Studies of Viral Virulence

I. Growth and Histopathology of Virulent and Attenuated Strains of Venezuelan Encephalitis Virus in Hamsters

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PATHOGENICITY AND VIRULENCE OF A VIRUS can be influenced by the host, the virus, or both; yet virulence is not a fixed viral characteristic because different strains of the same virus can be very different in virulence, and virulence can often be modified experimentally. It has been suggested that viral virulence is expressed through immunogenic means, interferon or temperature sensitivity of viral development.[1-3] The virulence of a virus may also be influenced by its ability to spread within a host and to reach specific target tissues. However, as yet, no viral attribute causing virulence or attenuation has been unequivocally identified with the precision and certainty of bacterial exotoxins.

An initial approach to investigating viral virulence is to compare growth of virulent and attenuated viruses in the same host species. If this is to be relevant to events as they occur naturally, one should employ a virus that causes disease in nature, and a host that, upon infection by a natural route, consistently becomes ill and dies with virulent virus but survives infection by attenuated virus. A virus-host system that meets these criteria is the Venezuelan encephalitis (VE) virus in the hamster. VE virus is transmitted in nature by bites of infected mosquitoes, and causes outbreaks of human and equine disease in the Americas. Hamsters can be infected by subcutaneous (sc) injection of small doses of virulent and attenuated strains; disease and death regularly occur with virulent virus,[4] whereas attenuated virus produces illness or death in less than 20% of hamsters.[5] This article compares growth curves of virulent and attenuated strains of VE virus in hamsters, and describes the resultant histopathology.

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Supported in part by International Post-Doctoral Research Fellowship 1 F05-TW-1174-01 from the US Public Health Service and in part by Research Contract DA-49-193-MD-2295 from the US Army Medical Research and Development Command, Department of the Army, under sponsorship of the Commission on Viral Infections of the Armed Forces Epidemiological Board.

Accepted for publication October 1, 1970.

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Materials and Methods

Viruses. The virulent strain of VE virus (63Z21) came from blood of a human who was accidentally infected in the laboratory with a strain of VE virus (63U2) isolated from tissues of a sentinel hamster exposed in Mexico.[5] It was passed once by intracranial inoculation of infected brain tissue into baby mice and once in cultures of guinea pig heart cells prepared from embryos. Preliminary subcutaneous (sc) and intracranial (ic) titrations of aliquots of culture fluid showed that all infected hamsters died within 5 days, because sera from survivors given high dilutions of virus were free of plaque-neutralizing (PN) antibody 19 days after inoculation.

The attenuated strain (TC83), kindly supplied by Dr. R. McKinney at Fort Detrick, was derived from the Trinidad burro strain of VE virus by serial passage in cultured guinea pig heart cells,[6] and was used as fluid from the eighty-third passage, as supplied for human vaccination. This virus readily infected hamsters when injected subcutaneously or intracranially, but caused overt disease in only 20% or less of those infected; the occurrence of illness was unrelated to virus-dose or route of injection.[5] Sera of survivors contained PN antibody 21 days after virus inoculation. Virus stocks were kept in aliquots at −60 C in an electric freezer, and each aliquot was used only once.

Hamsters. Female Syrian hamsters between 6 and 10 weeks old were injected either subcutaneously with 0.1 ml of virus suspension or intracranially with 0.05 ml. They were kept at a temperature of about 22 C.

Sampling of Tissues. Hamsters were anesthetized with ether and bled by cardiac puncture. Part of each blood specimen was heparinized (10 units/ml) for virus titration and part was allowed to clot for serum. Brain, spleen and mesenteric lymph nodes were removed and extracted separately to make 10% suspensions in 1% bovine albumin in Hanks' solution (BA). Bone marrow samples were obtained by crushing two femurs and extracting marrow in 1 ml of BA. All specimens for virus titration were stored at −60 C. For histopathology, tissues were fixed in 10% formalin and bone specimens were decalcified in nitric acid. Sections were stained with hematoxylin and eosin (H&E).

Virus Titrations. Stock viruses were titrated by sc inoculation of hamsters with $\log_{10}$ dilutions in BA; 50% endpoints were based on death, for virulent virus; and PN antibody in serum 21 days after inoculation, for attenuated virus.[7] Virus in tissues was titrated by counting plaque-forming units (pfu) on primary chicken embryo cell cultures maintained under agar medium.[8]

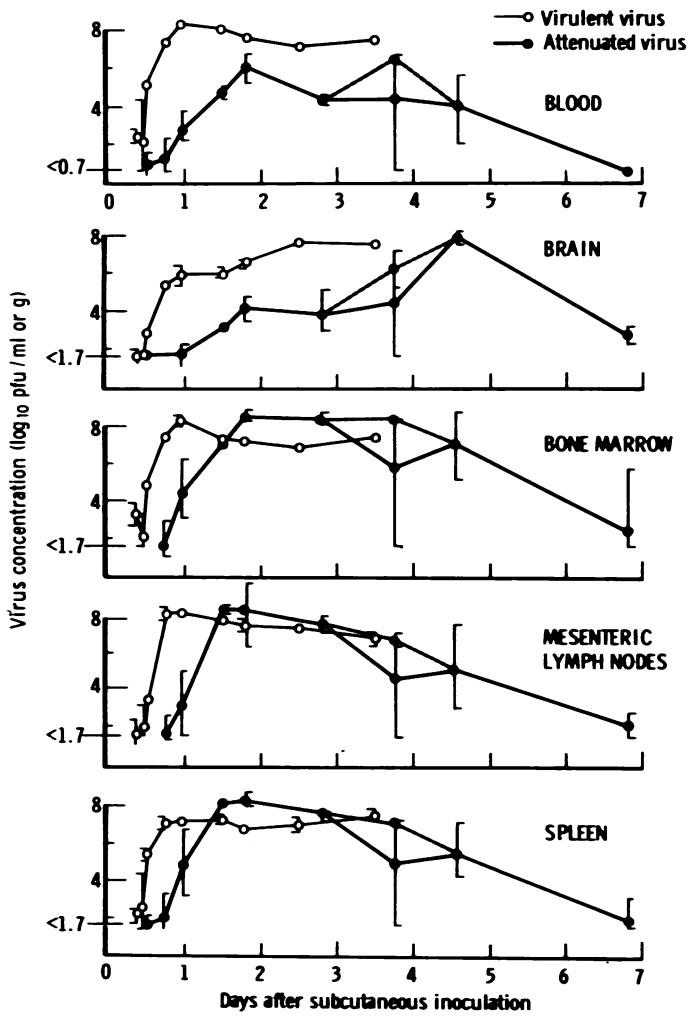
Virus Growth Curves. Groups of hamsters were injected with 1000 times the 50% hamster infective dose (HID_{50}) of either the virulent or attenuated strain of VE virus. At selected intervals 3 hamsters from each group were killed and tissue specimens taken for virus titration and histopathologic examination. The virus in each specimen was titrated separately, and the geometric mean of the 3 values for each tissue, at each time, was calculated.

Neutralizing Antibody Tests. Unheated sera were diluted at 0.5 $\log_{10}$ increments in BA and aliquots were mixed with equal volumes of homologous virus suspension containing 100 pfu/0.05 ml. After the serum-virus mixtures were incubated at 37 C for 1 hour, 0.1-ml volumes were inoculated onto sheets of chicken embryo cell cultures and adsorbed for 1 hour at 37 C before agar medium was added.[8] The highest dilution of serum that reduced the number of plaques 90% or more was taken as the serum antibody titer.

Results

Growth Curves of Virulent and Attenuated VE Viruses in Hamster Tissues after sc Inoculation

After subcutaneous injection of 1000 HID_{50} of either virulent or attenuated VE virus, each virus multiplied in brain, bone marrow, lymph nodes and spleen (Text-fig 1). The most obvious differences between the two strains were (1) the earlier multiplication of the virulent strain in all tissues and (2) its higher maximum titer in blood, though not in tissues. The dotted sections of the curves for attenuated virus between

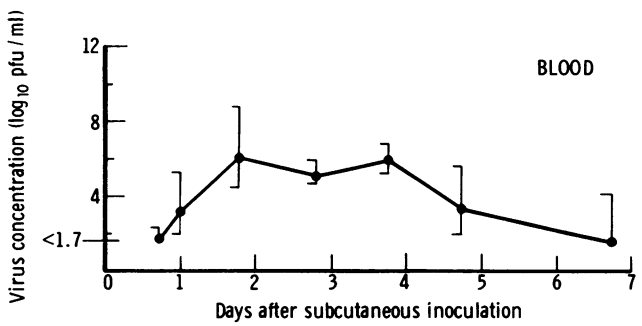


TEXT-FIG 1.—Virus levels in tissues of hamsters injected sc with 1000 HID_{50} of virulent or attenuated VE virus. Points indicate geometric means of 3 hamsters and vertical bars signify ranges. Dotted lines are explained in Results section of text.

3 and 5 days (Text-fig 1) show values derived by omitting one anomalous hamster from calculations of geometric means at 90 hours. This hamster's tissues were free of virus and there was detectable neutralizing antibody in its serum. Yet the other 2 or 3 hamsters sacrificed at 90 and 110 hours had high levels of virus in all tissues. Moreover, when another 11 hamsters were injected subcutaneously with 1000 HID_{50} of attenuated virus, and each hamster was bled by cardiac puncture at each interval, there was a high level of virus in the blood of each hamster 91 hours after injection (Text-fig 2).

Quantitative Relationship of VE Virus in Blood and Brain after sc Inoculation

For the first 2 days after inoculation, the amount of both viruses in the blood was more than 30 times the level of virus in the brain. Thus, the possibility existed that the virus found in the brain in the early stages of infection might represent merely contamination with blood. To test this possibility, a phage of *Pseudomonas aeruginosa* was injected into the heart cavities of 5 hamsters. After 3 or 4 minutes, the hamsters were killed and the phage in blood and brain samples was titrated by counting the plaques produced on a substrate of *Ps aeruginosa*. The amounts of phage in brain extracts were 0.5–1% of those in the blood. Thus, if the virus titer in the brain of a hamster was less than 1% of the virus titer in the blood of the same animal, then the virus in the brain extract probably represented blood contamination. This could account for VE virus in the brain extracts for the first 2 days after inoculation, but not thereafter (Text-fig 1). In contrast, the virus concentrations in marrow, lymph nodes, and spleen were equivalent to, or higher than, those in the blood, and, therefore, indicated virus growth in these tissues throughout the period studied.



TEXT-FIG 2.—Virus levels in bloods of 11 hamsters injected sc with 1000 HID_{50} of attenuated VE virus. Points indicate geometric means and vertical bars signify ranges.

Disease and Pathology of Virulent and Attenuated VE Virus Infections of Hamsters

Hamsters became listless about 48 hours after subcutaneous injection with 1000 HID_{50} of virulent VE virus (strain 63Z21). Their condition then deteriorated rapidly until, at 60 hours, they were hunched, obviously ill and characteristically had conjunctival discharge. Deaths began to occur at 72 hours, and by 84 hours all animals were either moribund or dead. None survived beyond 90 hours. These observations were like those seen with other virulent strains of VE virus.[4] The first pathology seen grossly was enlargement of brachial lymph nodes 24 hours after inoculation. Sometimes the gall bladder was enlarged at 48 hours, and at 60 hours the cut surface of the spleen was patchy, and there were hemorrhages in the intestinal wall and lumen. Terminally, by 4 days there were hemorrhages in the brain. Histopathologic changes were absent in hematopoietic tissues and brain at 15 hours after inoculation (Table 1), but by 40 hours, there was cellular necrosis in lymph nodes (Fig 1), lymphatic tissue of the intestinal wall (Fig 2), white pulp of the spleen (Fig 7) and bone marrow (Fig 11). The only changes in brain at 40 hours were small foci of possible necrosis (Fig 14). On day 4 (87 hours) when hamsters were moribund, there were hemorrhage and necrosis of intestinal wall (Fig 3) and brain (Fig 13); parenchymal cells of bone marrow, lymph nodes and spleen were almost completely destroyed except for occasional small foci of regenerating lymphoid cells in spleen (Fig 8). Skeletal muscle remained normal (Table 1).

Attenuated VE virus produced overt disease irregularly and in less than 20% of hamsters inoculated sc. Between 3 and 8 days after inoculation, some animals became listless and remained so for 5 or 6 more days before either recovering or dying. Deaths occurred from 5 to 23 days after infection. Rarely, an animal developed hind-limb paralysis, suggesting involvement of the central nervous system. No gross pathologic changes were seen. Histopathologic examinations were made of the tissues of 12 hamsters that were sacrificed in pairs 0.5, 2, 4, 7, 10 and 14 days after inoculation, and of 5 hamsters that died 5, 6, 9, 18 and 23 days after inoculation (Table 1). On the second day after injection, there seemed to be some depletion of lymphocytes in lymph nodes (Fig 4) and spleen, and proliferation of splenic reticuloendothelial cells (Fig 9). On the fourth day, lymph nodes were normal (Fig 5), and splenic lymphoid elements from the fourth to the seventh days were either normal or slightly hyperplastic (Fig 10). Bone marrow remained normal (Table 1 and Fig 12). No unequivocal histopathology was seen in brain (Table 1) although in 1 hamster there were slight increases in mononuclear cells associated with capillaries (Figs 15 and

Table 1. Extent and Temporal Development of Histopathology in 24 Hamsters Inoculated Subcutaneously with Virulent (63Z21) or Attenuated (TC83) Venezuelan Encephalitis Virus

		Degrees of histopathology at intervals after sc inoculation*											
		Hours					Days						
Tissue	Virus	12	15	40,43	65	87,90	5	6	7	9	10	14	18	23
Mesenteric lymph nodes	63Z21				+									
				+	+									
	TC83	—		±		—						—		
						—						—		
													—†	
Intestine wall lymphatic tissue	63Z21		—	+	+	+								
			—		+									
Intestine wall	63Z21		—	—	+	+								
			—	—	+									
Spleen	63Z21		—	+	+	+								
			—	+	+									
	TC83	—		±		±			—		—	—		
		—		±		±			±		—	—		
							—	—		—			—	—
Bone marrow (vertebral and rib)	63Z21		—	±	+	+								
			—	+	+									
	TC83	—		—		—			—		—	—		
		—		—		—			—		—	—		
							—	—		—			—	—
Brain	63Z21		—	±	±	+								
			—	±	±									
	TC83	—		—		—			—		—	—		
		—		—		—			—		—	—		
							—	—		—			—	—
Skeletal muscle	63Z21		—	—	—	—								
			—	—	—									
	TC83	—		—		—			—		—	—		
		—		—		—			—		—	—		
Liver	TC83						—	—						

* — = normal, ± = slight necrosis or hyperplasia, + = definite to severe necrosis, hyperplasia, congestion and hemorrhage. Each symbol signifies 1 hamster. Tissues were stained with H & E.

† Axillary node.

16), which might represent migrating lymphocytes and/or endothelial proliferation. The 5 hamsters that died after injection of attenuated virus showed no histopathology in hematopoietic or brain tissues and thus their deaths remained unexplained (Table 1). Skeletal muscle and liver were also normal in hamsters receiving attenuated virus (Table 1).

Antibody Responses of Hamsters Inoculated sc with Attenuated VE Virus

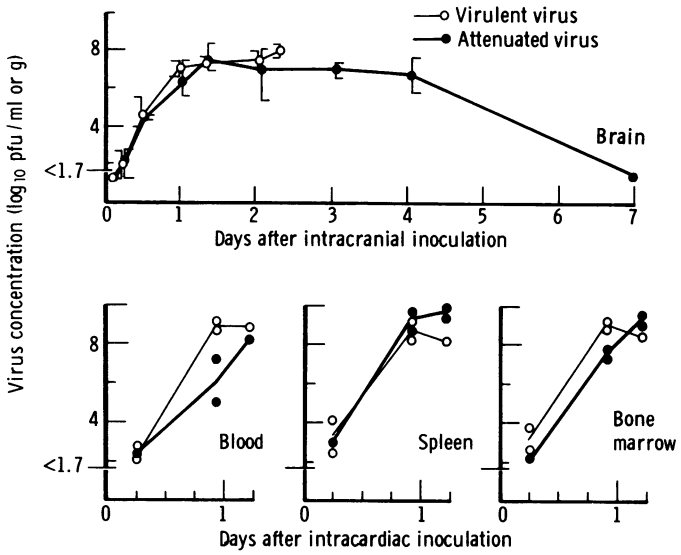
Since hamsters infected with virulent virus were viremic until death occurred, neutralizing antibody could not be measured in serum. Thus tests were restricted to sera of hamsters infected with attenuated virus. Six sera taken before the onset of viremia (12 and 17 hours after infection) were free of neutralizing antibody. By 7 days (163 hours) after infection, blood was free of infectious virus, and sera of 3 sacrificed hamsters had neutralizing antibody titers of 1:1000, 1:1000 and 1:3200. Sera of 9 hamsters killed between 9 and 14 days after infection all contained antibody with titers ranging from 1:320 to 1:3200.

Growth Curves of Virulent and Attenuated VE Viruses in Hamster Tissues after Intracranial (ic) or Intracardiac (ica) Inoculation

The results at this point in the investigations showed that attenuated VE virus given sc failed to produce significant disease or pathology in hematopoietic, brain and intestinal tissues which were targets of virulent virus. To learn whether this was related to the delayed growth and lower blood levels of attenuated as compared with virulent virus (Text-fig 1), each virus was inoculated ic or into the heart cavity to provide immediate contact with brain or hematopoietic tissues. In these experiments, both virus strains displayed similar growth curves (in brain after ic inoculation or in blood, spleen and marrow after ica inoculation) (Text-fig 3). Yet attenuated virus inoculated these ways still failed to kill hamsters any more often than sc inoculation (4 unsacrificed hamsters were still alive on day 4 and 2 on day 7 after ic inoculation, and only 1 of 3 died between 7 and 14 days after ica inoculation). In contrast, control hamsters given virulent virus ic were ill in 2 days and dead by 2.5 days.

Discussion

Both attenuated and virulent strains of VE virus multiplied readily after subcutaneous injection into hamsters. The virulent strain (63Z21) increased rapidly in tissues to reach maximum levels in blood, brain, bone marrow, lymph nodes and spleen within 24 hours. This was followed by massive destruction of cells in tissues of the hematopoietic system similar to that described in guinea pigs after intraperitoneal inoculation.[9,10] All hamsters infected with virulent virus suffered a short acute disease ending in death within 5 days of virus injection. The rate of virus multiplication and the distribution of virus in tissues was similar to those reported by Miller and Scherer for 2 other strains of virulent VE virus in hamsters.[4]



TEXT-FIG 3.—Virus levels in brain after ic inoculation, and in blood, spleen and bone marrow after ica inoculation, of 1000 HID_{50} of virulent or attenuated VE virus. Points for brain curve indicate geometric means of 2 or 3 hamsters and bars signify the ranges. For blood, spleen and bone marrow, each point represents one hamster.

The attenuated virus (TC83) caused few obvious clinical signs in hamsters. Some animals assumed a hunched posture and had ruffled fur for a few days, and there were occasional deaths between 5 and 23 days after infection, but most hamsters survived. The virus obviously multiplied in their tissues as the amount in blood, brain, bone marrow, lymph nodes and spleen increased and reached maximum levels in the blood and hematopoietic tissues within 42 hours, but not until 110 hours in the brain. The rapid decrease of attenuated virus in all tissues between the fourth and the sixth days was associated with the appearance of neutralizing antibody in the serum. There were no striking histopathologic changes.

Unfortunately, these results do not explain why one strain of Venezuelan encephalitis virus is virulent and rapidly fatal for hamsters after subcutaneous, intracranial or intracardiac inoculation, and another, attenuated strain rarely produces disease by these routes of inoculation. Virulence was not associated with selective growth in a particular tissue. Although growth of virulent virus in hamster tissues began earlier than attenuated virus after sc inoculation, both viruses reached essentially equal maximal concentrations in target organs though not in blood, and no differences in onset or rate of growth were seen after ic or ica inoculation. Thus it was clear that rapidity of virus growth or levels reached

in target organs were not critical determinants in virulence or pathology produced by VE viruses in hamsters.

Yet to be determined are the possibilities of more rapid antibody or interferon formation by attenuated virus or greater interferon sensitivity of attenuated virus, as explanations of the delayed growth of attenuated virus in comparison with virulent virus. However the eventually equal maximal concentrations of each virus in target tissues strongly suggest that factors suppressing virus growth, such as antibody and interferon, would not entirely explain the differences in histopathology and disease patterns. Of course one would expect the necrosis of lymphoid cells by virulent VE virus to reduce antibody production; that in turn would permit virus replication and cytonecrosis to continue unabated, whereas with attenuated virus, lymphoid tissues were not destroyed and thus antibody production occurred. Indeed, termination of attenuated VE viremia in hamsters was associated with appearance of neutralizing antibodies in the classical manner, and in fact, attenuated VE virus might have an enhancing effect on antibody production in hamsters as it does in guinea pigs.[11]

In summary, it is concluded that virulent VE virus causes disease and death in hamsters because it is cytodestructive for hematopoietic, brain and intestinal tissues whereas attenuated virus is not. Since many hamsters infected with virulent virus have terminal enterobacteremias presumably due to granulocytopenia from bone marrow necrosis, death is probably often due to bacterial infection and endotoxins. Nevertheless, the results of these studies indicate that in order to understand the mechanism of initial injury by virus, future investigations are now needed to determine the following: the proportion and types of cells supporting viral growth in target tissues, whether toxins are produced by cells infected with virulent virus, the biochemical events in infected cells, and the biophysical and biochemical properties of both virions.

Summary

Virulent Venezuelan encephalitis virus killed hamsters within 3–5 days of subcutaneous inoculation, whereas attenuated virus produced no disease in at least 80% of hamsters. Virulent virus began to grow sooner than attenuated virus in hamsters inoculated subcutaneously and reached higher titers in blood. Maximal titers of both viruses were similar in brain, bone marrow, lymph nodes and spleen, though peaks of growth curves occurred almost a day later with attenuated than with virulent virus. In contrast, both viruses grew equally fast and to similar titers in brain after intracranial inoculation, and in spleen and bone

marrow after intracardiac inoculation. Yet pathogenicity of attenuated virus was not increased by direct inoculation into the brain or heart cavity. The target organ of virulent virus in hamsters was principally the hematopoietic system, but lesions occurred in brain and intestinal wall late in the disease. Attenuated virus did not produce distinctive histopathologic lesions in hamsters whether or not they developed illness, and the hematopoietic and brain tissue changes seen were certainly insufficient in nature or extent to explain the occasional illness or death.

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Dr. Austin was on leave from the Medical Research Council of New Zealand's Virus Research Unit, Dunedin, New Zealand.

[Illustrations follow]

Tissues from hamsters given 1000 HID_{50} of virulent VE virus strain 63Z21, or attenuated VE virus strain TC83, subcutaneously, stained with H&E.

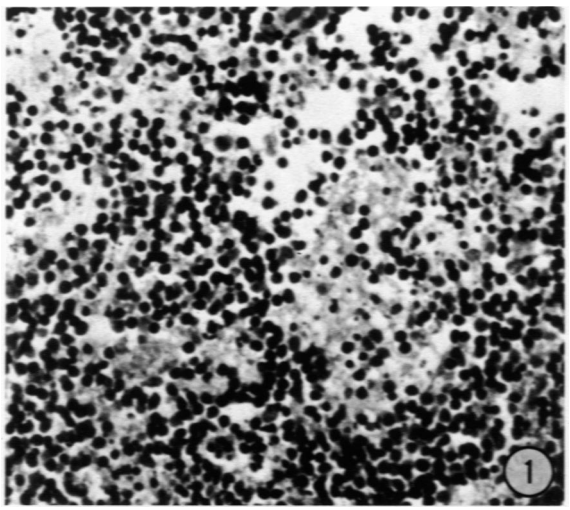
Fig 1.—Necrosis of cells in mesenteric lymph node, 40 hours after virulent virus (× 240).

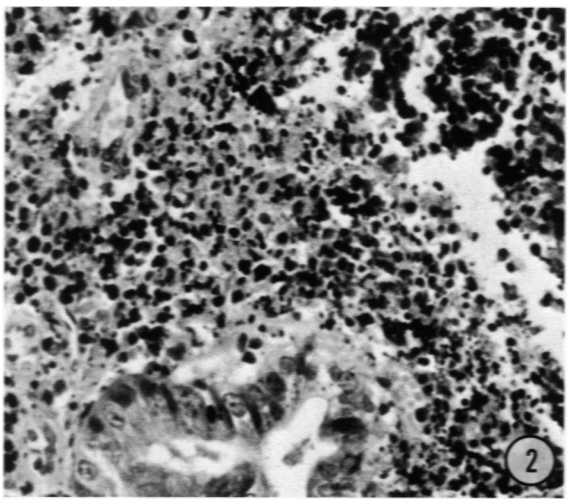
Fig 2.—Necrosis of cells of Peyer's patch, 40 hours after virulent virus (× 240).

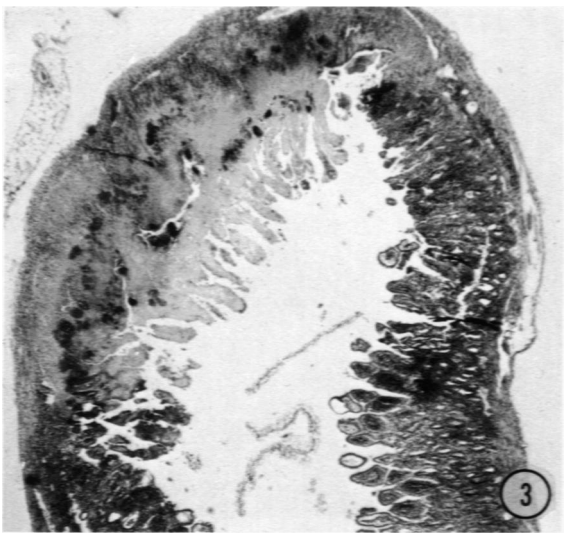
Fig 3.—Necrosis of segment of intestinal wall, 87 hours after virulent virus (× 20).

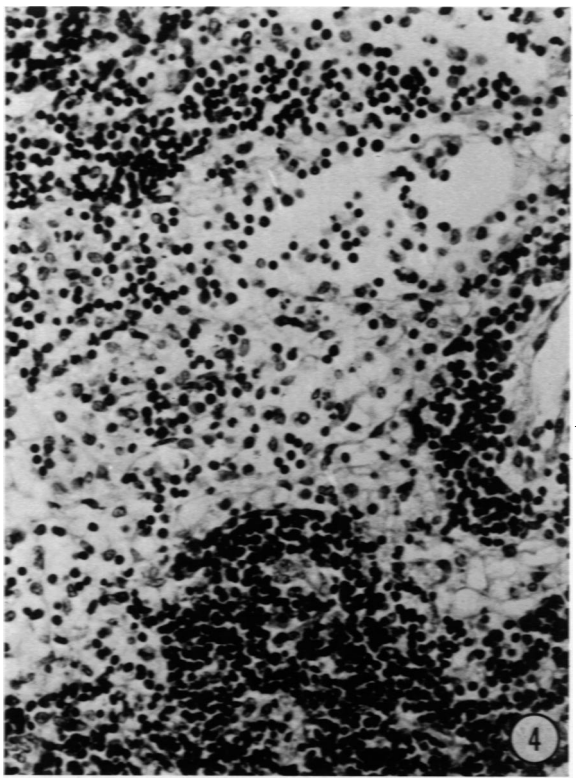
Fig 4.—Some depletion of lymphocytes in mesenteric lymph node, 43 hours after attenuated virus. (× 200).

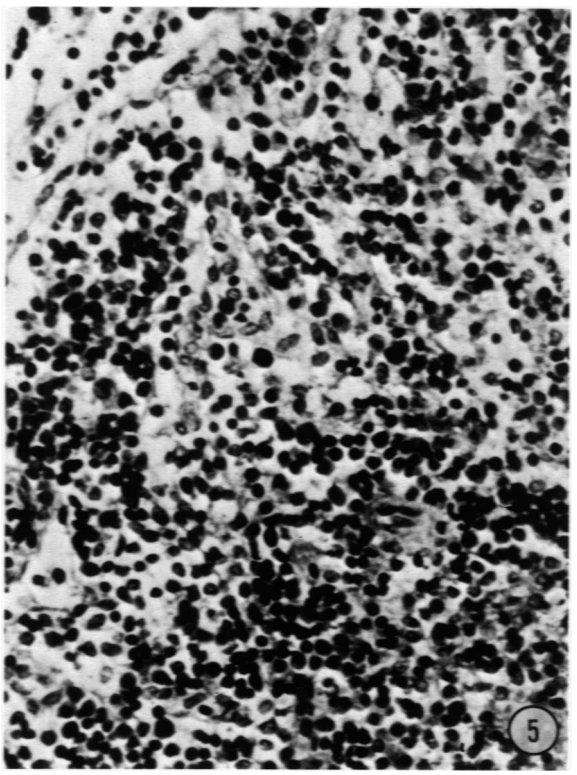
Fig 5.—Normal-appearing mesenteric lymph node, in healthy hamster sacrificed 90 hours after injection of attenuated virus (× 240).

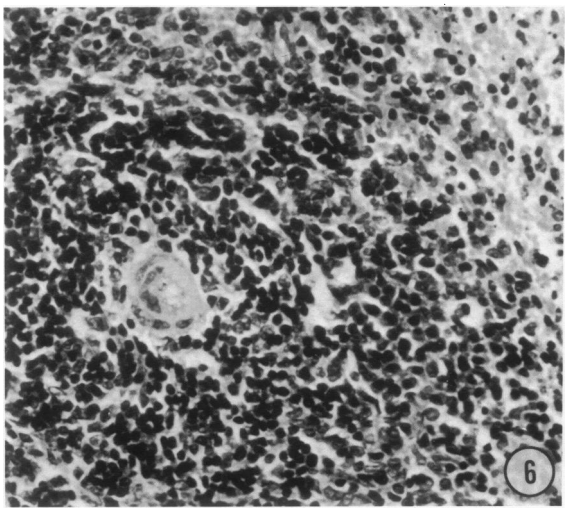








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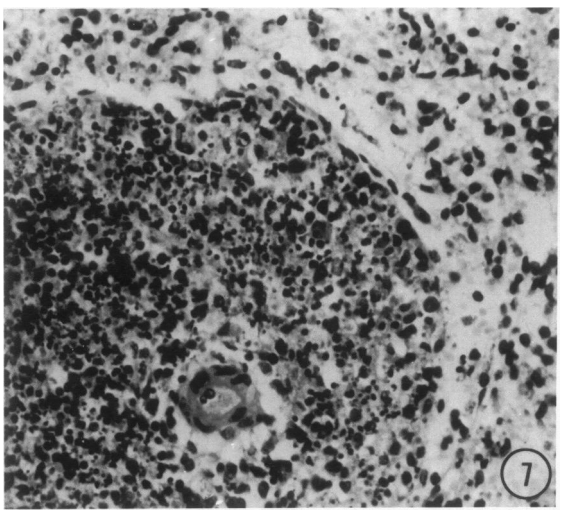
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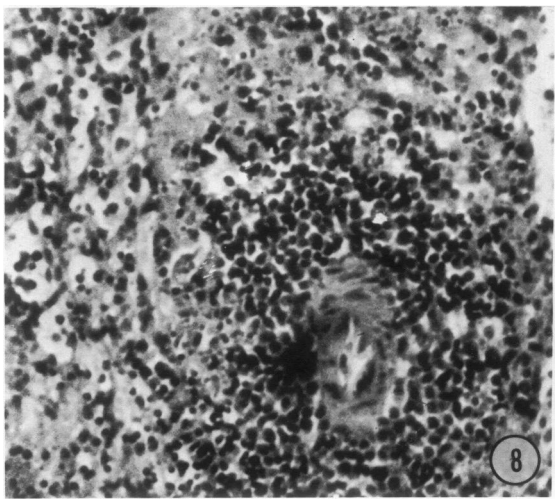
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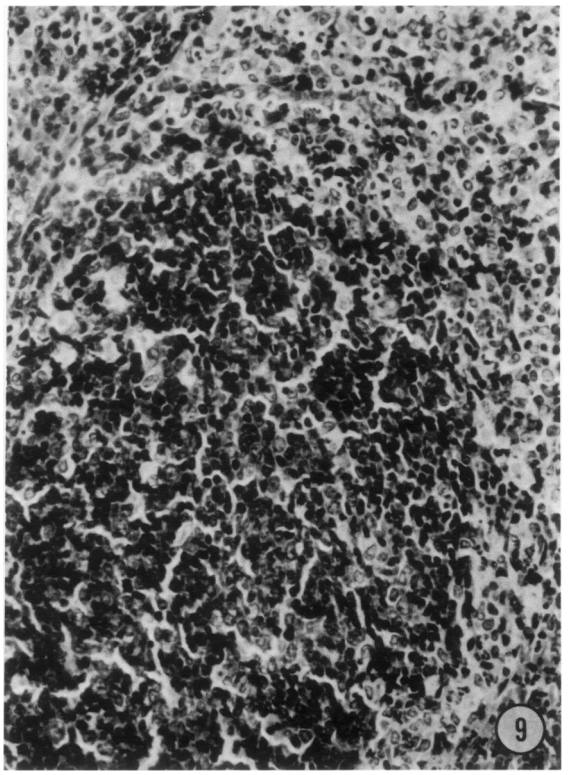
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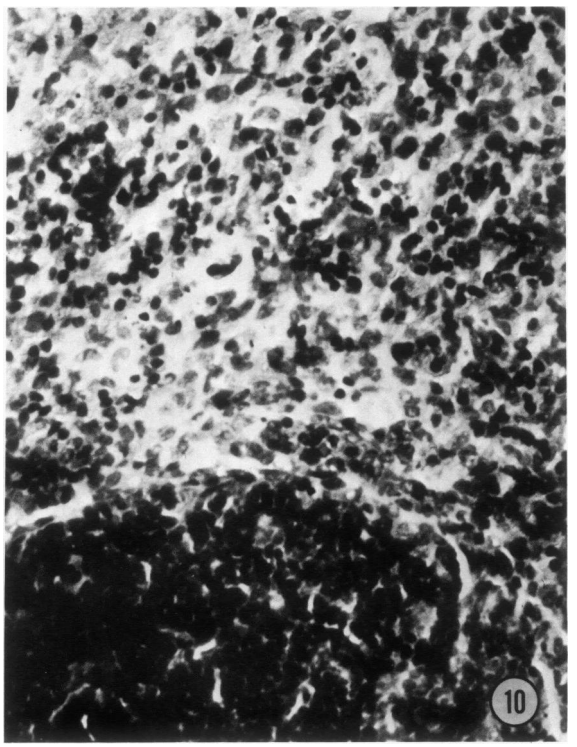
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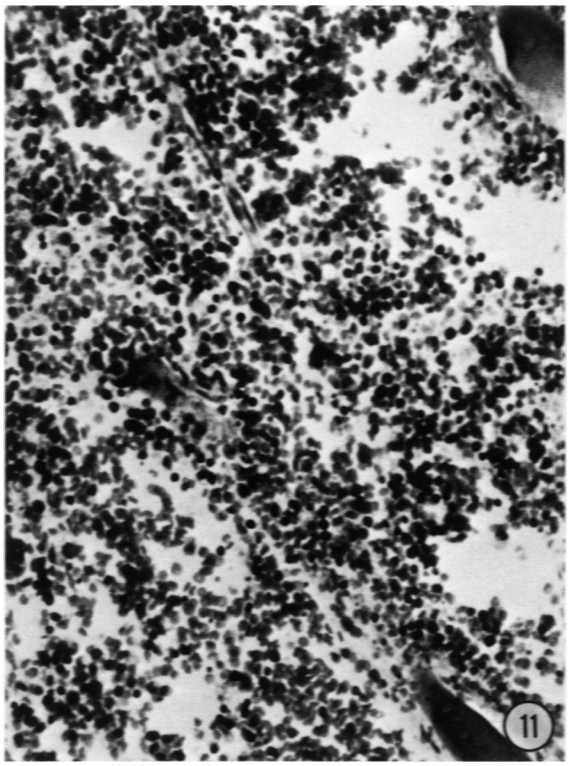

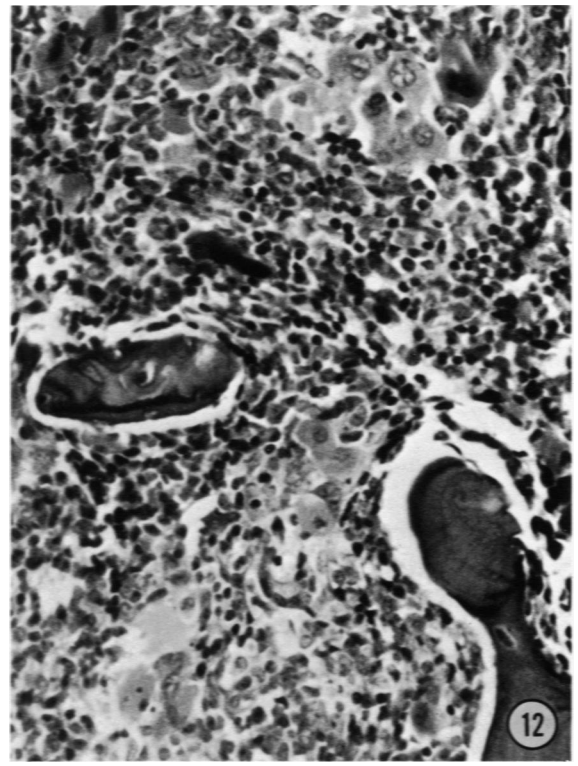

Fig 11.—Severe necrosis of bone marrow, 40 hours after virulent virus (same animal as Fig 1) (× 240).

Fig 12.—Normal-appearing bone marrow, in healthy hamster sacrificed 7 days after receiving attenuated virus (same animal as Fig 10) (× 240).

Fig 6.—Normal spleen from uninoculated hamster. (× 200).

Fig 7.—Necrosis of lymphoid cells in spleen, 40 hours after virulent virus (same animal as Fig 1) (× 200).

Fig 8.—Focal islets of regenerating lymphoid cells, 87 hours after virulent virus (same animal as Fig 3) (× 320).

Fig 9.—Some depletion of lymphocytes and proliferation of reticuloendothelial cells in lymph follicles 43 hours after attenuated virus (× 200).

Fig 10.—Spleen with many lymphocytes in follicles, 7 days after attenuated virus (× 240).

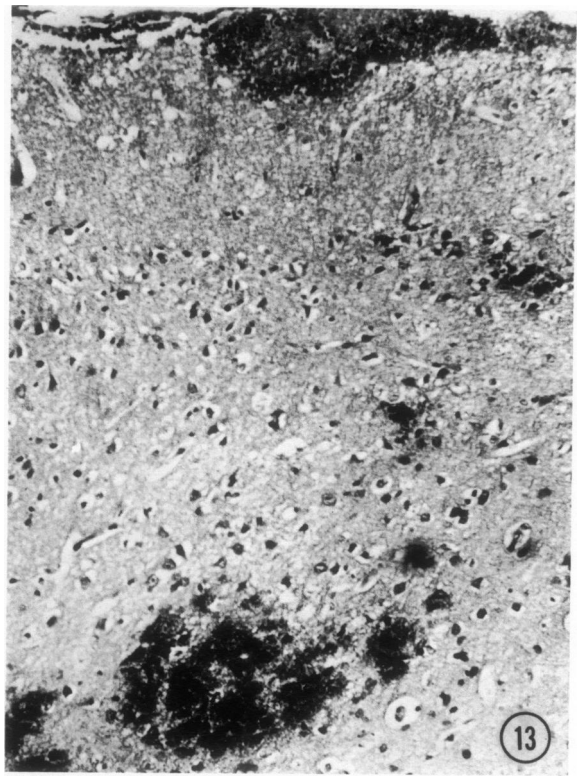

Fig 13.—Parenchymal and subarachnoid hemorrhage in brain, 87 hours after virulent virus (same animal as Fig 3) (× 120).

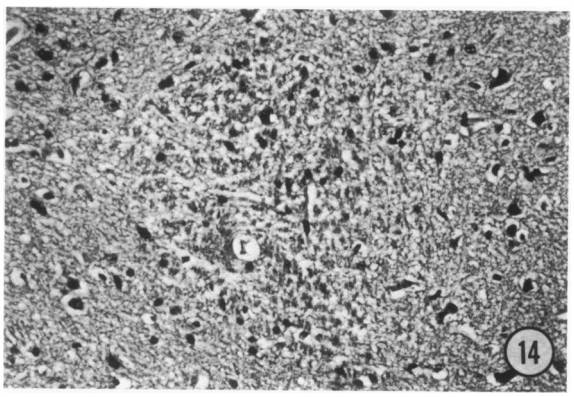

Fig 14.—Small focus of possible early necrosis in brain, 40 hours after virulent virus (same animal as Fig 2) (× 120).

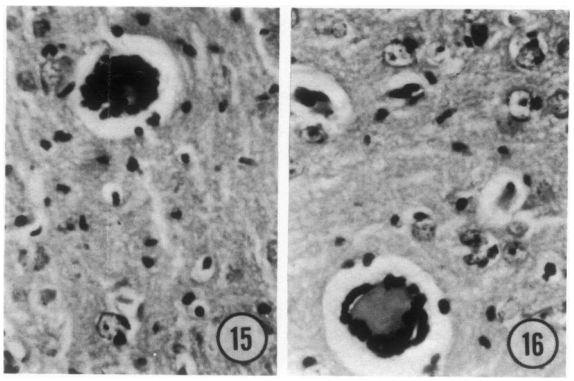

Fig 15 and 16.—Increased mononuclear cells in association with capillaries of brain, 6 days after attenuated virus (× 200).