

JAPANESE B ENCEPHALITIS VIRUS: ITS DIFFERENTIATION FROM ST. LOUIS ENCEPHALITIS VIRUS AND RELATIONSHIP TO LOUPING ILL VIRUS

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When encephalitis broke out in August and September of 1932 and 1933, centering in Illinois and Missouri, it was said to resemble Japanese B summer encephalitis (1). But after the St. Louis virus agent was discovered and found to be neutralized specifically by sera from convalescents, additional tests showed that this virus was not neutralized by sera from convalescents of Japanese B encephalitis (2). Further comparisons could not be made at that time because the etiological agent of Japanese B encephalitis had not yet been isolated, but the two diseases appeared to be immunologically distinct (2).

Japanese encephalitis recurred in epidemic form in 1934 and 1935, and from a number of cases virus was recovered. Hayashi, in 1934, reported the transmission of a virus from brain tissue of a fatal case to monkeys for several generations (3), and in 1935, October to December, Kawamura, Hashimoto, Kasahara, Kaneko, Takaki, Taniguchi, and Mitamura reported successful inoculations of brain tissue from fatal cases into mice, and occasionally into monkeys (4). Supplementing these early statements, further reports became available, in which the virus was related directly to the human disease (5-8).

The Japanese workers regarded their virus as similar in many respects to the St. Louis virus. Hence an exchange of Japanese and St. Louis strains and sera was effected between Drs. Kodama, Hashimoto, Takaki, Kasahara, and Mitamura and ourselves in the spring of 1936, and comparative studies were continued in Japan and in our laboratories.

More recently available reports from Japan (9-13) agreed that the

strains of Japanese virus recovered by various workers are identical; that they were neutralized by sera of a large percentage of tested convalescents and by sera of a few individuals without a history of encephalitis living in the epidemic areas, but not by sera of persons without encephalitis living in regions free of the disease (10, 11). Finally, they regarded the Japanese virus as generally similar but not identical with the St. Louis virus (9-11, 13).

The onset of the experimental disease in mice was said to be more frequently accompanied by paralysis of the posterior extremities (8, 9, 5, 7), and the virus following nasal instillation had a greater tendency to enter the blood stream (9) and a greater virulence when injected intraperitoneally (8, 9, 5, 7). In monkeys the virus was more virulent in that it induced a rapid and fatal cerebellar syndrome (9).

Kawamura and associates (9), and Kasahara and associates (13) found no cross-protection in hyperimmune Japanese and St. Louis rabbit sera nor in sera of convalescents of the two diseases. Kudo (10) and Takaki (11) observed no cross-protection of St. Louis hyperimmune rabbit sera but some crossing of Japanese immune rabbit sera.

The present report of our own studies on Japanese encephalitis, besides confirming for the most part those of the Japanese workers, shows certain relationships between this and other viruses associated with epidemic encephalitis in man.

Characteristic Reactions of Japanese Virus in Animal Species

The Japanese virus¹ induced reactions in animal species which were readily distinguished from those produced by St. Louis virus but approximated closely those of louping ill virus. It proved innocuous in rabbits and guinea pigs but induced in mice, monkeys, and sheep a fatal encephalitis.

Thus Swiss mice developed encephalitis following injections of virus by the intracerebral, intranasal, subcutaneous, and intraperitoneal routes. Following nasal instillation they showed, after 5 to 8 days, paralyzes of the posterior extremities, or occasionally tremors and convulsions. They became prostrate and died in 7 to 10 days. The experimental disease resembled that following inoculation

¹ Six strains received from five Japanese investigators proved similar in all respects as tested in our laboratory. They passed Seitz filters readily, were virulent when inoculated in 0.03 cc. amounts intracerebrally into Swiss mice to the 10^{-7} dilution, and when frozen and dried retained their virulence well.

with louping ill virus and with St. Louis virus except for a more frequent onset with paralysis. Histological examination of tissue of these mice showed a type and distribution of lesions similar to those produced by St. Louis and louping ill viruses. The brain showed perivascular and subdural accumulations of round cells plus specific necrosis of nerve cells in the olfactory tracts, Ammon's horn (Fig. 1, and Kawamura, Fig. 4 (9)), anterior limbic area, hypothalamus, and, at late stages, throughout the cortex. The spinal cord remained relatively normal. Virus injected intraperitoneally or even subcutaneously in relatively small doses, 100 to 1,000 times the minimum intracerebral dose, usually induced encephalitis. Louping ill virus was similar in this respect but St. Louis virus was innocuous by these routes unless massive doses were employed. Moreover, Japanese and louping ill viruses, inoculated subcutaneously or intraperitoneally, reached the circulating blood promptly and persisted longer than St. Louis virus. Finally, Japanese and louping ill (14) viruses, following nasal instillation, were readily recovered from the blood stream, while St. Louis virus was rarely found.

Macacus rhesus monkeys were susceptible to the Japanese virus inoculated intracerebrally (8, 9, 5, 7) or intranasally. Following nasal instillation they showed an elevation of temperature to 106° on the 4th day, and a severe cerebellar ataxia on the 5th or 6th day. They became prostrate and died within 10 days. Samples of blood drawn daily and injected intracerebrally into mice failed to show virus. Sections taken at autopsy showed lesions limited primarily to the brain and consisting of necrotic nerve cells scattered irregularly, plus foci of round cells surrounding small blood vessels. Necrosis of the Purkinje cells of the cerebellum was especially marked (Fig. 2, and Kawamura, Fig. 14 (9)). These cells in the superficial convolutions appeared enlarged, or with pyknotic nuclei and granular cytoplasm, or shrunken with deep staining cytoplasm. In the deeper crypts many of the Purkinje cells were entirely missing and the few remaining were small and distorted. Nearby, a local "gliosis" was not uncommon. There was very little change in the surrounding tissue. The reactions described above following nasal instillation are generally similar to those following intracerebral injection of the virus. They are also similar to those following intracerebral inoculation of the louping ill virus (15). Monkeys injected with St. Louis virus, on the other hand, either remained normal or developed a mild, non-fatal encephalitis. Lesions could not be demonstrated with certainty without multiple intracerebral injections.

Sheep inoculated intracerebrally or intranasally with Japanese virus developed an acute, fatal encephalitis.

Lambs weighing 40 to 60 pounds received an intranasal instillation of 1 cc. of the mouse brain virus diluted 1 to 100. Temperatures rose on the 4th day to 106.0°, the animals became quiet, lost appetite, their heads drooped and legs weakened, and by the 8th day they were unable to rise. No definite central nervous system signs were noted. Blood drawn daily and injected intracerebrally into mice showed no virus. At autopsy, brain tissue injected into mice brought them down promptly with the characteristic signs of the Japanese disease. Sections of brain tissue showed blood vessels generally engorged and surrounded by

many round cells. Foci of round cells were likewise scattered irregularly throughout the brain. Many Purkinje cells of the cerebellum were in various stages of necrosis and neighboring glial cells appeared abnormal (Fig. 3). Scattered nerve cells throughout the cortex and brain stem were likewise necrotic. As with monkeys, the reactions described above following nasal instillation are generally similar to those following intracerebral injection of the virus. They are also similar to those following intranasal and intracerebral inoculation of the louping ill virus (Fig. 4).

1 cc. of virus diluted 1 to 10 and injected subcutaneously into lambs proved harmless but immunized them against a later nasal injection fatal to unvaccinated controls.

TABLE I

Virulence of Central Nervous System Viruses of Man Inoculated Intracerebrally into Laboratory Animals

Virus	Rabbit, Guinea pig	Mouse	Macacus monkey	Young sheep
Rabies	++	++	++	++
Louping ill	0	++	++	++
Japanese B encephalitis				
Australian X (?)				
St. Louis encephalitis	0	++	0-±	0
Poliomyelitis	0	0	+	0

These reactions in animal species differentiate the Japanese B from the St. Louis virus but relate it closely to louping ill virus.

A presumptive differential diagnosis of Japanese B encephalitis and other viruses associated with a primary encephalitis of man may be carried out on the basis of the above reactions in animal species in the manner outlined in Table I.

Lack of Cross-Resistance of Immunized Mice

Mice immune to Japanese virus were not immune to St. Louis virus, and conversely, mice immune to St. Louis virus were not immune to Japanese virus. Tests were not made with louping ill virus.

Mice were immunized against Japanese and St. Louis virus by repeated subcutaneous injections of sublethal doses of the virulent homologous virus. Attempts to immunize mice against louping ill virus failed. Sublethal doses, even when repeated, did not immunize while larger doses, lethal to 20 to 30 per cent of vaccinated mice, left a selected group of survivors whose subsequent resistance

to homologous or heterologous virus could not be judged as due to specific immunity factors alone, but might have depended on initial, non-specific, inherited factors. For this reason these survivors were not considered proper material for testing and were discarded.

The Japanese and St. Louis virus mice were tested after 2 to 4 weeks with homologous and heterologous virus in graded intracerebral doses. Unvaccinated mice were likewise tested as controls. The protocol of one such experiment is shown in Table II, in which the Japanese virus proved fatal to unvaccinated and to St. Louis vaccinated mice through the 10^{-6} dilution but not to mice immunized with Japanese beyond the 10^{-5} dilution. Similarly, the St. Louis virus was fatal alike to unvaccinated and Japanese virus mice through the 10^{-6} dilution but not to St. Louis virus mice beyond the 10^{-4} dilution.

TABLE II

Absence of Cross-Resistance of Mice to Japanese B and St. Louis Encephalitis Viruses

Mice vaccinated against	Vaccinated mice tested with	Duration of life of tested mice in days		
		0.03 cc. of test virus diluted		
		10^{-5}	10^{-6}	10^{-7}
Unvaccinated controls	St. Louis virus	*5, 5, 5, 5	5, 5, 5, 6	†S, S, S, S
St. Louis virus	“ “ “	S, S, S, S	S, S, S, S	—
Japanese B virus	“ “ “	6, 6, 6, 7	5, 6, 6, 7	—
Unvaccinated controls	Japanese B virus	5, 5, 5	6, 6, 6, 7	7, 7, 8, S
Japanese B virus	“ “ “	8, 8, 8, 9	S, S, S, S	—
St. Louis virus	“ “ “	5, 5, 5, 5	7, 8, 9, S	—

* Mouse died of encephalitis 5 days following injection.

† Mouse remained well following injection. Discarded at 30 days.

Lack of Cross-Protection of Immune Sera

Protection tests disclosed no definite immune relation between Japanese B, St. Louis, and louping ill viruses.

Tests with hyperimmune sera were made in the following manner.

Monkeys were each given repeated subcutaneous and intraperitoneal injections of the respective living mouse brain viruses. Sera were drawn and mixed undiluted with various concentrations of mouse brain virus prepared in the usual manner in broth as a diluent. After standing at 37°C . for 2 hours and at 23°C . for 2 hours, the mixtures were each injected intracerebrally in 0.03 cc. amounts into four Swiss mice. Duration of life of the injected animals was recorded in days.

TABLE III
Lack of Cross-Neutralization of Japanese and St. Louis Encephalitis, and Louping Ill Viruses in Heterologous Sera

Test sera	Test virus	Duration of life of tested mice in days			
		Dilution of virus in serum mixtures. 0.03 cc. injected			
		10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷
Monkey	Japanese No. 2	*5, 5, 5, 7	7, 7, 8, 8	8, 8, 9	7, 7, 12
"	"	9, 11	S, S, S, S	S, S, S	—
"	"	—	7, 7, 8, 10	7, 8, 10	—
"	"	—	8, 9, 10, S	10, 11, S	—
"	St. Louis	—	5, 5, 7, 7	10, 10, 10, 11	—
"	"	7, 7, 9	S, S, S, S	—	—
"	"	—	9, 9, 10, 10	S, S, S, S	—
"	"	—	7, 9, 9, 9	11, 11, S, S	—
"	Louping ill	7, 7, 7, 7	7, 8, 8, 8	9, 9, 9, 10	8, 9, 9
"	"	S, S, S, S	S, S, S, S	S, S, S, S	—
"	"	—	7, 8, 9, 9	9, 9, 11, 11	—
"	"	—	9, 9, 9, 9	8, 9, 9, 9	—

S = mouse remained well 21 days.

— = dilution not tested.

* Mouse died of encephalitis 5 days following injection.

The protocol summarized in Table III shows Japanese virus No. 2 plus normal monkey serum fatal to 50 per cent or more of four mice per dilution through 10^{-7} , fatal through 10^{-4} when combined with homologous immune serum, and through 10^{-6} when mixed with St. Louis and louping ill immune sera respectively. Similarly, St. Louis virus mixed with normal monkey serum was fatal through the 10^{-6} dilution, with homologous immune serum through the 10^{-4} dilution, and with Japanese or louping ill, through the 10^{-5} and 10^{-6} dilutions respectively. Finally, louping ill virus mixed with normal monkey serum was fatal through the 10^{-7} dilution, with homologous immune serum, in less than the 10^{-4} dilution, and with Japanese and St. Louis sera through the 10^{-6} dilutions, respectively. In short, virus mixed with heterologous immune sera, although slightly less active than with normal monkey serum, was 10 to 100 times more active than when combined with homologous immune sera.

Sera from St. Louis convalescents did not protect against the Japanese virus nor did sera from Japanese convalescents protect against St. Louis virus. A curious instance of cross-protection, however, has been noted by Kuttner and confirmed in our laboratory.

Kuttner described two cases of encephalitis contracted by Europeans in China (16). Serum from W. protected against both Japanese and St. Louis viruses, and serum from G. protected against Japanese but was not tested against St. Louis virus. Further sera from these cases² were sent directly to our laboratory and were found to protect well against both Japanese and St. Louis and not against louping ill virus.

Finally, sera from Japanese and St. Louis convalescents did not protect against louping ill virus nor did sera from supposed convalescents of louping ill virus (17) protect against Japanese or St. Louis viruses.

DISCUSSION

The virus from Japan described above is regarded as the etiological agent of Japanese B summer encephalitis on the following grounds. During an epidemic outbreak, strains were recovered from brain tissue of fatal cases of encephalitis by several independent investigators in Japan at the same time and by the same technique. All strains have proved identical in so far as tested. They were said to be neutralized specifically by sera from convalescents and certain contacts. The virus proved similar to others in the encephalitis group

² Obtained through the kindness of Dr. Kuttner.

and yet ultimately distinct. Japanese B encephalitis is included, therefore, in the group of primary central nervous system infections of man of known virus etiology which occur in epidemic form in late summer. These infections, poliomyelitis, Japanese B encephalitis, Australian X disease, and St. Louis encephalitis, have features in common but are distinguishable by laboratory tests. Outbreaks of each are frequent and limited chiefly to hot weather, and cases of each are scattered throughout an infected community, usually not more than one per family. Clinically the diseases are often difficult to identify. Each may be recognized, however, by testing sera of convalescents for specific neutralizing properties against one of these virus agents, or by obtaining virus from brain tissue of fatal cases and testing its virulence for mice, monkeys, and sheep and its neutralization in specific antisera.

Possible relationships between Japanese B encephalitis, the sheep encephalitis of Scotland (louping ill), and poliioencephalitis of children in Australia (X disease) should be further explored. The sheep virus is probably infectious for man, judging from the fact that three investigators, shortly after commencing work with the virus (17), contracted encephalitis and later showed specific neutralizing antibodies in their sera. Moreover, louping ill virus has been related to the virus associated with X disease of children in Australia (18), and now has proved similar to the newly discovered Japanese B encephalitis virus. Further studies are needed to determine whether this relationship is merely superficial or of immediate epidemiological importance.

The question of mode of spread of this group of infections through a community and their portal of entry into the body are unknown and difficult to investigate. Laboratory tests on susceptible animals indicate clearly that the most vulnerable portal for the experimental introduction of these viruses is the nasal mucosa: this is the route of choice in mice for the St. Louis virus; in mice, monkeys, and sheep for the Japanese and louping ill (21) viruses; and in monkeys for the poliomyelitis virus. There is also evidence that spontaneous louping ill may occur both in man (17) and in mice (20) through the nasal mucosa. But contact experiments to induce spontaneous transfer of St. Louis, Japanese B, and poliomyelitis infections among susceptible

laboratory animals have failed and it is difficult or impossible to detect the virus in the upper respiratory tract of the diseased individual. On the other hand, subcutaneous injections of Japanese B and louping ill viruses in mice and of poliomyelitis virus in monkeys and possibly man may also induce disease. Moreover, reports state that in nature louping ill infection of sheep takes place subcutaneously by the bite of an insect (19). But experiments to detect virus in the blood of individuals following natural or experimental infection are either positive for brief periods only (louping ill, Japanese B viruses) or are completely negative (poliomyelitis, St. Louis viruses). Hence there is evidence for and against both the upper respiratory and subcutaneous routes of transmission of these infections and the question remains perplexing.

CONCLUSIONS

1. Japanese B encephalitis virus, obtained from Japanese investigators, has proved virulent for mice and monkeys, confirming the reports from Japan. It has also been found virulent for monkeys when instilled intranasally and for sheep when introduced intracerebrally or intranasally.
2. Japanese B encephalitis virus has been differentiated from St. Louis virus and found similar to louping ill virus according to its reactions in animal species. Serologically, however, it is distinct.
3. Japanese B encephalitis and its related group of primary virus encephalitides of man have been discussed with regard to their differentiation and mode of spread.

BIBLIOGRAPHY

1. Leake, J. P., *J. Am. Med. Assn.*, 1933, **101**, 928; Leake, J. P., Musson, E. K., and Chope, H. D., *J. Am. Med. Assn.*, 1934, **103**, 728.
2. Webster, L. T., Fite, G. L., and Clow, A. D., *J. Exp. Med.*, 1935, **62**, 827.
3. Hayashi, M., *Proc. Imp. Acad.*, Tokyo, 1934, **10**, 41.
4. These reports in the Japanese journals have not been available to the writer but are taken from later reports by Kasahara (5) and Kawamura (9).
5. Kasahara, S., and associates, *Kitasato Arch. Exp. Med.*, 1936, **13**, 48, 248.
6. Hashimoto, H., Kudo, M., and Uraguchi, K., *J. Am. Med. Assn.*, 1936, **106**, 1266.
7. Taniguchi, T., and associates, *Japan. J. Exp. Med.*, 1936, **14**, 185.
8. Kaneko, R., and associates, *Klin. Woch.*, 1936, **15**, 674.

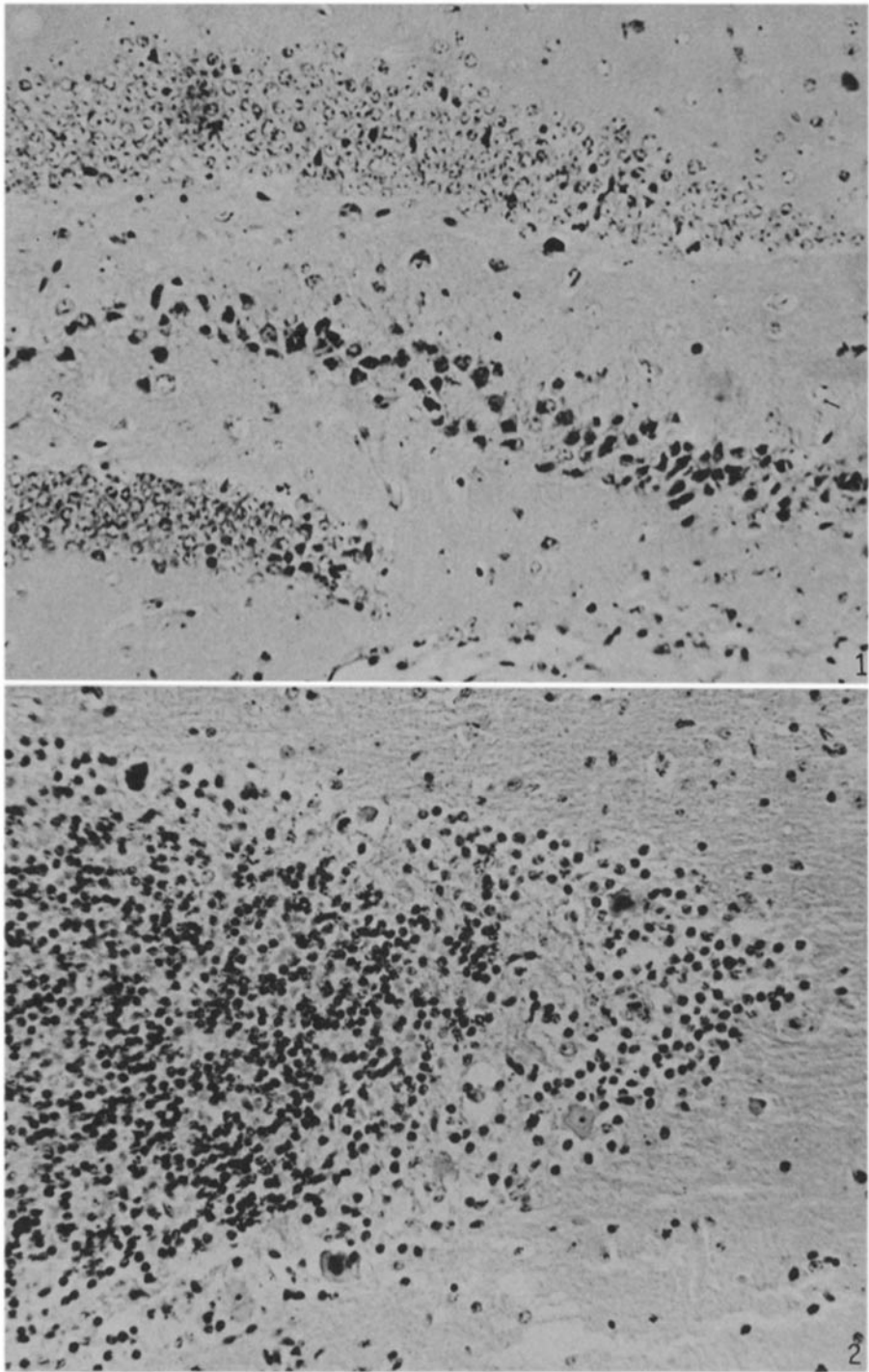
9. Kawamura, R., Kodama, M., and associates, *Kitasato Arch. Exp. Med.*, 1936, **13**, 281; *Arch. Path.*, 1936, **22**, 510.
10. Kudo, M., Uraguchi, K., Matsuda, S., and Hashimoto, H., *J. Immunol.*, 1937, **32**, 129.
11. Takaki, I., personal communication.
12. Kasahara, S., Yamada, R., Hamano, R., and Okamoto, Y., *Kitasato Arch. Exp. Med.*, 1937, **14**, 220.
13. Kasahara, S., Yamada, R., and Hamano, R., *Kitasato Arch. Exp. Med.*, 1937, **14**, 229.
14. Webster, L. T., and Fite, G. L., *Proc. Soc. Exp. Biol. and Med.*, 1933, **30**, 656; 1934, **31**, 695.
15. Hurst, E. W., *J. Comp. Path. and Therap.*, 1931, **44**, 231.
16. Kuttner, A., and Tung, T., *J. Clin. Inv.*, 1936, **15**, 525.
17. Rivers, T. M., and Schwentker, F. F., *J. Exp. Med.*, 1934, **59**, 669.
18. Perdrau, J. R., *J. Path. and Bact.*, 1936, **42**, 59.
19. McLeod, J., and Gordon, W. S., *J. Comp. Path. and Therap.*, 1932, **45**, 240.
20. Alston, J. M., and Gibson, H. J., *Brit. J. Exp. Path.*, 1931, **12**, 82; and unpublished observations by the author.
21. Webster, L. T., and Fite, G. L., *Proc. Soc. Exp. Biol. and Med.*, 1933, **30**, 656.

EXPLANATION OF PLATES

PLATE 26

FIG. 1. Swiss mouse. Section through Ammon's horn 6 days after nasal instillation of Japanese B encephalitis virus. The left and right vertical columns of pyramidal cells appear relatively normal. Cells in the central column are in various stages of necrosis. Eosin-methylene blue. $\times 275$.

FIG. 2. Macacus monkey. Section through cerebellum 8 days after nasal instillation of Japanese B encephalitis virus. The Purkinje cells are necrotic or entirely absent. Eosin-methylene blue. $\times 275$.



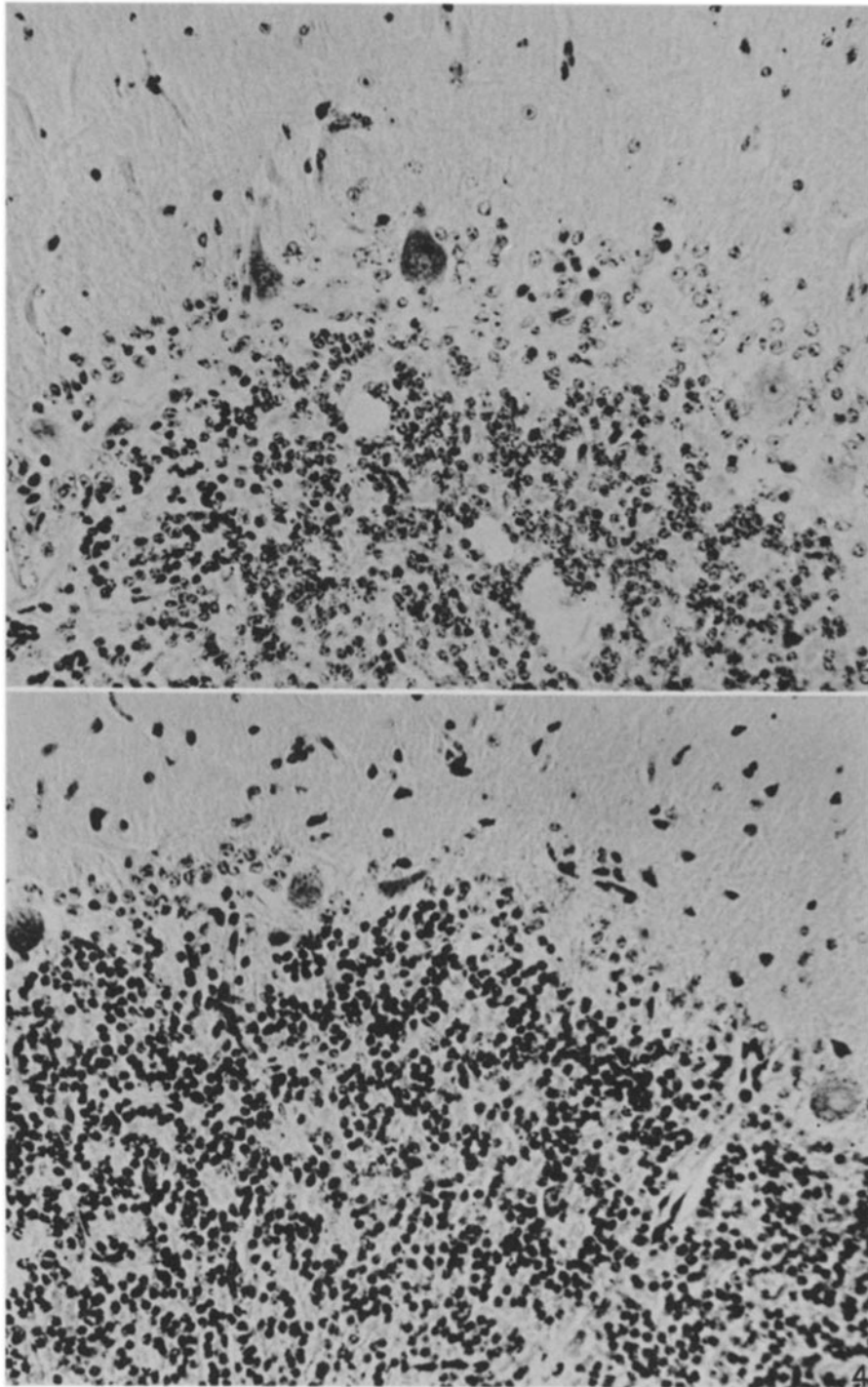
Photographed by Joseph B. Haulenbeck

(Webster: Japanese B encephalitis virus)

PLATE 27

FIG. 3. Young sheep. Section through cerebellum 9 days after nasal instillation of Japanese B encephalitis virus. The Purkinje cells are in early stages of necrosis. A few are missing entirely. There is some reaction of glial cells in the molecular layer. Eosin-methylene blue. $\times 275$.

FIG. 4. Young sheep. Section through cerebellum 8 days after nasal instillation of louping ill virus. The Purkinje cells are necrotic and there is reaction of neighboring glial cells. Eosin-methylene blue. $\times 275$.



Photographed by Joseph B. Haulenbeek

(Webster: Japanese B encephalitis virus)