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Growth Curves and Clearance Rates of Virulent and Benign Venezuelan Encephalitis Viruses in Hamsters

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Four virulent strains of Venezuelan encephalitis virus attained higher concentrations of infectious virus in bloods of adult hamsters than two benign strains when given subcutaneously. One benign virus, the attenuated TC83 vaccine strain, reached higher concentrations in bone marrow and Peyer's patches than virulent subtype I strains, and another benign virus, subtype IV, grew to lower levels. When inoculated intracranially, the two benign viruses attained high concentrations in brain, but did not significantly alter in lethality although morbidity was increased. Intracardiac inoculation failed to increase virus concentrations of two benign viruses in blood or hematopoietic tissues above those found after subcutaneous inoculation, nor did it increase lethalities to those of virulent virus. Three benign virus strains were cleared more rapidly from hamster plasmas than six virulent strains. Differences in clearance rates were apparently not due to destruction of benign viruses by blood or tissue components. Thus viral concentrations in blood correlated directly and clearance rates inversely with hamster virulence, whereas the rate and extent of growth of a VE virus in hematopoietic or brain tissues did not correlate with ability to kill hamsters.

Overt disease caused by virulent strains of Venezuelan encephalitis (VE) virus in nature is usually systemic in humans, and encephalitic in equines. Virulences of VE virus strains for humans seem to correlate with lethalities for hamsters, and thus hamsters have been used as model animals to study mechanisms of virulence. Virulent strains of VE virus inoculated subcutaneously kill hamsters within three to six days and produce extensive necrosis of hematopoietic tissues and Purkinje cells of brain, focal hemorrhages in cerebral cortex, and abundant viral antigen in hematopoietic tissues stained with fluorescent antibody (1, 4). Strains which kill less than 20% of hamsters given small doses of virus (termed benign strains), produce few lesions and little or no stainable viral antigen in hematopoietic or brain tissues (4). However, growth curves of one virulent and one benign strain in hematopoietic tissues were found to be similar in magnitude and to differ only slightly in timing (1). Therefore, a discrepancy seemed to exist between the similar quantities of infectious virulent and benign VE viruses in hematopoietic tissues and the dissimilar quantities of viral antigens and histopathologies caused by them. It thus became necessary to extend observations of viral growth in hematopoietic tissues to additional strains. Since quantities of virus in blood are influenced by uptake of virus by tissues, blood clearance curves were also determined. This article records growth curves of four virulent and two benign strains of VE virus in blood, bone marrow, and intestinal-associated lymphoid tissues, and clearance curves from plasma of six virulent and three benign strains. Growth curves were determined after subcutaneous, intracranial, or intracardiac inoculations, and clearance curves were determined after intracardiac inoculation.

MATERIALS AND METHODS

Viruses and virus assays. The six hamster-lethal VE virus strains employed were Trinidad burro of subtype I, variety A (designated VE-I.A), 69Z1 (VE-I.B), 63U2 (VE-I.E), Fe 5-47et (VE-II), BeAn8 (VE-III), and 52049 (VE-III). The three VE strains of low hamster virulence were TC83 (VE-I.[A]), BeAr 35645 (VE-IV), and BeAr40403 (VE-IV). This antigenic subtyping and variety classification is that of

Young and Johnson (11). The Trinidad burro strain (11) was obtained from Fort Detrick labeled VE-I-2AC-8, after isolation from the brain of a burro; it had been passed an undetermined number of times in guinea pigs, and 14 times in chicken embryos. Strain 69Z1 was from human blood in Guatemala (10) and had been passed once in suckling mouse brain and twice in primary chicken embryonic cell cultures (CEC). Strain 63U2 was in the sixth suckling mouse brain passage from the brain of a sentinel hamster in Mexico (8). Strain Fe 5-47et was in the second mouse brain passage from a pool of *Aedes taeniorhynchus* collected in Florida (12). BeAn8 and 52049 were strains of Mucambo virus used as the ninth mouse brain passage from blood of a sentinel *Cebus* monkey in Brazil or as first Vero African green monkey cell cultural passage from *Zygodontomys brevicauda* in Trinidad (11). Strain TC83 was reconstituted human vaccine lot 3-2-L6. Strains BeAr35645 and BeAr40403 were Pixuna viruses in the fourth mouse passage from pools of *Anopheles nimbus* and of *Trichoprosopon digitatum*, respectively, in Brazil (11). To prepare stock virus suspensions, each virus was passed once in CEC prepared and used as described previously (7). Viruses were titrated by counting plaque forming units (PFU) in CEC grown in 8-cm^2 wells of plastic plates and maintained under agar medium (9) for 72 h at 37 C.

Inoculation of hamsters and harvest of tissues. Male hamsters were obtained from Lakeview Hamster Colony, Newfield, N.J. and were 5 to 8 weeks old for growth curve experiments unless indicated otherwise in the text. They were older (140–160 g) for clearance experiments to permit numerous serial bleedings.

For growth-curve experiments, each hamster was given 1000 PFU of virus in 0.2 ml of 1% bovine albumin in Hanks solution, pH 8.0, (BA) subcutaneously (sc). Volumes were 0.05 ml and given intracranially (ic) and intracardiacally (1 ml) (ica). Blood was obtained from orbital sinuses or hearts of ether-anesthetized hamsters, by using plastic syringes wet with heparin solution (200 U/ml) and 22-gauge needles. Organs for virus titrations were removed aseptically and ground in mortars in a small volume of BA with sterile Alundum (90 mesh). Enough BA was then added to make 10% (wt/vol) suspensions. To obtain bone marrow suspensions, both femora were cleaned of muscle tissue, split longitudinally with a sterile razor blade, marrow was scraped out with the tip of a fine forceps, and then ground with Alundum in 1.5 ml of BA. Suspensions of cells from Peyer's patches were made by dissecting five well-delineated areas of lymphoid tissue from the wall of the ileum, and grinding them in 1.5 ml of BA. Both marrow and Peyer's patch suspensions were considered to be approximately 1% (wt/vol). (Actual measurements of a few marrow suspensions showed this to be a valid assumption.) All suspensions were centrifuged at 10,000 $\times$ g for 30 min at 0 C. Bloods or supernatant fluids from tissue suspensions were stored in screw-capped vials (15 by 48 mm) in multiple samples at −60 C in an electric freezer.

For clearance experiments, hamsters were inoculated under light ether anesthesia via the intracardiac route by using plastic syringes and 23-gauge needles. At 1, 5, 10, 20, 40, 80, and occasionally 120 min after inoculation of $10^{6.8}$ to $10^{7.0}$ PFU of virus in 1 ml of BA containing 1 mg of Evan's blue, 0.3 to 0.5 ml of blood was taken from the orbital sinus of each hamster under light ether anesthesia by using plastic syringes wet with heparin (200 U/ml) and 22-gauge needles. Three to eight hamsters were successfully inoculated and serially bled for each virus tested. Hamsters which experienced cardiac tamponade, as determined when dead hamsters were autopsied, were excluded in calculations of clearance rates.

Measurements of virus and Evan's blue concentrations in blood for clearance curves. Dilutions (1:30) of the virus inoculum and each blood sample were made by adding 0.2 ml to a chilled (0 C) test tube containing 5.8 ml of sterile Hanks solution without phenol red. Test tubes were stored up to one-half hour at 0 C before they were centrifuged at 400 $\times$ g for 10 min at 0 C. Supernatant fluids were decanted into two screwcapped vials; an equal volume of BA was added to one vial to stabilize infectious virus for storage at −60 C in an electric freezer. The other sample was stored for up to 4 h at 0 C and then the concentration of Evan's blue was measured spectrophotometrically at a wavelength of 610 μm. The standard curve was linear between 0 and 130 μg of Evan's blue per ml.

The dilution of the virus inoculum which occurred after inoculation into the vascular system was calculated by dividing the Evan's blue concentration of the virus inoculum by that of each plasma. The inoculum virus concentration was then divided by this dilution factor (usually 60–90) to yield the theoretical virus concentration (Vo) in blood if no virus were cleared. Actual virus concentrations (V) of diluted bloods were determined by counting PFU on CEC monolayers under agar and multiplying by 2 to compensate for the 1:2 dilution made by addition of BA to plasma before storage for virus assay. These virus concentrations were then divided by Vo to yield the fraction of virus cleared by mechanisms other than dilution. The $-\log_{10}$ of the fraction V/Vo is mathematically equivalent to the $\log_{10}$ PFU of virus cleared. Significances of differences in slopes of calculated regression lines were compared by an analysis of covariance at the $P < .05$ level and differences in V/Vo at 10 min were evaluated by use of the Student t test ($P < .05$) with the aid of an Olivetti Programma 101 computer.

RESULTS

Growth curves of virulent and benign VE viruses in blood and hematopoietic tissues after subcutaneous inoculation of hamsters. Three virulent VE-I strains reached higher titers in blood than benign viruses, VE-I.(A) and VE-IV (strain BeAr34645) (Fig. 1). Viremia curves for the virulent VE-I.A and I.B viruses studied herein were similar to that previously published for virulent subtype I.E strain 63Z21 (1). Therefore, data for five hamsters with each virus were combined and plotted as one curve in

Fig. 1, with range bars indicating maximum variations.

Benign VE-IV virus produced low titers in bone marrow and Peyer's patches, but benign VE-I.(A) virus reached and maintained high titers in marrow and Peyer's patches (Fig. 1). Previously reported curves of virulent VE-I.E virus in bone marrow and mesenteric lymph nodes are plotted here for reference (1). The curves of VE-I.(A) virus in bone marrow and Peyer's patches represent data from new experiments, but they are similar to curves previously reported (1). It is noteworthy that the levels which VE-I.(A) virus attained in bone marrow

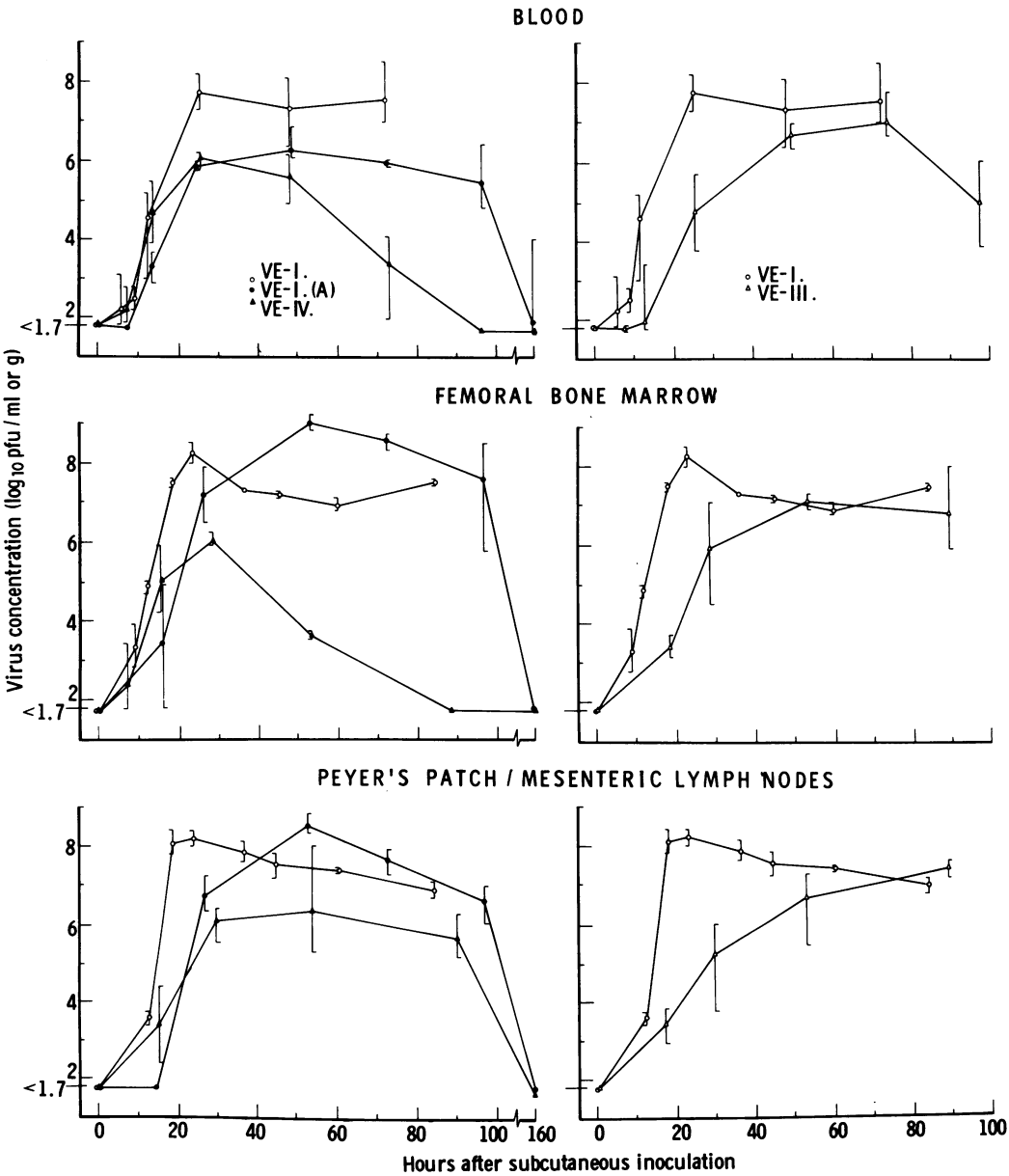

FIG. 1. *Concentrations of infectious VE viruses in bloods, femoral bone marrows, and Peyer's patches or mesenteric lymph nodes of hamsters after subcutaneous inoculation. Each point represents a geometric mean titer of five hamsters except VE-I in blood which is based on 15 hamsters and VE-I in marrow and mesenteric lymph nodes which represent three hamsters per point.*

did not represent blood contamination since titers were 100 times greater than those in blood.

The viremia curve of virulent VE-III virus, strain BeAn8, ascended less rapidly and reached lower levels than that of virulent VE-I viruses. Yet hamsters sickened and died by 90 h after sc inoculation. Growth of VE-III virus in marrow and Peyer's patches was also delayed in comparison with virulent VE-I.E virus, but titers eventually reached similar levels (Fig. 1).

Lethalities and virus titers of virulent and benign VE viruses in brain after intracranial inoculation of hamsters. Previous studies suggested that although attenuated VE-I.(A) virus given ic reached titers in brain comparable to virulent VE-I.E virus, there was no increase in mortality (1). Since the sample of hamsters was small in that experiment, and the implications were important, the experiment was repeated twice with larger numbers of hamsters and with another benign virus, VE-IV.

In the first experiment, 15 hamsters, 6 weeks of age, were inoculated ic with 0.05 ml containing 1,000 PFU of benign VE-I.(A) virus, 14 with VE-IV virus, and 5 with virulent VE-I.A virus as a control. Five hamsters of each group inoculated with the benign viruses were sacrificed 50 h after inoculation, by which time VE-I.(A) virus titers in brain could be expected to have reached maximal levels (1). No animal inoculated with either benign virus appeared sick at that time. In contrast, by 50 h virulent VE-I.A virus had killed one of five hamsters and the other four were moribund when sacrificed. The geometric mean virus titers in brains of these hamsters were $10^{6.5}$ PFU per g for attenuated VE-I.(A), $10^{5.7}$ for benign VE-IV, and $10^{6.8}$ for virulent VE-I.A. During 2 weeks of observation, only 1 of 10 hamsters inoculated with benign VE-I.(A) died on day 11 although 6 were ill between days 7 and 10. Three of 10 animals inoculated with benign VE-IV virus died on days 4, 7, and 8 and another was sick between days 6 and 10, but recovered completely.

In the second experiment, 55 hamsters, 10 weeks of age, were inoculated ic with 0.05 ml containing 1,000 PFU of VE-I.(A), 55 with VE-IV, and 25 with virulent VE-I.E virus. Five hamsters were selected at random from each group daily, and blood and brain samples were taken for virus titration. Of the remaining hamsters observed for 2 weeks, only 2 of 20 inoculated with VE-I.(A) died (days 5 and 7 after inoculation) and 1 of 20 with VE-IV (day 6), whereas 10 of 10 died with virulent VE-I.E virus (days 3 and 4). All hamsters appeared ill 4 to 6 days after receiving VE-I.(A) virus and 3 to 5 days after VE-IV virus. Each virus grew to high titers in brain (Fig. 2). Even though maximal titers of the benign strains of VE virus were lower than virulent VE-I.E in brain after ic inoculation, they were similar to levels of VE-I.E virus in brain before death after sc inoculation (1).

Thus, concentrations of benign VE viruses in brain did not explain their failure to kill hamsters uniformly like virulent strains. Intracranial inoculation increased morbidity of two benign VE viruses but lethalities were not increased to the level of uniform and rapid death caused by virulent VE-I viruses.

Lethalities and virus titers of virulent and benign VE viruses in hematopoietic tissues and blood after intracardiac inoculation of hamsters. Hamsters were given 1,000 PFU of virulent VE-I.B or benign VE-I.(A) or VE-IV viruses via the ica route in 1 ml of BA. As controls, some hamsters were similarly inoculated sc. Hamsters were sacrificed for quantitation of virus in organs when those given virulent virus were sick at 36 h. Virulent VE-I.B virus produced high titers of infectious virus in blood, marrow, and spleen after ica and sc inoculation (Table 1). Deaths occurred more rapidly after ica than after sc inoculation. Mean titers of benign VE-I.(A) virus at 36 h after ica or sc inoculation were comparable in bone marrow, Peyer's patches, and spleen, but the virus titer in blood was slightly higher, and deaths occurred in ica inoculated animals (Table 1).

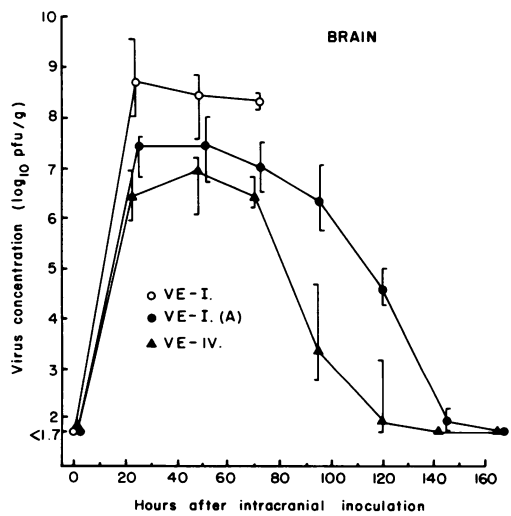

FIG. 2. *Concentrations of infectious VE viruses in brains of hamsters following intracranial inoculation. Each point represents a geometric mean titer for five hamsters.*

TABLE 1. *Lethalities and virus levels in blood, femoral bone marrow, Peyer's patch, and spleen 36 h after intracardiac or subcutaneous inoculation of hamsters with virulent (I.B) or benign (I.[A] and IV) VE viruses*

Virus and strain	Route of inoculation	Fraction of hamsters dying	Days of death	Virus concentration as $-\log_{10}$ PFU per g or ml[a]			
				Blood	Marrow	Peyer's patch	Spleen
VE-I.B	Intracardiac	3/3	1.5–2[b]	8.2	8.1		8.2
69Z1	Subcutaneous	10/10	3–5	8.0	7.3	7.8	7.2
VE-I.(A)	Intracardiac	5/13	5–13[c]	7.0	8.2	8.7	8.1
TC83	Subcutaneous	0/10		6.0	8.3	8.4	8.1
VE-IV	Intracardiac	0/8		5.1	5.0	5.6	5.3
BeAr35645	Subcutaneous	0/10		6.0	4.8	6.2	5.8

[a] Geometric means of five hamsters six weeks of age.
[b] Moribund at 36 h after inoculation and sacrificed at this time.
[c] Died on days 5, 6, 8, 12, and 13. Cardiac tamponade was excluded by autopsy.

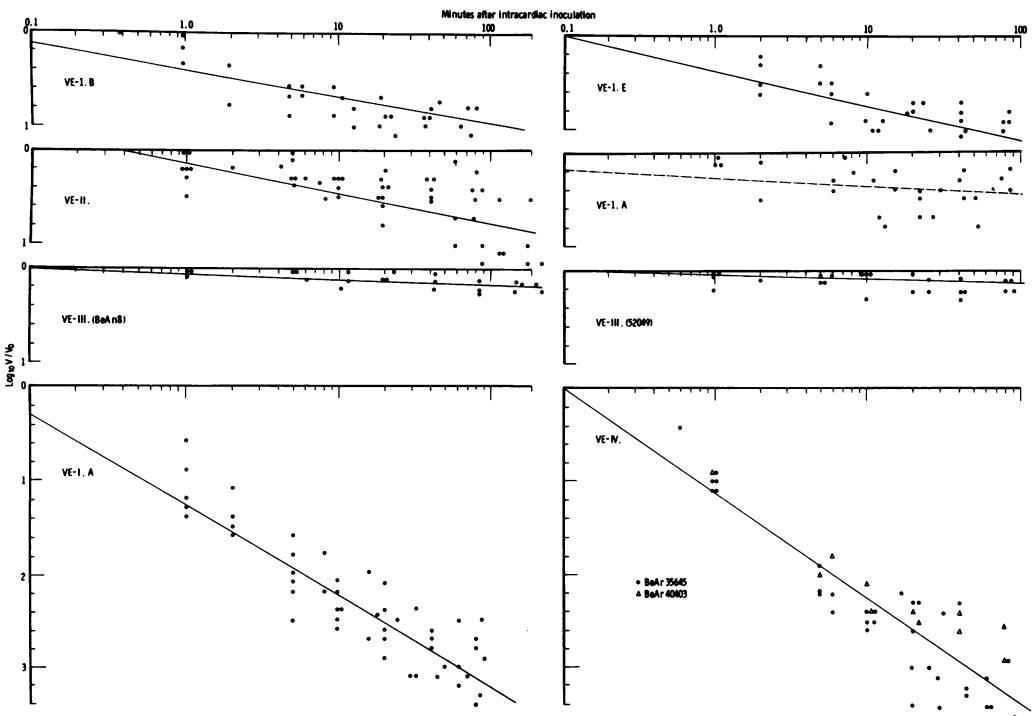

FIG. 3. *Clearance rates of nine strains of VE virus of subtypes I–IV from hamster plasmas after intracardiac inoculation. Each point represents the value V/Vo (or $-\log_{10}$ PFU cleared) for one blood specimen from one hamster.*

VE-I.(A) virus titers in spleen and marrow were like those of virulent VE-I.B virus. Virus titers of benign VE-IV virus in blood and hematopoietic tissues were similar after ica and sc inoculation, but were significantly lower than those of benign VE-I.(A) or virulent VE-I.B virus (Table 1). VE-IV virus did not kill hamsters after ica or sc inoculation of 1,000 PFU.

Clearance rates from plasma of virulent and benign VE viruses after intracardiac inoculation of hamsters. Six strains of virulent VE virus of subtypes I, II, and III were cleared at slow rates from hamster blood (Fig. 3). Two VE-III strains were essentially not cleared within 100 min. In contrast, three benign VE viruses (the benign TC83 strain and two strains

of subtype IV) were cleared rapidly. Correlation coefficients of seven of the eight regression lines obtained when $-\log_{10}$ V/Vo values were plotted versus $\log_{10}$ min after inoculation were >0.79, corresponding to $P < 0.01$. The curve of VE-I.A which had a correlation coefficient of only 0.234 ($P > 0.10$) was drawn as a dashed line. When differences in slopes of calculated regression lines were compared at $P < 0.05$ level by analysis of covariance, clearance rates of virulent strains VE-I.B, I.E., II, and III were significantly lower than those of three benign strains. When the amounts of each virus cleared in 10 min were compared using the Student t test, rapid clearance of virus from hamster plasma again correlated with low hamster virulence. Ten minutes after intracardiac inoculation, 2.2 and 2.3 $\log_{10}$ PFU of benign VE-I.(A) and VE-IV viruses had been cleared, whereas values for the virulent viruses were VE-I.E, 0.8; I.B, 0.7; II, 0.5; III (BeAn8), 0.1; and III (52049), 0.1 $\log_{10}$ PFU cleared.

Tests for differential inactivation of virulent and benign VE viruses by hamster blood or tissues. To test the possibility that different clearances of VE viruses from hamster plasmas were due to different rates of inactivation of virus by blood or tissues, 10^6 PFU of three virulent strains (VE-I.E, I.B, and III) and of two benign strains (VE-I.(A) and IV) were mixed separately with 2-ml samples of either whole, freshly-obtained hamster blood containing heparin at final concentration about 10 U per ml or a 10% (wt/vol) suspension of hamster liver in BA. The mixtures were incubated at 37 C in a water bath and 0.5-ml samples were removed from duplicate tubes at 1, 10, 20, and 60 min for the blood experiment and 2, 20, 40, and 80 for the liver suspension experiment. Blood and liver suspensions were centrifuged before titration of virus in plasma, packed erythrocytes, or supernatant fluids of liver suspensions. No inactivation of virus was observed with any of the strains at any of the times tested (Table 2). Virus titers of erythrocyte fractions were comparable to those of plasmas, indicating no significant adsorption of virus to blood cells.

DISCUSSION

It is apparent from these and previous results (1) that lethalities of VE viruses for hamsters were not related directly to rates of ascent or heights of growth curves of infectious virus in hematopoietic tissues which develop major histopathologic lesions. Only viremia correlated with virulence since virulent VE viruses reached higher levels in blood than benign strains. Intracranial inoculation of two benign VE viruses into hamsters increased morbidities of both, but mortalities did not rise to the 100% levels of virulent VE-I and VE-III viruses. Intracardiac inoculation of two benign VE viruses which provided rapid contact with hematopoietic tissue, did not change lethalities to those of virulent strains even though one benign strain of virus reached titers in marrow, Peyer's patch, and spleen, like those of a virulent strain.

The discrepancy between similar concentrations of infectious virulent and benign VE viruses in hematopoietic and brain tissues and the striking differences in content of viral antigens detected by fluorescent antibody (4) may provide a key to understanding the mechanisms of virulence of VE viruses for hamsters. Viral antigen in disproportion to infectious virus could mean that abortive viral infection was occurring in cells of target tissues and that accumulation of incomplete virus might relate to cellular destruction, histopathology, illness, and death.

Clearance of infectious virus from plasma correlated inversely with virulence. These results were similar to findings with Mengo virus which showed that an avirulent strain was more rapidly cleared from mouse plasma than a virulent strain (2). However, in that system the avirulent strain was inactivated by a mouse liver homogenate, whereas benign VE viruses were not inactivated by hamster blood or liver homogenate.

The factors which produced differences in viral clearance rates among VE viruses are unknown. Since benign viruses were cleared

TABLE 2. *Failure of hamster blood or liver suspension to inactivate virulent (I.E, I.B, and III) or benign (I.[A] and IV) VE viruses*

Virus and strain	Titers[a] of virus as $-\log_{10}$ PFU per 0.2 ml of			
	Plasma	Erythrocytes	Liver suspension	Control BA
VE-I.E	4.5	4.6		4.4
63U2			4.3	4.4
VE-I.B				
69Z1			4.6	4.8
VE-III	4.2	4.3		4.3
BeAn8				
VE-I.(A)	4.4	4.4		4.5
TC83			4.7	4.6
VE-IV	4.6	4.6		4.6
BeAr35645			4.4	4.3

[a] Means of two mixtures of blood or liver with virus after 60 or 80 min, respectively, at 37 C.

rapidly and virulent viruses slowly, it is possible that host defense mechanisms react more rapidly to benign than virulent VE viruses to result in prevention of illness and death of hamsters. Benign VE viruses might be taken up faster than virulent viruses by tissue cells including phagocytes since benign VE-I.(A) virus has been found to adsorb more efficiently than a virulent VE-I.E strain to monolayers of BHK21 hamster kidney cells in culture (9), and in another system, a low virulence clone of encephalomyocarditis virus was shown to have an increased affinity for mucopolysaccharides (3). Degrees of aggregation of virulent and benign virions might differ to influence phagocytosis and clearance in vivo. Low clearance rates of virulent strains could be due to saturation of receptors by noninfectious particles, such as described for poliovirus type I (4) although saturation of receptors was not responsible for the low level of clearance observed with a strain of Sindbis virus in mice (5). Further studies of these possibilities and of host defense mechanisms including interferon production and blockade of the reticuloendothelial system obviously need to be done in hamsters infected with virulent and benign VE viruses. To date only preliminary experiments have been done with hamsters pretreated with Thorotrast (1 ml of 25% wt/vol of colloidal thorium dioxide inoculated intraperitoneally) and inoculated with 1,000 PFU of attenuated VE-I.(A) virus. This type of blockade of the reticuloendothelial system produced deaths of three of four hamsters in one experiment and eight of ten in another; Thorotrast alone was not lethal.

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