts 2-infected mice will be the subject of another report (Y. Iwasaki, and H. F. Clark, manuscript in preparation).

Composition of ts 2 virus populations. Because the pattern of disease observed after both central and peripheral inoculation of ts 2 virus into mice was unique to our experience, it was of special interest to determine whether this pathogenic potential was a stable characteristic of all virions comprising the substrain population. To test this question, seven clones were selected from a BHK-21 cell-propagated stock (10th passage) of ts 2 virus and tested for temperature sensitivity and mouse pathogenicity.

Surprisingly, each clone was completely nonpathogenic for weanling mice after the two cell culture passages necessary to produce new cloned stocks. (Clones of the parental CVS stock have never been observed to lose pathogenicity for i.c.-inoculated weanling mice after any number of passages in BHK-21 cells.) Significant levels of pathogenicity for weanling mice were not observed until cloned stocks were passed twice in i.c.-inoculated suckling mice. The temperature sensitivity and mouse pathogenicity patterns characteristic for each clone are illustrated in Table 3.

It was apparent that the parental ts 2 virus stock contained a mixture of ts and revertant virions. Clones 2 and 5 were temperature sensitive and less cytopathic than the five revertant clones identified although, unlike most ts viruses, clones 2 and 5 consistently produced approximately 10-fold higher titers of infectious virus in cell culture or mouse brain than did the revertant clones. None of the clones exhibited pathogenic potential similar either to that of the parental ts 2 virus stock or to that of parental CVS virus stock. However, ts clones 2 and 5 were less pathogenic in weanling mice than the revertant clones.

Stability of ts 2 virus pathogenic potential. The apparent consistency of the unique ts 2 virus pathogenic pattern, i.e., selective pathogenicity via peripheral route when administered in high concentration, was surprising in view of the apparently mixed genotypic composition of the virion population. The stability of ts 2 pathogenicity for weanling mice, therefore,

TABLE 3. *Characteristics of clones selected from ts 2 BHK-21 cell-propagated virus*

Clone no.	BHK-propagated (2×) clones			Suckling mouse brain-propagated (2×) clones		
	Temperature sensitivity[a] (rct 40.5)	Mortality induced in weanling mice[b]		Temperature sensitivity (rct 40.5)	Mortality induced in weanling mice[c]	
		IC	IP1		IC	IP1
1	(−) 0.0056	0/5	0/5	(−) 0.0050	1/5	0/5[b]
2	(+) <0.00000010	0/5	0/5	(+) 0.0000041	0/5	0/5
3	(−) 0.025	0/5	0/5	(−) 0.0015	4/5	3/5
4	(−) 0.022	0/5	0/5	(−) 0.0050	4/5	0/5
5	(+) <0.00000050	0/5	0/5	(+) <0.0000010	0/5	0/5
6	(−) 0.012	0/5	0/5	(−) 0.00012	3/5	0/5
7	(−) 0.010	0/5	0/5	(−) 0.00026	3/5	0/5
ts 2 BHK_{10}[d]	(−) 0.0052					
CVS parental virus	(−) 0.0096					

[a] rct 40.5 value = yield of released virus at 48 h postinfection in BHK-21 cell culture incubated at 40.5 C divided by the yield of virus from a replicate infected cell culture incubated at 33 C (3, 5).

[b] Titers of virus stocks (inoculated nondiluted) ranged from 5.0×10^5 to 2.6×10^7 PFU/ml.

[c] Titers of virus stocks (inoculated nondiluted) ranged from 1.0×10^5 to 1.0×10^6 PFU/ml. There was one case of nonfatal paralysis.

[d] Tenth passage in BHK cells.

was tested after each of 10 consecutive passages in suckling mouse brain (Table 4).

The basic pattern of pathogenicity was similar at all virus passage levels with the exception of an unexplained lack of pathogenicity at the ninth passage level. Intracerebrally inoculated mice usually exhibited mild signs of encephalitic illness that only exceptionally progressed to death (12.5%). Virus at virtually all passage levels caused a majority of i.pl.-inoculated mice to develop paralysis characteristic of rabies (incidence 81.6%) which progressed irregularly to death (28.7%). A preliminary analysis of nine clones of virus isolated from the fifth suckling mouse brain passage population of ts 2 indicated the presence of a mixed population of virions of different pathogenic characteristics, a phenomena similar to that described above for the seven clones selected from BHK-21 cell-origin virus.

The pathogenicity of ts 2 virus recovered from brains of symptomatic weanling mice was also tested. Virus from mice dead or moribund as a result of infection introduced i.c. or i.pl. was passaged once in suckling mice and tested in weanling mice. The results (Table 5) were very inconsistent. The recovered virus was often fatal for i.c.-inoculated adult mice, particularly virus originating from the 10th suckling mouse brain passage ts 2 stock, but inoculation periods were invariably longer than that characteristic of parental CVS virus. Virus from a single adult mouse (no. 3) exhibited the typical ts 2 characteristic of selective pathogenicity when administered i.pl. Four of the seven isolates induced at least one case of nonfatal paralysis in animals inoculated i.pl., a phenomenon never observed with the parental CVS virus.

Interfering capacity of ts virus. The failure of ts 2 virus to consistently induce fatal disease when inoculated i.c. in high concentration suggested that ts 2 virus may interfere with replication of infectious virus in the brain. This possibility was tested in an experiment in which serial dilutions of CVS virus were mixed with equal volumes of a viable ts 2 virus population and subsequently inoculated into weanling mice (Table 6).

The ts 2 virus effected a potent interference with CVS virus-induced disease, leading to a prolonged incubation period when mixed with high concentrations of CVS virus and complete protection when mixed with lower concentrations of CVS virus. In several mice the mixed infection led to survival with paralytic sequelae.

To determine whether ts 2 virus might have an increased propensity to exhibit "autointerference," possibly mediated by defective interfering (DI) virus particles, ts 2 virus was compared with CVS virus and with three ts 2 clonal derivative viruses in an experiment designed to determine the effect on virus yield in vitro of serial passage at high and low multiplicities of infection (Table 7). It was evident that autointerference was not readily demonstrable in this

TABLE 4. *Pathogenicity for weanling mice of ts 2 rabies virus after serial passage in suckling mouse brain*

Passage level and determination		Intracerebral inoculation		Intraplantar inoculation		
		Deaths	Day of death	Paralysis incidence	Deaths	Mean day of death
A. Passages 2-11	Titer PFU/ml					
2	4.0×10^4	0/10		10/10	9/10	11.4
3	1.0×10^5	2/5	9, 14	5/5	4/5	11.5
4	7.0×10^5	0/10		8/10	0/10	
5	3.2×10^6	0/5		5/5	5/5	11.0
6	5.0×10^6	1/5	12	5/5	0/5	
7	1.4×10^7	1/5	10	5/5	1/5	10.0
8	2.2×10^6	1/5	10	4/5	0/5	
9	5.5×10^5	1/5	12	0/5	0/5	
10	1.8×10^4	1/10	12	9/10	2/10	11.5
11	3.1×10^7	2/5	10, 14	7/7	3/7	14.3
B. Comparison of early- and late-passage virus within a single experiment						
2		0/10		6/10	1/10	15.0
10		1/5	8	7/10	0/10	
C. Summation A and B		10/80 (12.5%)		71/87 (81.6%)	25/87 (28.7%)	

TABLE 5. *Pathogenicity for weanling mice of virus recovered from mice with lethal disease induced by ts 2 rabies virus*[a]

Source animal no.	Original inoculum and route of inoculation	Day of brain harvest	Pathogenicity of recovered virus				
			i.c.		i.pl.		
			Deaths	Mean day of death	Deaths	Mean day of death	Nonfatal paralysis
1	ts 2 smb_5, ND[b]; i.pl.	12	2/5	11.0	1/5	15.0	0/5
2	ts 2 smb_5, ND; i.pl.	12	3/5	9.3	0/5		1/5
3	ts 2 smb_5, ND; i.pl.	12	0/4		1/5	17.0	3/5
4	ts 2 smb_5, $10^{-6.0}$; i.c.	25	0/5		0/5		1/5
5	ts 2 smb_{10}, ND; i.c.	7	5/5	12.2	2/5	14.5	0/5
6	ts 2 smb_{10}, ND; i.c.	8	5/5	9.4	4/5	14.2	1/5
7	ts 2 smb_{10}, ND; i.pl.	8	2/5	13.5	0/5		0/5

[a] Virus from brains harvested from mice dead or moribund after infection with ts 2 rabies virus was passaged once in suckling mouse brain (smb); a 10% smb suspension was inoculated either i.c. or i.pl. into weanling mice. Titers of suckling mouse brain-origin stock were from $10^{5.0}$ to $10^{7.0}$ PFU/ml.

[b] ND, Nondiluted.

TABLE 6. *Effect on mouse mortality of the addition of ts 2 virus to CVS virus inoculated intracerebrally*[a]

CVS dilution	Virus diluted 1:2 in:			
	Cell culture medium		ts 2 virus	
	Mortality	Mean day of death	Mortality	Mean day of death
ND[b] (final brain 1:20)	5/5	8.0	5/5	9.2
−1	5/5	7.8	4/5[c]	8.8
−2	4/5	11.2	1/4[c]	10.0
−3	5/5	10.3	0/5[c]	
−4	4/5	13.2	0/5	
−5	3/5	9.0	0/5	
−6	0/5		0/5	
−7	0/5		0/5	
CVS titer[d]	$10^{6.6}$		$10^{3.7}$	

[a] Aliquots of serial dilutions of CVS were added to equal volumes of cell culture medium (control) or ts 2 virus (10^{-1} suckling mouse brain suspension: 3.2×10^6 PFU/ml). The resultant mixtures were inoculated intracerebrally into weanling mice.

[b] Nondiluted.

[c] One surviving mouse developed paralytic signs.

[d] Expressed as mean lethal dose per gram of brain tissue.

TABLE 7. *Infectious virus yields in BHK-21 cells of CVS, ts 2, and ts 2 clones after serial passage with nondiluted or dilute (10^{-3}) inocula*[a]

Virus	Dilution	Passage				
		1	2	3	4	5
CVS	ND	8.1[b]	7.1	6.1	6.8	7.5
	10^{-3}	6.7	6.7	6.7	6.4	6.6
ts 2	ND	7.4	6.2	6.7	6.8	7.3
	10^{-3}	7.4	7.7	6.6	5.7	5.9
clone 2	ND	>9.0	8.4	8.3	7.1	8.2
	10^{-3}	8.8	7.1	7.1	5.5	6.4
clone 5	ND	>8.7	>8.7	8.1	7.0	7.3
	10^{-3}	7.9	6.7	6.9	4.7	4.7
clone 4	ND	7.1	7.0	6.2	6.4	6.9
	10^{-3}	7.8	7.0	6.8	6.8	6.7

[a] BHK-21 cell monolayers were infected with virus stocks of BHK-21 cell origin nondiluted (ND) (multiplicity of infection at passage 1 = 1.0 to 10.0) or diluted 10^{-3}-fold. Released virus was harvested after 3 days of incubation at 33 C and used as inoculum for the successive passage nondiluted or again diluted 10^{-3}-fold.

[b] Log_{10} PFU of released virus per milliliter.

system with any of the viruses tested. The only consistent effect of cell multiplicity of infection upon virus yield was a marked reduction in yield of cells infected at low multiplicity with the two ts clones of virus, clones 2 and 5.

DISCUSSION

An apparently revertant virus population derived from a chemical mutagen-induced ts mutant of CVS rabies virus was observed to have a unique capacity for selectively inducing paralytic disease after peripheral inoculation in high doses into adult mice. This observation contradicts the traditional dogma that "neurotropic" viruses induce disease most efficiently when inoculated directly into the central nervous system. The possibility that such a phenomenon may not be unique is of special public health importance, since i.c. inoculation of inactivated virus preparations is considered the ultimate safety test for many inactivated virus vaccines. A survey of several attenuated rabies serogroup viruses in our laboratory, however, failed to reveal other strains exhibiting this

propensity. Furthermore, i.c. pathogenicity induced by the ts 2 mutant is unimpaired in newborn mice.

The erratic dose-response pattern of disease induced by ts 2 virus administered i.c. is reminiscent of patterns occasionally observed with other rabies virus strains. Flury HEP fixed rabies virus of chicken embryo origin was reported to be selectively pathogenic for guinea pigs and hamsters at less than maximum (10% tissue) concentrations, although virulence for other hosts (including mice) was not enhanced by dilution (12). Both the ERA strain of rabies virus and several substrains of the rabies serogroup Lagos bat virus have been noted to cause death curves in i.c.-inoculated mice that do not follow a regular dose-response relationship (6; T. J. Wiktor and H F. Clark, unpublished data). However, unlike ts 2 virus, the abovementioned viruses are not pathogenic at any concentration when inoculated into adult mice by peripheral routes.

The mechanism explaining the failure of high doses of ts 2 virus to cause fatal disease when inoculated i.c. is unknown. Apparently some portion of the component virus population interferes with the potentially fatal disease-inducing capacity of other virions present in the ts 2 virus stock or in artificially added normally lethal CVS rabies virus preparations. A role of the immune response in this phenomenon may be indicated by evidence that the incidence of lethal disease caused by ts 2 virus given i.c. is enhanced in mice immunosuppressed by treatment with cyclophosphamide (10), although 7 of 14 mice given ts 2 virus i.c. after immunosuppression nevertheless survived (10).

Interference by means of DI virus production until a protective immune response is initiated is an attractive hypothesis. DI virus interference with vesicular stomatitis virus-induced pathogenicity in mice has been reported (8, 9), and DI rabies virus interference with virus replication (7, 11) and cytopathogenicity (11, 20) in vitro have been described. However, our failure to demonstrate autointerference in cell cultures serially inoculated with ts 2 at high multiplicity of infection suggests that ts 2 interference may not be entirely analogous to that described in vesicular stomatitis virus-infected systems. The possible role of DI virus particles, as well as possible alternative mechanisms (enhanced immunogenicity, alternative forms of viral interference, etc.) are being further explored in an attempt to explain ts 2 pathogenic properties following central nervous system inoculation.

The frequent induction by peripherally administered ts 2 virus of self-limited nonfatal persistent paralytic disease is also of interest. Induction of nonfatal paralytic disease was not a characteristic of the parental CVS strain virus, although such a disease pattern has been observed with other rabies viruses in this study (vide infra) and others (13). Permanent paralytic sequelae have also been noted in normally fatal experimental rabies infections aborted by immune intervention in guinea pigs (Wiktor, unpublished data), by inferference of peripherally initiated infection by means of attenuated virus given i.c. (19), or by maintenance of infected mice at elevated ambient temperature (1).

The stability upon serial passage of the pathogenic properties of ts 2 virus is surprising. Whereas the study of ts mutants in vivo has often been discouraged because of the expectation of enhanced reversion rates, consistent association of the ts state with reduced pathogenicity has now been demonstrated with the vesicular stomatitis virus (17, 18) and Mokola and Lagos bat rhabdoviruses (6), as well as a variety of viruses of other groups (reviewed in reference 15).

The relative stability of the pathogenic expression of a mutant-revertant virus mixture is described in this report. Preliminary characterization of several clones selected from this mixture suggests that none of the individual component clones of the mixture has a phenotypic expression in vivo identical to that of the mixture. "Reconstitution" experiments in which mice are inoculated with artificially prepared mixtures of these clones are in progress.

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