Histopathology and Distribution of Viral Antigens in Hamsters Infected with Virulent and Benign Venezuelan Encephalitis Viruses

P. B. Jahrling, PhD and W. F. Scherer, MD

Lethalities and virulences of Venezuelan encephalitis (VE) viruses for hamsters were found to correlate with severity of histopathologic lesions in hematopoietic and brain tissues. Highly virulent strains (subtype I) destroyed marrow and lymphoid cells rapidly and produced intestinal wall damage; focal brain hemorrhages and destruction of Purkinje cells also occurred within the 4 to 5 days between subcutaneous inoculation and death. Like subtype I virus, a slightly less virulent strain (subtype II) also caused necrosis of bone marrow and brain lesions, but only minimal lymphoid cell damage occurred. The less virulent subtype III VE virus, which killed hamsters between 4 and 14 days after inoculation, usually caused no lesions in hematopoietic tissues, and deaths were related chiefly to hemorrhagic brain lesions and necrosis of Purkinje cells. Two VE viruses, benign for hamsters (the TC-83 attenuated vaccine strain and subtype IV), usually caused no necrosis of hematopoietic or brain tissues; focal extravasations of blood and swollen glial cells were found in brains of the rare hamsters that died. The degrees of necrosis seen in tissues stained with hematoxylin and eosin correlated with the quantities of viral antigens detected by fluorescent antibody, except in pancreas and small intestinal smooth muscle and glands, where antigens of subtype I virus were present without morphologic damage (Am J Pathol 72:25–38, 1973).

VENEZUELAN ENCEPHALITIS (VE) viruses are currently considered to exist in nature as four antigenic subtypes which cross-protect but are distinguishable by *in vitro* immunologic tests.[1] In nature, virulences of VE viruses relate to the diseased hosts, man and equines; obviously, extensive studies of virulence in significant numbers of these hosts are impractical. Therefore animal models must be employed; the hamster is useful because hamster lethalities of VE viruses correlate quite well, thus far, with human morbidity though not with equine mortality. Histopathologic lesions in hamsters were

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initially studied after infection by a human virulent strain of VE virus, subtype I, variety E and a human-attenuated vaccine strain (TC83).[2] The primary target organ of the virulent virus was found to be the hematopoietic system, although hemorrhagic lesions occurred in brain and intestinal wall late in the course of disease; attenuated virus produced minimal or no lesions. Subsequently it was recognized that VE viruses in nature display a spectrum of virulence for hamsters. Therefore, pathologic studies were extended to other strains and subtypes, and fluorescent antibody was used to search for viral antigens. This article records the histopathologic lesions and distributions of viral antigens in tissues of hamsters infected with virulent VE viruses of subtypes I, II and III and benign viruses of subtypes I and IV.

Materials and Methods

Viruses and Virus Assays

The five hamster-lethal VE virus strains employed were Trinidad burro, subtype I variety A (VE-I.A); 69Z1, subtype I variety B (VE-I.B); 63U2 (VE-I.E); Fe 5-47et (VE-II); and BeAn8 (VE-III).[1,3] The two benign VE strains were TC-83 [designated VE-I.(A)] and BeAr35645 (VE-IV).[1] The Trinidad burro strain was obtained from Fort Detrick as VE-I-2AC-8; it had been passed an undetermined number of times in guinea pigs and 14 times in chicken embryos after isolation from the brain of a burro. Strain 69Z1 was from human blood obtained in Guatemala and had been passed once in suckling mouse brain and twice in primary chicken embryonic cell cultures (CEC). Strain 63U2 was in sixth suckling mouse brain passage from brain of a sentinel hamster exposed in Mexico. BeAn8, a strain of Mucambo virus, was used as ninth mouse brain passage from blood of a sentinel *Cebus* monkey in Brazil. Strain TC83 was reconstituted human vaccine lot 3-2-L6. Strain BeAr 35645 (Pixuna virus) was in fourth mouse passage from a pool of *Anopheles nimbus* collected in Brazil. To prepare stock suspensions, each virus was passed once in CEC which was prepared and used as described previously.[4] Viruses were titrated by counting plaque forming units (PFU) in CEC grown in 8 sq cm wells of plastic plates and maintained under agar medium [5] for 72 hours at 37 C.

Hamsters and Their Inoculation

Male Syrian golden hamsters were obtained from Lakeview Hamster Colony, Newfield, NJ and were used when they were 5 to 8 weeks old. Hamsters were inoculated subcutaneously over the back with 0.2 ml of 1% bovine albumin in Hanks' solution pH 8.1, containing 1000 PFU of virus.

Harvests of Tissues for Histopathologic or Immunofluorescence Examination

Hamsters were sacrificed with chloroform or were autopsied within several hours of death. Tissues were placed directly in 10% formalin, embedded in paraffin and sectioned (not less than 3 days after fixation) before staining with hematoxylin and eosin. Femurs were fixed for at least 24 hours in 10% formalin and were then decalcified for 3 to 5 hours in 7% nitric acid, before being cut longitudinally with a

razor blade and returned to 10% formalin for 24 hours or more. Tissues for immunofluorescence were immersed in Cryoform (International Equipment Co) and rapidly frozen in the cryostat within ½ hour of removal from the hamster. Bone marrow was sectioned by scraping cells from split femurs, suspending them in 1 ml of 1% bovine albumin and centrifuging at 400g for 10 minutes in a 3 × 10 mm plastic test tube (Beckman "microtube"). The entire tube was mounted and frozen in Cryoform and sectioned through the packed cell pellet. Frozen sections 6 μ thick were transferred to glass slides and allowed to dry in air. They were then immersed in cold 0.1 M phosphate-buffered sodium chloride (0.15 M) at pH 7.0 (PBS) for 10 minutes, fixed in cold (0 C) analytical grade acetone for 30 seconds, then reimmersed in PBS for 10 minutes. Sections were usually stained with fluorescent reagents immediately. Occasionally, fixed and washed frozen sections were dried in air and either stored at 5 C for up to 1 week or at −20 C for longer periods. Every fifth frozen section was stained with hematoxylin and eosin.

Immunofluorescence Staining of VE Viral Antigens

The indirect procedure employed immune anti-VE virus guinea pig serum and fluorescein-labeled anti-guinea pig globulin prepared in goats (Cappel Laboratories, Downington, PA). Antiserum was pooled from 2 guinea pigs that had been inoculated subcutaneously at three 1-week intervals with 10^6 PFU of live attenuated VE-I.(A) virus, and challenged 2 weeks later by subcutaneous inoculation of 1000 PFU of virulent VE-I.A virus strain Trinidad burro. Guinea pigs were bled 2 weeks after challenge. In a virus-dilution plaque-reduction neutralization text in CEC, a 1:10 dilution of pooled antiserum neutralized 6.4 $\log_{10}$ PFU of VE-I.(A) virus. The $\log_{10}$ neutralization indices against the other viruses tested were VE-I.A, 4.7; VE-I.B, 4.3; VE-III, 2.8; VE-IV, 2.4.

To suppress nonspecific fluorescence, the guinea pig antiserum was absorbed with normal hamster liver powder, prepared by acetone precipitation,[6] and with normal lyophilized hamster plasma. Goat anti-guinea pig globulin preparation labeled with fluorescein isothiocyanate (designated FG for fluorescein globulin) was also absorbed with hamster liver powder and lyophilized hamster plasma. Specificity of FG was checked by immunoelectrophoresis against guinea pig, hamster and calf sera; only guinea pig serum precipitated with FG.

Frozen sections were flooded with a 1:4 dilution of hyperimmune or normal (control) guinea pig serum in PBS at pH 7.0, and incubated in moist chambers at 37 C for 30 minutes. Antiserum was then blotted off, and sections were washed in groups of ten for 10 minutes in each of three 600-ml changes of PBS on a rotary shaker. Sections were then flooded with a 1:2 dilution of FG in PBS (pH 7.0) mixed with an equal volume of rhodamine-conjugated albumin (Microbiological Associates, number 50-798), incubated for 30 minutes at 37 C and washed as above. Coverslips were then mounted with a semipermanent mounting medium consisting of 20% Elvanol® in buffered glycerol (pH 7.2) prepared as described elsewhere.[6] Stained sections were examined under a Leitz Ortholux fluorescent microscope equipped with an HBO-200 Orzan mercury arc lamp, BG-12 exciter filter, OG-1 barrier filter, a dry darkfield condenser (D = 0.80) and achromatic objectives. Black and white photographs were obtained with a Yashica TL camera back, a 12 × objective, and Tri-X film (Kodak, ASA-400), exposed for 20 to 60 seconds.

A positive control, to test the ability of the fluorescence reagents to detect the presence of viral antigens, was performed as follows. Hamster embryonic cell monolayers, grown on coverslips and infected with a multiplicity of approximately 10, were stained 30 hours after virus inoculation. Intense fluorescence was seen in the

cytoplasm of infected cells for all four viruses tested, although the fluorescence with VE-IV was somewhat less intense than for VE-I.B, VE-III, or VE-I.(A).

The following negative controls for specificity were run for each tissue section tested: a) infected tissue + normal guinea pig serum (undiluted) + FG; b) infected tissue + rabbit anti-VE-I.(A) serum + FG; and c) normal tissue + immune guinea pig serum + FG. Only when these three controls were negative for fluorescence was the test considered valid.

Results

Histopathologic Lesions in Hamsters Infected with Lethal VE Viruses of Subtypes I, II and III or with Benign Viruses of Subtypes I and IV

Hematopoietic Tissues

Virulent VE-I.A and I.B viruses produced extensive necrosis of femoral bone marrow, white pulp of spleen and Peyer's patches similar to that already described for VE-I.E virus.[2] Cellular depletion and necrosis also occurred in thymus of young 6-week-old hamsters infected with VE-I viruses (Figure 1).

VE-II virus caused lesions in bone marrow similar to VE-I viruses, but in 3 dead and 1 moribund hamster examined 4 days after inoculation, only minimal or no necrosis was found in white pulp of spleen or Peyer's patches.

The tissue lesions produced by lethal VE-III virus were different from those of VE-I or VE-II viruses. In 6 of 7 hamsters dying 4 to 14 days after subcutaneous inoculation, femoral bone marrow was normal or only slightly congested (Figure 2), and there was only partial necrosis of marrow and Peyer's patch in the seventh dead hamster (Figures 3 and 4; Table 1). In the sick hamster sacrificed on day 4 after subcutaneous inoculation, there also was minimal necrosis of marrow and Peyer's patch cells. White pulp of spleen was normal in all hamsters examined after infection with VE-III virus.

The benign attenuated VE-I.(A) strain produced no hematopoietic lesions except transient depletion of lymphoid tissue followed by hyperplasia, as reported previously.[2] Autopsies of healthy hamsters sacrificed 1, 2, 3, 4 and 7 days after subcutaneous inoculation of benign VE-IV virus revealed no lesions in hematopoietic organs, including thymus. The low mortality rate (2%) of VE-IV virus and unpredictable times of death[7] made it difficult to examine significant numbers of dead hamsters histopathologically.

Brain and Other Tissues

Detailed examinations of brains from hamsters moribund from infection with VE-I.A, I.B or I.E viruses revealed histopathologic lesions

Table 1—Extent and Temporal Distribution of Histopathologic Lesions in 10 Hamsters Inoculated Subcutaneously with Venezuelan Encephalitis Virus Subtype III (VE-III)

			Tissue lesions							
Hamster No.	Status	Day*	Femoral bone marrow	Peyer's patch	Spleen	Brain†	Intestinal wall	Kidney	Liver	Heart
1	N	2	—	—	—	—	—	—	—	—
2	N	3	—	—	—	±	—	—	—	—
3	S	4	±	±	—	±	—	—	—	—
4	D	4	—	—	—	+	—			
5	D	5	—	—	—	+	—	—	—	—
6	D	5	±	±	—	+	—			
7	D	6	—	—	—	+	—	—	—	—
8	D	7	—	—	—	+	—	—	—	—
9	D	11	—	—	—	+	—	—	—	—
10	D	14	—	—	—	+	—	—	—	—

* Time after subcutaneous inoculation with VE-III

† Severe necrosis of blood vessels and hemorrhage, without inflammatory cells were seen on days 5 to 7; perivascular cuffing with mononuclear and plasma cells was seen on days 11 and 14

N = normal, S = sick, D = dead; — = normal, ± = slight necrosis, + = severe necrosis

not reported previously.[2] Purkinje cells were often hyperchromatic or decreased in number as early as 48 hours after subcutaneous inoculation, like those shown at 72 hours in Figure 5. Perivascular cuffing was seen when survival was prolonged for 1 or 2 additional days by replacement of electrolytes and fluids or by preinoculation of the interferon inducer poly I:C before VE-I.E virus. VE-II virus also produced focal hemorrhages in brain and necrosis of Purkinje cells in 4 of 4 dead or moribund hamsters. With VE-III virus, various degrees of hemorrhage were present in brains of 7 dead animals (Figure 6), and perivascular cuffing was seen in 2 hamsters surviving to 11 and 14 days (Figure 7; Table 1).

Contrary to our previous report,[2] lesions were present in brains of the occasional hamsters that died after infection with attenuated VE-I.(A) virus. There were focal extravasations of blood cells around small vessels in the brains of 7 hamsters dying on days 5, 5, 6, 6, 9, 17 and 18; swollen glial cells were widely distributed throughout the cerebral cortex in the 3 hamsters dying late on days 9, 17 and 18 (Figure 8). No neuronal damage was recognized. No lesions were seen in brains of 5 well hamsters sacrificed as mentioned above after inoculation of VE-IV virus. However, brain from 1 hamster dying 8 days after infection with VE-IV virus showed swollen glial cells similar to those noted with VE-I.(A) virus (Figure 8).

Focal infarctions of liver were occasionally noted with VE-I viruses. VE-II and VE-III viruses produced no lesions of intestinal wall, nor did they produce lesions of kidney, liver or heart muscle (Table 1).

VE Viral Antigens in Tissues of Hamsters Infected with Lethal Viruses of Subtypes I and III or with Benign Viruses of Subtypes I and IV

Hematopoietic Tissues

Antigens of VE-I.A, I-B and III viruses were detected by use of fluorescent antibody in femoral bone marrow, Peyer's patch and spleen within 24 to 48 hours of subcutaneous inoculation (Table 2). Almost all nucleated cells in bone marrow showed specific fluorescence 24 hours after infection with VE-I.A and VE-I.B viruses; by 48 hours, large quantities of fluorescent cell debris were observed (Figure 9). VE-I.A and VE-I.B viral antigens were likewise found in most lymphoid cells of Peyer's patch (or intestine-associated lymphoid tissue) within 24 hours of infection (Figure 10). White pulp of spleen contained large quantities of VE-I.A and VE-I.B viral antigen by 24 hours. By 48 hours fluorescence was still concentrated in white pulp, but was widely distributed in red pulp as well (Figure 11). Cellular debris which was prominent in hematoxylin and eosin sections of spleen was intensely fluorescent with both virulent VE-I viruses.

VE-III viral antigens were seen in only two of six bone marrow sections, and in less than 1% of nucleated cells. VE-I.(A) antigens were likewise detected in only one of eight hamster spleens as a narrow band of intensely fluorescent cells and necrotic cell debris around the periphery of one area of white pulp.

VE-IV viral antigens were not detected in any hematopoietic tissue obtained from 2 hamsters 48 hours after subcutaneous inoculation (Table 2).

Brain and Other Tissues

By 48 hours after subcutaneous inoculation of VE-III virus, clusters of Purkinje cells fluoresced, and after 72 hours, almost all Purkinje cells contained antigens of VE-I or III viruses (Figure 12). VE-I viral antigens were detected in brain cells associated with small blood vessels although there was no unequivocal immunofluorescent evidence that endothelial cells in brain were infected (Figure 13).

There was intense focal fluorescence in pancreases of all 14 hamsters infected with VE-I virus strains (Table 2). Fluorescence was usually confined to small foci of acinar cells (Figure 14), but occasionally islet

Table 2—Detection of Viral Antigen by Immunofluorescence in Tissues of Hamsters Inoculated Subcutaneously with Lethal or Benign VE Viruses

Tissue	Hours after inoculation	No. specimens **positive** for VE viral antigen/ No. tested*				
		Lethal			Benign	
		I.A	I.B	III	I.(A)	IV
Marrow	24	**2**/2	**2**/2	0/2	0/2	
	48	**2**/2	**2**/2	1/2	1/2	0/2
	72	**2**/2		1/2	0/2	
Peyer's patch	24	**1**/1	**2**/2	0/1	0/3	
	48	**4**/4	**4**/4	**1**/2	1/3	0/2
	72	**3**/3		1/2	0/2	
	96	**1**/1				
Spleen	24	**1**/1	**2**/2	0/2	1/3	
	48	**3**/3	**4**/4	1/2	1/3	0/2
	72	**3**/3		1/2	0/2	
	96	**1**/1		0/2		
Brain	24	**1**/1		**1**/1	0/1	
	48	**2**/2	**2**/2	**2**/2	0/1	0/2
	72	**2**/2	**1**/1	**2**/2	0/2	
	96	**1**/2		**2**/2		
Pancreas	24	**1**/1	**2**/2	1/2	0/3	
	48	**3**/3	**4**/4	**1**/2	1/3	0/2
	72	**3**/3		0/2	0/2	
	96	**1**/1		1/2		
Intestinal wall smooth muscle	24	0/1	**1**/2	0/1	0/3	
	48	3/4	**4**/4	**1**/2	1/3	0/2
	72	**3**/3		0/2	0/2	
	96	**1**/1		0/2		
Brunner's glands	24	**1**/1	**2**/2	0/1	0/3	
	48	**4**/4	**4**/4	**1**/2	0/3	0/2
	72	**3**/3		1/2	0/2	
	96	**1**/1		1/2		

* Positive specimens are shown in **bold face** if viral antigen was detected in >10% of cells in the entire section. In addition, one or two specimens of the following tissues were tested for each virus, at 48 and 72 hours after subcutaneous inoculation: liver, lung, skeletal muscle, myocardium, kidney and thymus. All were uniformly negative, except for occasional isolated cells in liver and lung.

cells contained viral antigens (Figure 15). VE-I viral antigens were also detected in smooth muscle cells of small intestine in 12 of 15 specimens examined 24 to 96 hours after subcutaneous inoculation (Figure 16; Table 2). Antigens were seen in glandular tissue (Brunner's

glands) of small intestine in all of 15 specimens (Figure 16). VE-III viral antigens were detected in small clusters of smooth muscle cells in only one of seven specimens examined and in one of seven sections of intestinal glands. Smooth muscle fluoresced in one of eight sections of intestine examined after VE-I.(A) virus infection. VE-IV viral antigens were not detected in these tissues of 2 hamsters 48 hours after inoculation (Table 2).

Discussion

These experiments and a previous histopathologic study of hamsters infected with one virulent and one attenuated VE virus [2] have revealed that virulent VE viruses have two target tissues in hamsters, and that development of histopathologic lesions correlated with the degree of virulence of the virus strain, as measured by its lethality and incubation period. The two target tissues were the hematopoietic system (chiefly bone marrow, lymph nodes including Peyer's patches, splenic white pulp and thymus) and the brain. With highly virulent subtype I VE viruses, all hamsters died within 3 to 5 days after subcutaneous inoculation, and the major lesions were necrosis of marrow and lymphoid elements of the hematopoietic system, hemorrhages in brain and damaged Purkinje cells. Peyer's patch lesions were usually severe and resulted in intestinal wall damage. Focal infarcts of liver were possibly caused by emboli from thrombi in intestinal vessels. As virulence diminished slightly with subtype II and III VE viruses, hamster illnesses began later, on the third to fifth days after inoculation; occasional hamsters survived for 10 to 20 days. In these animals, less damage occurred to lymphoid cells, and with subtype III virus, intestinal lesions were absent. Subtype II virus caused severe necrosis of bone marrow, whereas subtype III did not in most hamsters. Both viruses caused focal brain hemorrhages and injured Purkinje cells, but in a few hamsters dying late with subtype III virus, perivascular cuffing also occurred. Thus the pathogenesis of subtype I-VE virus infections of hamsters involved lesions of marrow, lymphoid and brain tissues. With subtype II virus, lymphoid lesions were minimal or absent, but marrow and brain lesions were still usually sufficient to cause rapid death. With subtype III virus, hamsters died of brain lesions, usually without detectable damage to hematopoietic cells.

The benign VE viruses of subtype IV and the attenuated TC83 vaccine strain derived from subtype I, variety A, caused no severe lesions in hamsters. There was transient depletion of hematopoietic

tissues followed by hyperplasia with TC-83 virus; in the few hamsters that died, there were focal extravasations of blood around small vessels of brain and swollen glial cells in cerebral cortex.

The results of staining tissues with fluorescent antibody confirmed the viral origin of lesions in marrow, Peyer's patch and spleen caused by subtype I viruses, since these damaged tissues fluoresced intensely. Purkinje cell damage and perivascular accumulations of cells in brain also correlated with detectable viral antigens, since these cells fluoresced after infection with lethal subtypes I and III viruses. In contrast, the normal marrows, Peyer's patches and spleens of hamsters infected with lethal subtype III or the two benign subtype I.(A) and IV viruses fluoresced only minimally or not at all. Yet the fluorescent reagents employed detected antigens of these viruses grown in hamster cell cultures, and VE-I.(A) virus was used to make the antibody.

Fluorescent antibody staining also detected subtype I viral antigens in some tissues without inflammation or degeneration, namely, acinar and islet cells of pancreas and smooth muscle and Brunner's glands of small intestine. Detection of VE viral antigens in pancreatic acinar cells has been previously reported for mice [8] and guinea pigs.[9] These cells fluoresced uncommonly after infection with subtype III or TC-83 virus. How infection of these tissues without histopathologic changes influences virulence and pathogenesis is unknown. Nevertheless, with this exception, the development of lesions in virulent VE virus infections of hamsters is now understood sufficiently to begin studies of viral components and the mechanisms responsible for pathologic changes.

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Sections of tissues obtained from 5- to 8-week-old hamsters inoculated subcutaneously with VE viruses and stained with hematoxylin and eosin or by the indirect fluorescent antibody technic. The hamsters shown in Figures 2 and 6 were dead; the others were sick when sacrificed.

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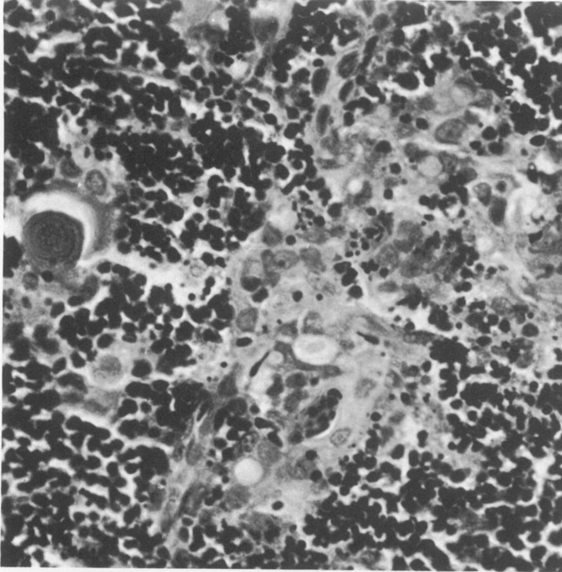

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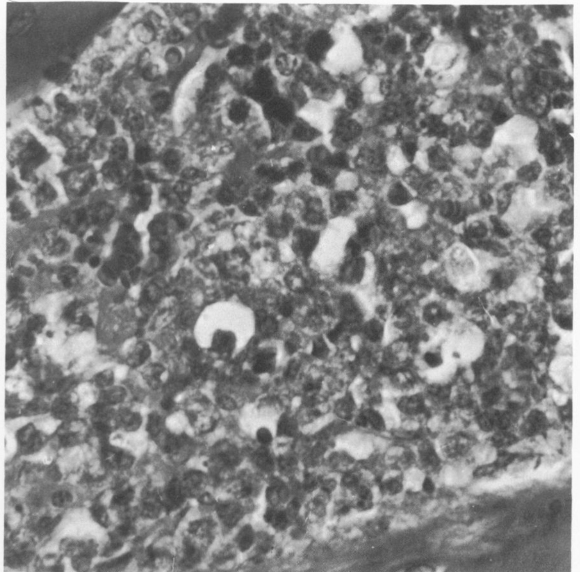

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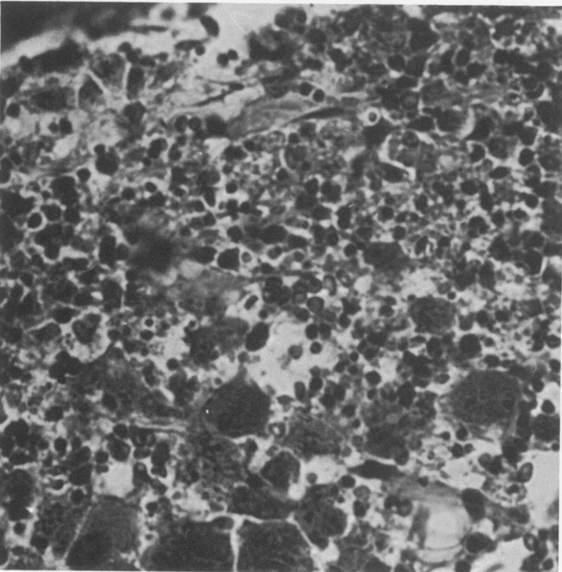

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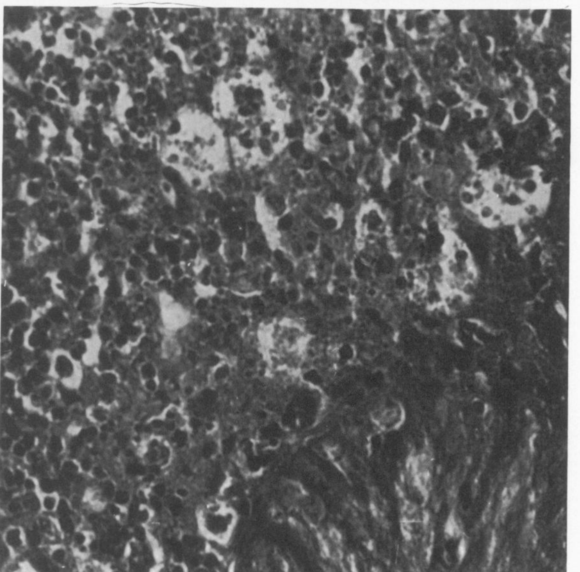
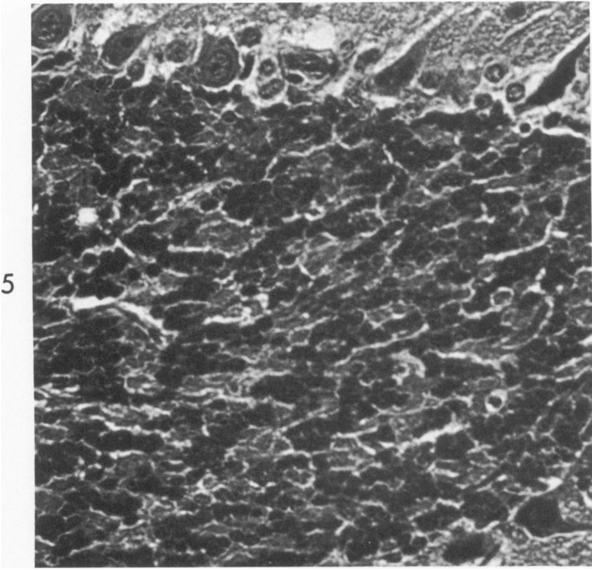


Fig 1—Depletion and necrosis of thymus, 72 hours after VE-I.A virus (× 560). **Fig 2**—Normal appearing femoral bone marrow, 5 days after VE-III virus (× 560). **Fig 3**—A focus of necrosis in femoral bone marrow 5 days after VE-III virus (× 560). **Fig 4**—Moderate necrosis of lymphoid cells of Peyer's patch and intact mucosum of ileum. Same hamster as Figure 3 (× 560).

Fig 5—Degenerating Purkinje cells, 72 hours after VE-I.A (× 560). **Fig 6**—Hemorrhage in brain and absence of inflammatory cells 5 days after VE-III (× 350).

Fig 7—Perivascular cuffing of blood vessel wall in brain of a hamster dying with central nervous system signs 14 days after VE-III (× 560). **Fig 8**—Swollen glial cells in brain of a rare hamster which died 9 days after attenuated VE-I.(A) (× 560).



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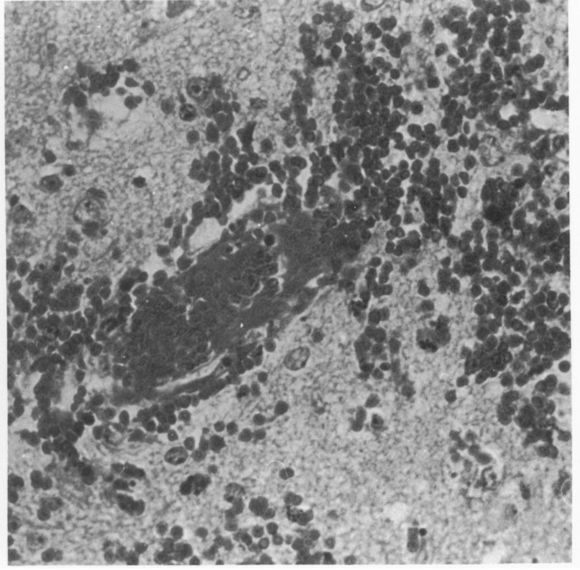

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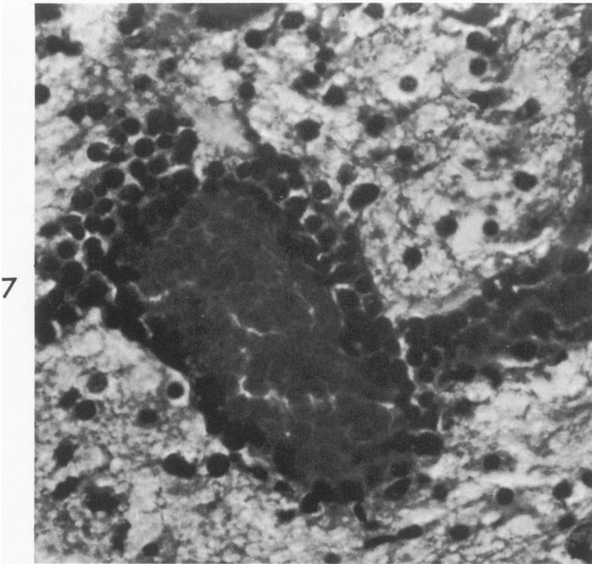

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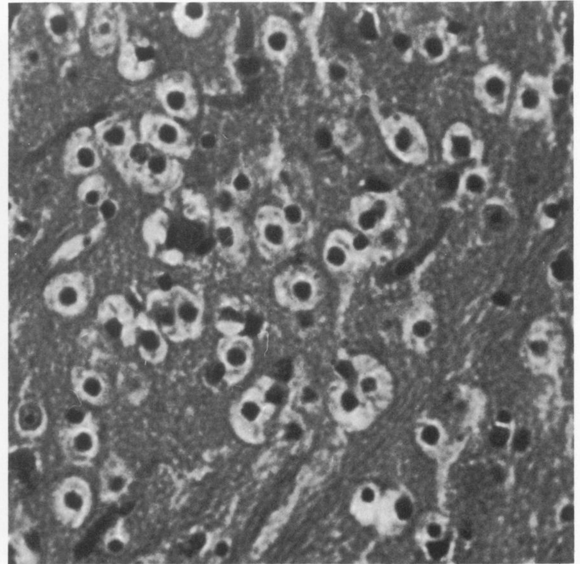

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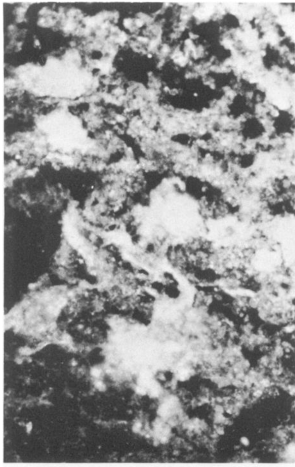

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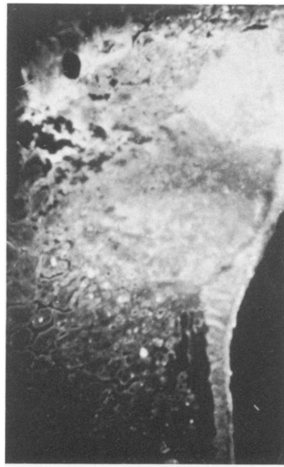

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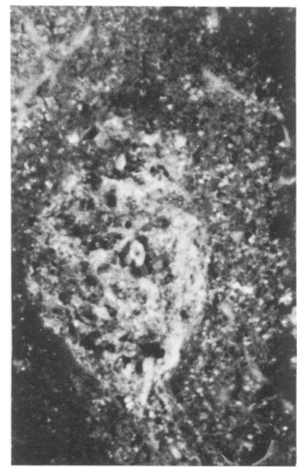

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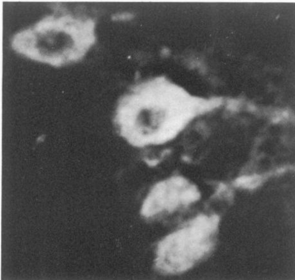

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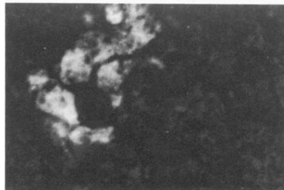

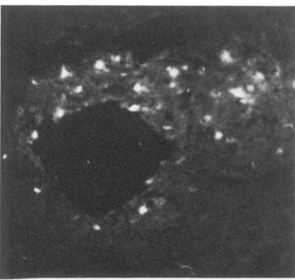

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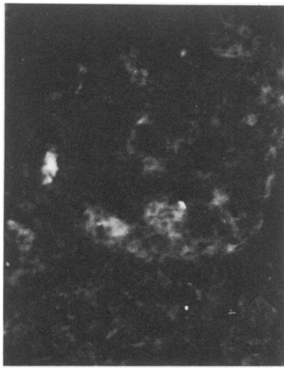

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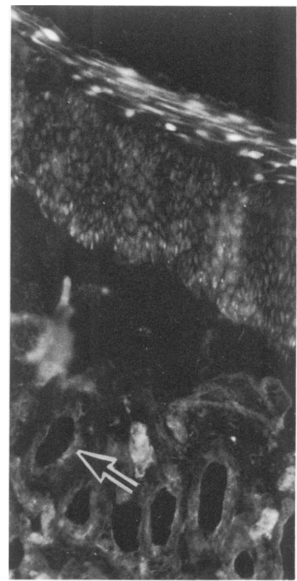

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Fig 9—Generalized, intense fluorescence in femoral bone marrow cells and cell debris, 48 hours after VE-I.B (× 540). **Fig 10**—VE-I.A antigen in lymphoid elements of Peyer's patch of a hamster sacrificed 24 hours after infection (× 540). **Fig 11**—VE-I.A antigen concentrated in splenic white pulp, but widely distributed in red pulp 48 hours after inoculation (× 240). **Fig 12**—VE-III antigen in Purkinje cells, 72 hours after infection (× 1070). **Fig 13**—VE-I.A antigen in unidentified cells in association with small cerebral blood vessels, 48 hours after infection (× 220). **Fig 14**—Intense fluorescence in small foci of acinar cells of pancreas, 48 hours after VE-I.A (× 220). **Fig 15**—VE-I.B antigen localized in an islet of Langerhans, 48 hours after infection (× 220). **Fig 16**—VE-I.B antigen in smooth muscle and glandular structures (*arrow*) of ileum, 48 hours after infection (× 350).