Factors Influencing Virulence and Plaque Properties of Attenuated Venezuelan Equine Encephalomyelitis Virus Populations

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A minority of stable large-plaque virus increased proportionally in stored unstable attenuated (9t) Venezuelan equine encephalomyelitis virus populations. L-cell-grown progeny (9t2) of stored 9t showed large amounts of large-plaque virus and increased virulence. Small-plaque virus inhibited large-plaque virus but not the reverse. Serial passage of small-plaque virus from 9t2 yielded a strain (20t) that was more attenuated than 9t.

When it became necessary to replenish attenuated Venezuelan equine encephalomyelitis (VEE) virus working seeds, L-cell cultures were inoculated with 10^{-7} or 10^{-8} dilutions (multiplicities of infection = 0.000001 to 0.000001) of attenuated virus as a precaution against introducing nonattenuated particles previously found to be present in attenuated viral stocks (1). Surveillance of our small-plaque attenuated 9t and 10t preparations disclosed, however, that after 9 months of storage at -60°C , attenuated virus titers began to decline, whereas the concentration of the nonattenuated particles remained relatively unaffected. By using "aged" attenuated virus as inocula, with its increased proportion of the more stable nonattenuated virus, replication of large-plaque nonattenuated particles attained a sufficient level so that the progeny population behaved as nonattenuated virus. An example of such a virus population, 9t2 with its plaque and virulence properties, is shown in Table 1 (line 3). For comparison, virulence and plaque characteristics of the attenuated 9t virus and the nonattenuated PES virus from which the 9t strain was derived are shown on lines 2 and 1, respectively.

In an attempt to analyze the composition of 9t2 virus in greater detail, L-cell fluid culture preparations of virus from several large plaques (L_1 to L_4 , Table 1) were examined and found to induce large plaques. They showed no evidence

of attenuation for young adult mice, because the intraperitoneal (IP) titers were within 1 log of the intracerebral (IC) titers. Virus from small plaques from 9t2, tested in the same manner, induced small plaques with 5 to 10% large plaques and showed $\log_{10}$ IC and IP titer differences (IC-IP) ranging from 4.3 to 6.2, which suggested that, while the small-plaque virus present in 9t2 was more attenuated than the PES strain, it had become less attenuated than the original 9t strain.

In the next series of tests, 10 plaque-forming units (PFU) of virus obtained from individual attenuated "aged" 9t small plaques and nonattenuated PES large plaques were inoculated singly and dually into duplicate Leighton tube cultures of L-cells (multiplicity of infection = 0.0001). The 9t small-plaque virus yielded a maximal titer of $7.3 \log_{10}$ small PFU/ml at 48 hr (Table 2); at the same time, this culture contained $5.0 \log_{10}$ large PFU/ml. L-cell cultures inoculated dually with small- and large-plaque virus yielded similar results at 48 hr, but the large PFU titer increased to $6.1 \log_{10}$ by 72 hr. Inoculation of only large-plaque virus resulted in a maximal large-plaque titer of $7.3 \log_{10}$ PFU/ml at 72 hr; the small-plaque titer was $< 3.0 \log_{10}$. By 96 hr, however, while the large-plaque titer remained at $7.1 \log_{10}$, the small-plaque virus attained a titer of $6.2 \log_{10}$ /ml. All cultures were lysed by 120 hr.

These data show the marked capability of these small-plaque or large-plaque virus types to produce substantial concentrations of the other upon inoculation into L-cells. When large- and small-plaque types were used as a mixed inoculum,

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TABLE 1. Mouse intracerebral and intraperitoneal LD₅₀ titers of virulent parent, attenuated (9t) variant, and various substrains derived from the 9t strain

Virus type	Titer (log ₁₀ /ml) and route of injection			Plaque type
	Intracerebral	Intraperitoneal	IC-IP ^a	
Parent ^b	10.3	9.8	0.5	Large (>2 mm)
9t ^c	8.2	<0.6	>7.2	Small (≤1 mm)
9t2 ^d	8.7	5.6	3.1	Mixed
L ₁ to L ₄ ^e	9.8 to 9.9	8.8 to 9.6	0.3 to 1.0	Large
S ₁ to S ₄ ^f	7.7 to 9.0	2.1 to 3.9	4.3 to 6.2	Small ^g
20t ^h	<1.5	<1.6		Small ⁱ

^a Titers in mice injected intracerebrally minus titers in mice injected intraperitoneally.

^b Nonattenuated parent egg-seed strain.

^c Attenuated strain that was obtained after nine serial passages in L-cell fluid culture.

^d Virus harvested 48 hr after inoculation of "aged" 9t strain in L-cell fluid culture.

^e Virus from four representative large (>2 mm) plaques (L₁ to L₄) was selected, passed in L-cell fluid cultures, and then plaqued in L-cells, selected, and passed again for a total of three times. Values shown represent range of titers obtained during the third passage in fluid culture.

^f Virus from four representative small (≤1 mm) plaques (S₁ to S₄) was selected and treated according to the procedure described in footnote *e*.

^g Small with a few (5 to 10%) large plaques.

^h Virus from the S₃ substrain was selected and given 20 passages by using the procedures described in footnote *e*. Despite the lack of intracerebral or intraperitoneal lethality, this virus showed a plaque titer of 8.2 log₁₀ PFU/ml.

ⁱ Small, same as with 9t strain.

TABLE 2. Replication of small-plaque (9t) and large-plaque (PES) VEE virus inoculated singly or dually into L-cell fluid cultures

Inoculum plaque type	Progeny plaque type ^a	Time postinoculation and log ₁₀ PFU/ml			
		24 hr	48 hr	72 hr	96 hr
9t ^b	Small	5.5	7.3 ^c	7.0	5.3
	Large	<3.0	5.0	5.4	<3.0
9t and PES	Small	5.4	7.5	6.9	5.4
	Large	<3.0	4.7	6.1	<3.0
PES ^d	Small	<3.0	<3.0	<3.0	6.2
	Large	<3.0	6.2	7.3	7.1

^a Virus assays expressed as PFU/ml of both large (>2 mm) and small (0.5-1mm) plaque titers in each virus harvest sample.

^b Diameter, 0.5 to 1.0 mm.

^c Italic value represents the maximal PFU titer of each growth curve.

^d Diameter, 3.0 to 4.0 mm.

however, large-plaque replication was restricted to titers that were always at least 1 log less than those found after inoculation of large-plaque virus alone. In contrast, small-plaque viral replication appeared unaffected by the presence of large-plaque virus.

Finally, our efforts were directed toward reisolation "pure" attenuated small-plaque virus. S₃ virus (Table 1) was selected for further passage because it showed one of the greatest IC-IP

values (6.2 log₁₀). After the 20th passage, our tests showed that the L-cell fluid culture harvest, designated 20t, consisted of small-plaque virus and had lost its lethality for mice by the IC as well as the IP route (Table 1, bottom line). The immunizing (infectivity) titer of the new 20t substrain was comparable to its plaque titer.

In summary, an attenuated (9t) VEE virus population contained small numbers of virulent particles. The proportion of virulent virus increased relative to the attenuated virus upon storage because of the comparatively poor storage stability of the latter. When stored 9t virus was passed in L-cells, the progeny viral population (9t2) showed evidence of IP lethality for mice and contained many large-plaque virulent viral types along with a majority of small-plaque types. Small-plaque virus from the stored 9t strain inhibited the growth of large-plaque (PES) virus in L-cell fluid cultures; the reverse did not occur. This provides evidence for a possible mechanism by which attenuated VEE virus is selected over virulent virus *in vitro*. Finally, by deliberate plaque selection and passage in L-cells of small-plaque virus from stored 9t virus, a new 20t substrain possessing a high level of plaque homogeneity and attenuation exceeding that of the 9t strain was obtained without any loss in immunogenicity.

LITERATURE CITED

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