Lesions of the Central Nervous System Induced in Nonhuman Primates by Live Influenza Viruses

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ABSTRACT

Intracerebral and intraspinal inoculations of non-neuropathic and neuropathic strains of influenza virus into rhesus, patas and cercopithecus monkeys resulted in an acute focal ependymitis, choroiditis and meningitis followed by focal ependymal denuding without parenchymal involvement. Aqueductal stenosis and moderate hydrocephalus developed in two animals as sequelae of ependymal cell loss.

RÉSUMÉ

L'inoculation intra-cérébrale et intra-rachidienne de souches neuropathogènes et non neuropathogènes du virus de l'influenza à des singes rhésus, patas et cercopithèques se traduisit par des foyers d'épendymite, de choroïdite et de méningite aiguës, ainsi que par des foyers de desquamation des cellules épendymaires, sans atteinte du parenchyme. Deux des singes rhésus développèrent une obstruction de l'aqueduc de Sylvius et une hydrocéphalie modérée attribuables à la desquamation des cellules épendymaires.

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INTRODUCTION

In man, influenza virus has been tentatively implicated in acute and chronic forms of encephalitis (18, 19, 22-24). Experimentally, lesions of the central nervous system (CNS) have been reported largely in rodents (2, 9-13, 15, 17, 21) and recently in birds (16). However, attempts to initiate influenza virus infection of the CNS in monkeys appear to have been reported only in squirrel monkeys receiving immunosuppressive therapy (14).

The purpose of the present paper is to describe the histological lesions induced in nonhuman primates following intracerebral and intraspinal inoculations of different strains of influenza virus.

MATERIALS AND METHODS

VIRUSES

The influenza strains and their sources are shown in Table I. All strains were assayed after inoculation of ten to 12 day chick embryos using hemagglutination activity to detect virus after three days incubation at 33°C. Infectivity was expressed as the reciprocal of the dilution causing infection in 50% of eggs (EID/50).

Monkeys

Rhesus *(Macaca mulatta)*, cercopithecus *(Cercopithecus aethiops)* and patas *(Erythrocebus patas)* monkeys were used for the inoculation experiment. They had been quarantined for four weeks before the experiment and their sera had been tested for hemagglutination inhibition (HI) activity. The number of animals inoculated with each strain is given in Table I.

Animal Inoculation

The procedure of neurovirulence safety test of live attenuated measles vaccine in monkeys was used (3). Virus inocula prepared from infected allantoic fluid contained $10^{7.5}$ or $10^{7.0}$ EID/50 and, in some experiments, different dilutions of the stock virus strains were inoculated (Table I). Undiluted noninfected allantoic fluid served as a control material. Virus containing and control materials were inoculated intrathalamically (0.5 ml), intraspinally (0.1 ml) and intramuscularly (0.5 ml) at the same time into each monkey after anesthesia. Each monkey was isolated in a separate cage and observed three times daily for neuromuscular disability. Clinical assessment was made by agitating the animal within the cage.

Tissues

When moribund or at the end of the observation period (19-21 days), the animals were anesthetized, exsanguinated and tissues were removed for histological and virological studies. Tissues for histological studies were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6μ and stained with hematoxylin and eosin. Selected sections were stained with Luxol fast blue cresyl violet. The following regions of the CNS were examined histologically: frontoparietal and occipitotemporal cortex, thalamus, caudate nucleus, putamen, hippocampus, pons, medulla oblongata, cerebellum and thoracic and lumbar regions of spinal cord, each cut into six blocks. Sections of lungs, spleen, heart and kidneys were also examined.

Virology

Procedures of virus isolation and hemagglutination test have been described elsewhere (4). Briefly, 10% clarified brain and lung suspensions were inoculated separately into ten to 12 day chick embryos for hemagglutination activity on Vero, Hep-2 and primary rhesus monkey kidney cells for hemadsorption activity and intracerebrally into 18 to 20 g CD-1 mice for histological studies and virus isolation.

Hemagglutination-Inhibition Test (HI)

The first blood sample was collected from each monkey before infection and the second one was taken from each of them when moribund or on day 17-19 after infection.

TABLE I. Influenza Virus Strains Used and Species of Nonhuman Primates Inoculated

Strain	Reference	Dose EID/50 Log 10	Number of Animals Inoculated		
			Rhesus	Patas	Cercopithecus
A/Hong Kong/1/68 (H3N2) E[a]17	Beare and Bynoe (1)	7.5	32	4	3
		6.5			3
		5.5			4
		4.5	3		
A/Hong Kong/68 (H3N2) E ?.....	Smorodintsev (20)	7.0	3		
A/Aichi/2/68 (H3N2) E 51.....	NIH[e]	7.0	5	4	
A/NWS/33 (H0N1) TC[b]200 <		7.0	5		
Mb[c]18 E 4.....	Minuse[f]	3.0	4		
A/PR8/198/34 (H0N1) M[d] 593... E 170<	Minuse[f]	7.0	3		
Normal allantoic fluid.....		—	8		

[a]Egg passages
[b]Passages in tissue culture
[c]Passages in mouse brain
[d]Passages in mouse
[e]NIH, Division of Biological Standards, Respirovirus Unit, Bethesda, Maryland
[f]Miss E. Minuse, Ann Arbor, Michigan

TABLE II. Mortality, Lesions of the Central Nervous System, Virus Isolation and Seroconversion Following Intracerebral and Intraspinal Inoculation of Nonhuman Primates with Live Influenza Viruses

Virus Strain	Dose EID/50 Log 10	Monkey Species	Mortality	Histopathology: parenchyma	Histopathology: leptomeninges, choroid plexus, ependyma	Virus isolation (day)	Seroconversion (mean geometric HI titer)
A/Hong Kong/1/68 (H3N2)[a]	7.5	Rhesus	26/32	0/32	32/32	1/32 (2)	5/6
	4.5	Rhesus	0/3	0/3	0/3	0/3	0/3
	7.0	Patas	0/4	0/4	2/4	0/4	4/4 (16)
	7.5	Cercopithecus	0/3	0/3	0/3	0/3	3/3 (291)
	6.5	Cercopithecus	0/3	0/3	3/3	0/3	3/3 (160)
	5.5	Cercopithecus	0/4	0/4	0/4	0/4	4/4 (53)
A/Hong Kong/68 (H3N2)[b]	7.0	Rhesus	1/3	0/3	3/3	1/3 (5)	2/2
A/Aichi/2/68 (H3N2)	7.0	Rhesus	4/5	0/5	5/5	0/5	3/3
	7.0	Patas	0/2	2/2[c]	2/2	0/2	1/2 (4)
A/PR8/34 (H0N1)	7.0	Rhesus	0/3	0/3	3/3	0/3	3/3
A/NWS/33 (H0N1)	7.0	Rehsus	2/5	0/5	5/5	1/4 (6)	4/4
	3.0	Rhesus	2/4	0/4	4/4	0/4	3/3
Normal allantoic fluid		Rhesus	0/8	0/8	0/8	0/8	0/8

[a]Attenuated by Beare and Bynoe (1)
[b]Attenuated by Smorodintsev (20)
[c]Mild perivascular infiltration

Sera were heat inactivated (56°C for 30 minutes) and treated with receptor destroying enzyme (RDE) overnight. HI was performed with four hemagglutinating units of antigen.

RESULTS

Table II summarizes the mortality, histological and serological findings and virus isolation.

Clinical Signs and Mortality

None of the monkeys inoculated with noninfected allantoid fluid showed evidence of clinical disease during the 21 days of observation. Approximately 80% of the rhesus monkeys inoculated with either strain of virus developed clinical evidence of CNS involvement and 63% died (Table II). These signs appeared usually five to seven days after inoculation. The animals first exhibited hyperesthesia which was soon followed by lethargy, hunched posture and ataxia. The animals died or were sacrificed within a few hours after the appearance of the clinical signs. Mortality was not recorded in rhesus monkeys inoculated with the A/PR8/34 strain, in patas monkeys inoculated with A/Hong Kong/1/68 and A/Aichi/2/68 strains and in cercopithecus monkeys inoculated with the A/Hong Kong/68 attenuated strains.

Pathology

Apart from the stab wound, none of the monkeys inoculated with control material had histological lesions and gross and histological lesions were not recorded in visceral organs of control and infected monkeys. Coronal sections of the brains showed moderate hydrocephalus in two rhesus monkeys sacrificed 21 days after inoculation (Fig. 1). Histopathological findings were limited to the CNS of infected animals and these lesions were remarkably consistent in all monkeys inoculated whether with the A/Hong Kong/68 attenuated strains, the

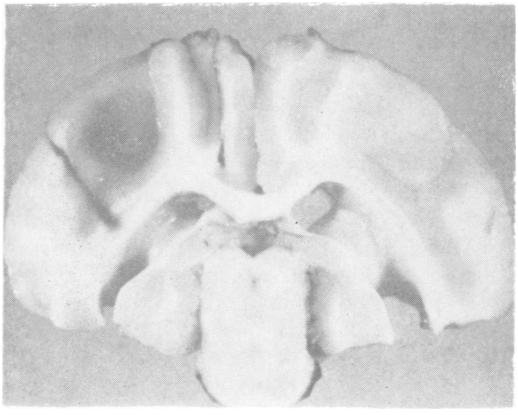

Fig. 1. Lateral ventricles of a rhesus monkey inoculated intracerebrally with A/Hong Kong/68 attenuated strain 21 days previously showing mild hydrocephalus.

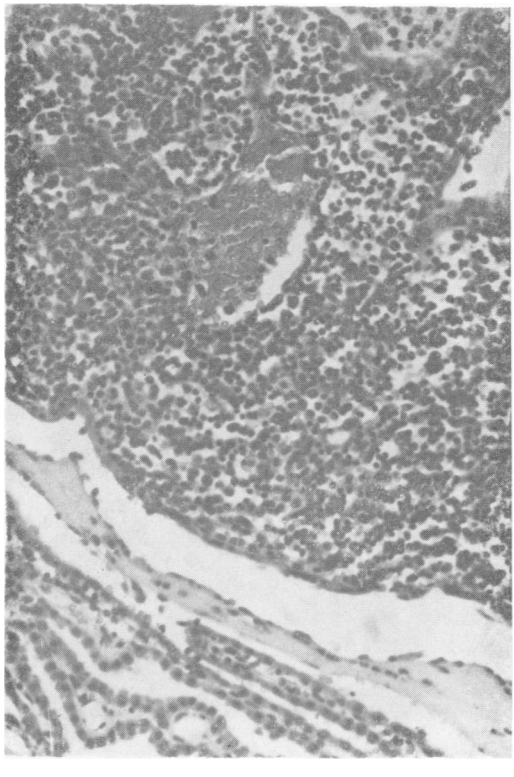

Fig. 2. Choroid plexus of lateral ventricle. Rhesus monkey, five days after intracerebral inoculation with A/Hong Kong/68 attenuated strain showing marked inflammatory response. H & E. X100.

A/PR8/34 and the A/Aichi/2/68 strains or with the mouse neurotropic A/NWS/33 strain. In monkeys dead or sacrificed in the acute phase of infection (five to seven days postinoculation) the histological changes consisted primarily of intense in-

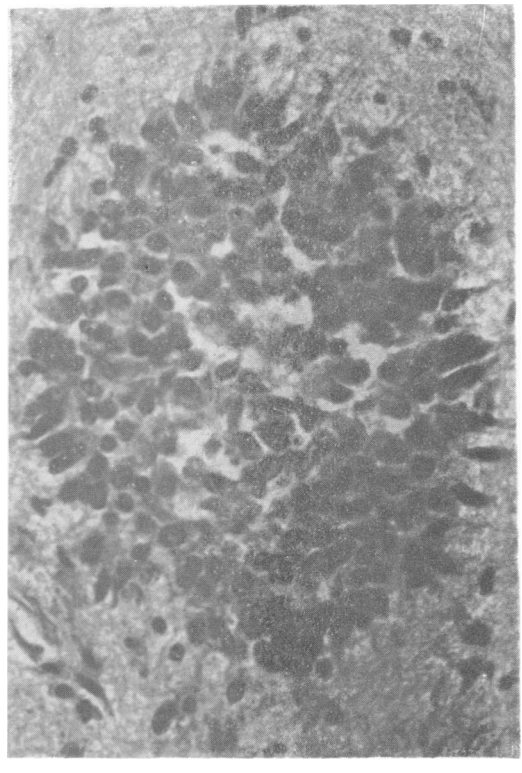

Fig. 3. Central canal of lumbar region. Rhesus monkey, seven days after intraspinal inoculation with A/Hong Kong/68 strain. Note inflammatory response, disruption of ependyma and ependymal cells free in lumen of canal. H & E. X250.

filtration of the choroid plexus (Fig. 2), ependyma, central canal (Fig. 3) and to a lesser degree of the leptomeninges. Mononuclear cells predominated (Figs. 3 and 4) and only occasional polymorphonuclear cells were present. Loss of ependymal cells lining the ventricular, aqueductal and central canal surfaces was also a constant feature (Fig. 5) and ependymal cells were free in the ventricular lumen and central canal. Perivascular inflammatory response was occasionally observed in vessels near the ependymal surfaces (Fig. 6) but inflammation in other areas of the brain and spinal cord was not observed.

In monkeys sacrificed 19-21 days postinoculation the choroid plexus infiltrates were still present (Fig. 7) but the inflammatory response had markedly diminished particularly in the leptomeninges. Patchy ependymal loss was still evident in the ventricles, aqueduct and central canal. The nervous tissue underlying the denuded areas frequently presented a loosened fib-

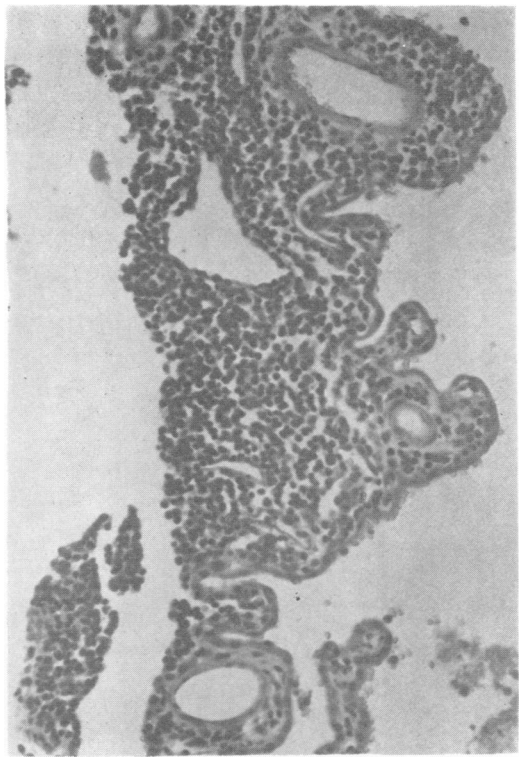

Fig. 4. Choroid plexus of lateral ventricle. Rhesus monkey, seven days after intracerebral inoculation with A/Hong Kong/68 attenuated strain. Mononuclear cells predominate in the inflammatory response. H & E. X100.

rous appearance. In two patas monkeys inoculated with the A/Aichi/2/68 strain, perivascular and parenchymal cellular infiltrates were also found in the pons. Marked narrowing of the aqueduct of Sylvius was evident in two of the six rhesus monkeys killed 21 days postinoculation as a sequella of ependymal cell loss and inflammatory reaction (Fig. 8).

Recovery of Influenza Virus

As indicated in Table II, virus was isolated from the brain of three monkeys that died between the second and the sixth day. Virus was not recovered from the lung of any of the animals inoculated.

All mice inoculated with the brain suspensions of infected monkeys survived an observation period of seven days before they were killed. Histological lesions were not seen and influenza virus was not isolated.

Serological Conversion

All but one of the rhesus monkeys surviving seven days after inoculation of $10^{7.5}$ EID/50 of the virus strains showed a fourfold rise of the HI antibody titer. A dose of $10^{3.0}$ EID/50 of the A/NWS/33 virus strain induced a similar rise in this monkey species.

Patas monkeys produced low titers with the same large inoculum, while cercopithecus monkeys produced higher titers than rhesus monkeys and responded to a dose of $10^{5.5}$ EID/50 of the A/Hong Kong/68 strain.

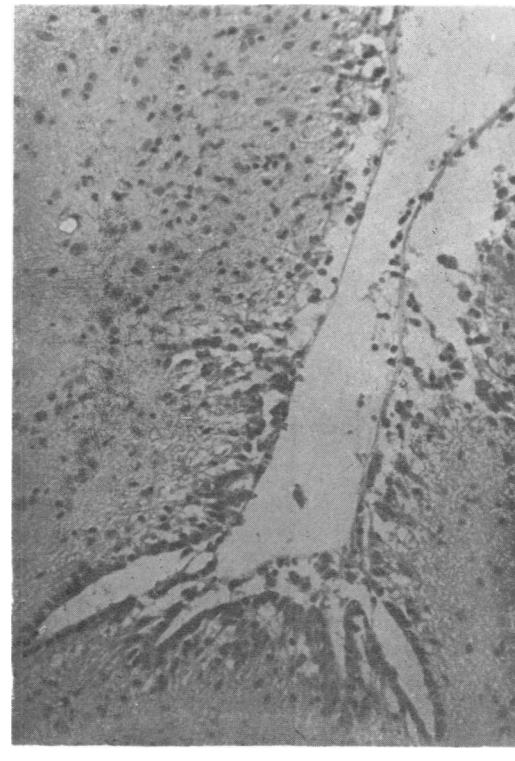

Fig. 5. Ependyma of lateral ventricle. Rhesus monkey, seven days after intracerebral inoculation with A/Hong Kong/68 attenuated strain. There is almost total loss of ependymal cells and increased periventricular gliosis. H & E. X100.

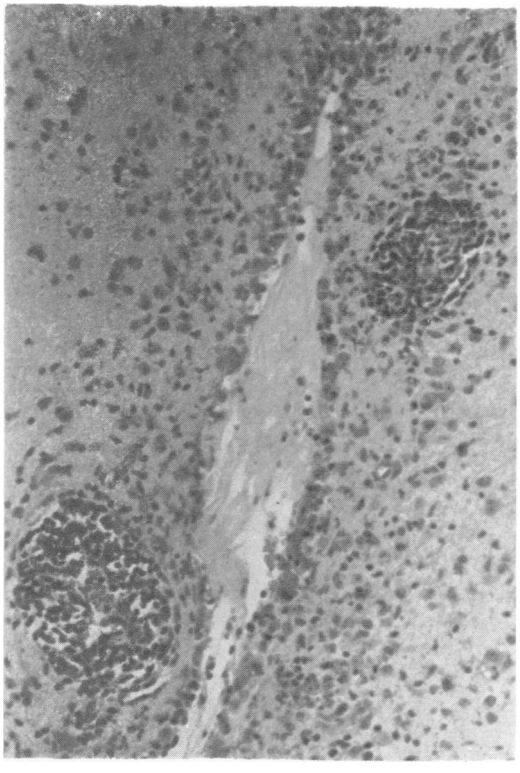

Fig. 6. Ependyma of the third ventricle. Rhesus monkey, seven days after intracerebral inoculation with A/Hong Kong/68 attenuated strain. Acute ependymitis with denudation of epithelium and extension of the inflammatory reaction to the subventricular zone. H & E. X100.

DISCUSSION

Intracerebral and intraspinal inoculations of attenuated, nonattenuated and neuroadapted strains of influenza virus into rhesus, cercopithecus and patas monkeys resulted in an acute, often fatal infection of epithelial cells of ependyma and choroid plexus. All the influenza virus strains tested, including the A/NWS/33 mouse neurotropic strain and the attenuated strains induced similar qualitative and quantitative histological lesions in the CNS of all three species of monkeys. These lesions were characterized by the destruction of the ependymal lining of the ventricles and the central canal and by cellular infiltration of the choroid plexus and leptomeninges.

The affinity of influenza virus for ependymal cells of rodents and birds has been recorded by previous workers (5, 8, 10, 12, 13, 16). There were no reports, however, providing details as to the histopathology of influenza virus in nonhuman primates. The present study demonstrates the susceptibility of the ependymal surfaces of monkeys to influenza virus. Lesions induced by the neurotropic strains A/NWS/33 were limited to the ependyma and extension of infection to the brain parenchyma as it occurs in mice (8, 13) did not occur in monkeys.

The susceptibility of ependymal cells to viral infections appears to be different from other CNS cells (11). This difference may be explained by the fact that the ependyma lining the cerebral ventricles, the central canal and covering the choroid plexus is morphologically and functionally distinct from the underlying parenchyma (6, 7).

A feature brought out in this report is the ability of influenza virus to produce ependymitis leading to hydrocephalus. Mims

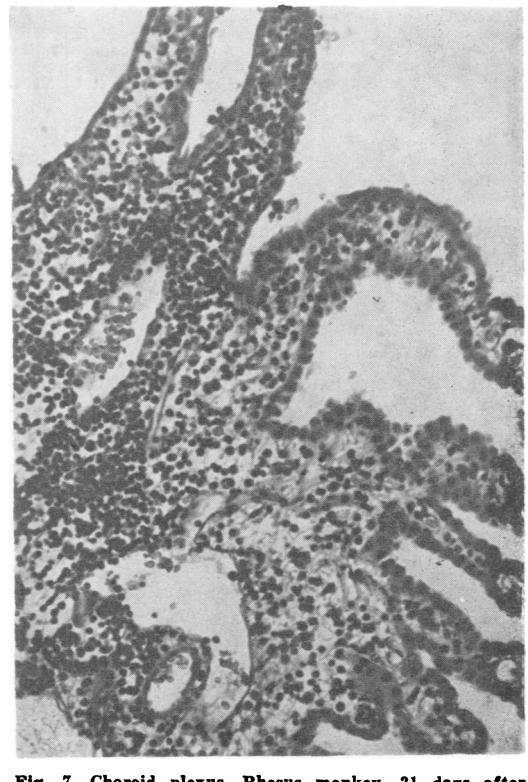

Fig. 7. Choroid plexus. Rhesus monkey, 21 days after intracerebral inoculation with A/Hong Kong/68 attenuated strain. Moderate choroiditis. H & E. X100.

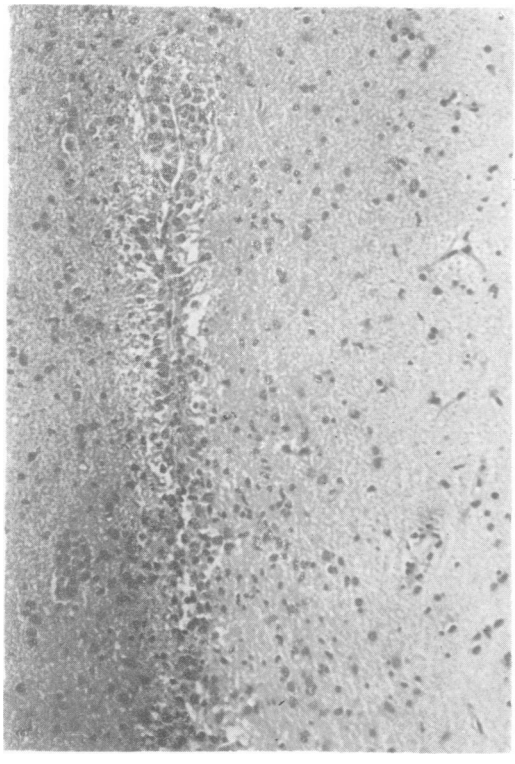

Fig. 8. Coronal section through the midbrain of a rhesus monkey 21 days after intracerebral inoculation with A/Hong Kong/68 attenuated strain. Occlusion of the lumen of partially ulcerated ventricle. Moderate involvement of surrounding parenchyms is visible. H & E. X100.

(13) has previously reported on the occurence of acute hydrocephalus in mice following intracerebral inoculation of influenza virus type A.

Mims (13) has also demonstrated that unadapted type A influenza virus undergoes a single cycle of replication in ependymal and meningeal cells of mice following intracerebral inoculation yielding noninfectious viral components and has shown that neuroadapted strains undergo successive cycles of multiplication in ependymal and meningeal cells with subsequent invasion of underlying parenchymatous tissue. In the present study, virus was recovered on only three occasions from animals that died between the second and the sixth day after inoculation. These isolates are believed to represent residue of the inoculum.

In spite of many studies made on influenza virus, we are still ignorant of what disease of the CNS occurs in the course of infection with this virus in nature. The present studies show that ependymitis, choroiditis and meningitis can be produced in monkeys with different strains of influenza virus and that moderate hydrocephalus can develop as a sequela of the infection. The affinity of influenza virus for ependyma of nonhuman primates may have relevance to disease in man.

ACKNOWLEDGMENTS

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ADDENDUM

Since the completion of this paper, Huygelen *et al* have reported that monkeys (species not mentioned) inoculated by the intrathalamic route with $10^{4.7}$ EID/50 failed to develop symptoms or microscopic lesions. However, monkeys inoculated with $10^{7.9}$ EID/50 failed to show symptoms but develop perivascular infiltrations around the inoculation sites and inflammatory reactions around the ventricules and in the vicinity of the choroid plexuses. These results are in agreement with our observations in cercopithecus monkeys except that a seroconversion was observed in our case.

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