Japanese Encephalitis Virus Neurotropism Is Dependent on the Degree of Neuronal Maturity

AKIHIKO OGATA,¹ KAZUO NAGASHIMA,¹ WILLIAM W. HALL,² MASUMI ICHIKAWA,³
JUNKO KIMURA-KURODA,⁴ AND KOTORO YASUI^{4*}

Division of Infectious Disease, North Shore University Hospital, Manhasset, New York 11030,² and Department of Pathology, Hokkaido University School of Medicine, Sapporo-shi, Hokkaido 060,¹ and Departments of Developmental Neuroanatomy³ and Microbiology,⁴ Tokyo Metropolitan Institute for Neurosciences, 2-6, Musashidai, Fuchu-shi, Tokyo 183, Japan

Received 16 July 1990/Accepted 13 November 1990

In a study on Fischer rats, all animals infected with Japanese encephalitis virus (JEV) before the age of 13 days died, but animals infected after the age of 14 days did not die, confirming the age-dependent resistance to JEV infection in the rat brain. A study of the kinetics of JEV infection in the developing rat brain disclosed that JEV antigen disappeared in a particular pattern, i.e., from the deeper layers to the upper layers of the motor cortex, which paralleled neuronal maturation in the cortex. Fifteen-day-old rats, which were resistant to JEV infection, received intracerebral transplants of neurons taken from 19-day embryos. When these animals were infected with JEV after transplantation, viral antigen was detected only in the embryonal neurons soon after transplantation. Thus, it can be concluded that the susceptibility to JEV infection in the rat brain is closely associated with neuronal immaturity.

The capacity of viruses to selectively infect specific tissues depends on an interaction between viral genes or proteins and host factors. Binding of viral attachment proteins to host cell receptors is a well-established phenomenon of viral tissue tropism. For example (16, 24), human immunodeficiency virus type 1 specifically attacks T-helper/inducer cells by usurping CD4 molecules, Epstein-Barr virus selectively attacks B cells by using complement receptor type 2 as its own receptor, and hepatitis B virus infects only hepatocytes via the hepatocyte receptor for polymerized serum albumin. However, the interaction of a virus and its receptor cannot explain the mechanism of viral tissue tropism sufficiently. Poliovirus destroys only human motoneurons, although its receptor gene is known to be expressed in tissue widely (18), and human rhinovirus infects only nasal mucosa although its receptor, intercellular adhesion molecule 1, is distributed throughout the whole body (5, 22). In addition to the interaction between cell receptors and viral attachment proteins, viral enhancers have been known to play a role in tropism. The human papovavirus, JC virus, the agent responsible for the demyelinating disease progressive multifocal leukoencephalopathy is known to proliferate only in primary human fetal glial cells. In this case, the viral enhancer region present in the JC virus genome is the major determinant of viral tropism (14).

Japanese encephalitis virus (JEV), a member of the flaviviruses, is the most common cause of arthropod-borne human encephalitis worldwide. The complete nucleotide sequence of JEV genome RNA has recently been determined (23), and the biological functions of virus-encoded proteins have been analyzed. However, the neurotropism and intracerebral disease expression of JEV have not been examined. Early experimental studies of JEV infection in rats suggested that the younger an animal is, the more vulnerable it is to JEV infection (2). It has been previously reported that both adult and newborn mice are susceptible to

intracerebral inoculation with JEV; however, the effect was dose dependent, and younger mice were more susceptible than older mice to the lethal effects of JEV (6). Such an age-dependent susceptibility of brain to viral infection has also been noted with the other flaviviruses, St. Louis encephalitis virus (19, 20) and yellow fever virus (21). In the present study, we analyzed JEV infectivity in developing rats and examined the vulnerability of transplanted neurons to JEV. The results demonstrate that JEV infectivity is closely associated with neuronal immaturity.

MATERIALS AND METHODS

Animals and virus. The animals used in the study were albino rats, Fischer strain, obtained from Nippon SLC (Tokyo, Japan). The supplier allowed mating to occur overnight, and the following morning was considered to be embryonic day 1 for females with vaginal plugs, with birth usually occurring on day 21. The virus used was the JaGAr-01 strain of JEV. The supernatants from 10% homogenate of the infected brains of mice (infective titer of 10^9 PFU/ml) were diluted 10 times with 20% hemacel (Hoechst) in Eagle minimum essential medium and were stored at -70°C until use. Virus solutions (0.03 ml containing 3×10^6 PFU) were inoculated intracerebrally with a specially designed two-step thin syringe (Hoshimorido Iryoki KK, Tokyo, Japan) which is 27 gauge in diameter with a stopper 3 mm from the tip. The brain site used was located at the midpoint of the line connecting the left eye to the midpoint between the right and left ears. Hemacel (20%) in Eagle minimum essential medium was injected into controls.

Preliminary experiments. Animals were infected with JEV at each day after birth, and mortality rates were estimated (Fig. 1). The 7-day-old rats exhibiting 100% mortality were chosen for further study. After infection, they were sacrificed on days 1, 2, and 3 under anesthesia with ether by perfusion fixation via the aorta with 4% freshly prepared paraformaldehyde in 0.1 M phosphate buffer. Because in both frozen and paraffin-embedded sections, JEV antigen

* Corresponding author.

FIG. 1. Relationship of age at inoculation and mortality in rats infected intracerebrally with JEV. A striking age-dependent decrease in susceptibility is evident.

was clearly demonstrated at the third day after inoculation by immunohistochemical methods described below, the third day after inoculation was used for further pathological studies.

Kinetic study. Animals (5 to 10 in each group) were inoculated with JEV on days 1, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 17, 19, 21, and 49 after birth. Each animal was sacrificed 3 days after inoculation under ether anesthesia by perfusion fixation. Coronal brain sections (2 mm wide) were taken from the frontal tip to the spinal cord, embedded in paraffin, and stained with hematoxylin-eosin, Luxol fast blue-cresyl violet (Klüver-Barrera method), and anti-JEV antibody (described below).

Neuronal transplantation and infection. Pieces of embryonic cerebral cortex were sterilely taken from the fetus at the 19th gestational day by using a magnifying microscope, dissected in 10 ml of phosphate-buffered saline containing 2 mg of cysteine salt, 2 mg of bovine serum albumin, 50 mg of

glucose, and 2 μ l of papain (Boehringer), and incubated for 30 min at 37°C. They were centrifuged for 10 min at 70 $\times$ g, and the pellets were resuspended in Dulbecco modified Eagle medium (DMEM) supplemented with antibiotics. The tissues were dispersed by pipetting, and DNase I (Sigma) was added at a final concentration of 0.05%. After repeated centrifugation and pipetting, the dispersed cells were labeled by inoculation with the DMEM solution containing 0.001% fast blue (Sigma) for 15 min. The labeled cells were washed three times with DMEM and adjusted to 10⁷/ml, and 0.03 ml was transplanted into the brain of a 15-day-old rat at the right cerebral cortex by the same method used for the viral inoculation. Six rats were infected with JEV 3 days after neuronal transplantation, and nine rats were infected 9 days after transplantation. Both groups were sacrificed at the third day of virus infection. Fast blue-labeled neurons were surveyed under UV light with a fluorescence microscope.

Immunohistochemistry. The avidine-biotin-peroxidase complex (ABC) method was used in the present study. After deparaffinization, the specimens were quenched with 0.3% H₂O₂-methanol to suppress the endogenous peroxidase activity, incubated with 10% normal goat serum for 20 min, and allowed to react with anti-JEV rabbit serum. Incubation with preimmune rabbit serum was used as negative control. They were then reacted with biotinylated anti-rabbit goat immunoglobulin G (Vecstain), and ABC reaction products were visualized by using 3,3'-diaminobenzidine tetrahydrochloride (Sigma).

RESULTS

The mortality of rats infected with JEV is shown in Fig. 1. All rats infected when they were from 2 to 12 days old died. However, the mortality rate of animals infected when they were older than 12 days decreased with age, with 13-day-old rats having 50% mortality (2 of 4) and 14-day-old rats having 8.3% mortality (1 of 12). Animals inoculated when they were older than 17 days showed 0% mortality (17-day-old rats [0 of 4], 19-day-old rats [0 of 6], and 21-day-old rats [0 of 14]). Although both 2-day-old and 9-day-old rats showed 100% mortality, younger animals died more rapidly. All 2-day-old rats died within a week, whereas 9-day-old rats started dying at day 7 after inoculation until day 14. This showed that the older the rat was at the time of infection, the longer it survived.

The distribution of JEV antigen in the brain was examined

TABLE 1. Topographical distribution of JEV antigen in the rat brain

Sample location	Immunostaining ^a of samples from rats at the following age (days):											
	1	3	4	5	6	7	8	9	10	12	14	17
Caudate putamen	+	+	+	+	+	+	+	+	+	+	±	—
Substantia nigra	+	+	+	+	+	+	+	+	+	+	±	—
Thalamus	+	+	+	+	+	+	+	+	+	±	—	—
Amygdaloid nuclei	+	+	+	+	+	+	+	+	+	±	—	—
Mamillary body	+	+	+	+	+	+	+	+	—	—	—	—
Retrosplenial cortex	+	+	+	+	+	+	+	+	—	—	—	—
Dorsal cortex of inferior colliculus	+	+	+	+	+	+	+	+	—	—	—	—
Ammon's horn	+	+	+	+	+	+	+	±	—	—	—	—
Pontine nuclei	+	+	+	+	+	+	—	—	—	—	—	—
Nucleus solitary tract	+	+	+	+	+	+	—	—	—	—	—	—
Cerebellum	+	+	+	+	±	±	—	—	—	—	—	—
Olfactory bulb	+	+	+	—	—	—	—	—	—	—	—	—

^a Intensity for immunostaining: +, definitely positive; ±, faintly positive; —, negative.

FIG. 2. Motor cortex of rat infected with JEV and stained with anti-JEV. ABC method. Magnification, $\times 100$. (a) Motor cortex of a rat infected at the age of 4 days. Diffuse infection involving all neurons in the cortex is observed. (b) Motor cortex of a rat infected at the age of 6 days. Neurons in the upper layers still contained viral antigen, but most of those in deeper layers were not infected. (c) Motor cortex of a rat infected at the age of 8 days. Neurons in the superficial position of layer II were stained, but staining of the remaining neurons was negative. (d) Motor cortex of a rat infected at the age of 9 days. No JEV-positive neurons were present in the cortex.

in animals of various ages (Table 1). Up until the age of 5 days, all areas of the brain except the olfactory bulb were infected, showing panencephalitis. In the 7-day-old rats, antigen could not be found in the pons, medulla oblongata,

and cerebellum, and in the 10-day-old rats, antigen was undetectable in the cerebral cortex, and only neurons of the subcortical nuclei, such as caudate nuclei, putamen, substantia nigra, and thalamus, were infected. In 14-day-old

FIG. 3. Layer II of the motor cortex from a rat infected with JEV at the age of 7 days. (a) Slight shrinkage of neurons and lymphocytic infiltration of the meninges were seen. Klüver-Barrera stain was used. Magnification, $\times 400$. (b) Immunohistochemical study with anti-JEV demonstrated that several neurons in the superficial areas of layer II contained JEV antigen. Immunoreactive material was found in the apical cytoplasmic portion of the neurons. ABC method. Magnification, $\times 400$.

FIG. 4. Neurons taken from an embryo on day 19 (gestational age) were transplanted into the brain of a 15-day-old rat. This was followed by inoculation of JEV on the third day after transplantation. (a) The transplanted neurons stained darker than the original cortical host neurons and destroyed the arrangement of the normal cortical architecture. Hematoxylin-eosin stain was used. Magnification, $\times 150$. (b) Transplanted cells labeled with fast blue and visualized by fluorescence microscopy. Magnification, $\times 150$. (c) Transplanted cells shown to be infected with JEV, with original host neurons uninfected. ABC method. Magnification, $\times 150$.

rats, JEV antigen was confined to the caudate nuclei, putamen, and substantia nigra, and from 17 days onward, no viral antigen was detected anywhere in the brain. The data showed that JEV-susceptible neurons changed with age in the brain.

When the distribution of JEV antigen in the motor cortex was examined in detail, it became clear that there was a pattern of laminar infectivity; that is, up until day 5, neurons of all layers of the cortex were diffusely infected (Fig. 2a), but in the 6-day-old rats neurons in layers II and III were severely infected, with uninfected neurons in layers IV and V (Fig. 2b), although a few neurons in layer VI contained viral antigens. In the 8-day-old rats, JEV-positive neurons were found only in the superficial area of layer II (Fig. 2c). Virus antigens were not found in the motor cortex in the 9-day-old rats (Fig. 2d). Pathological examination at higher magnifications clearly showed that in rats infected with JEV at the age of 7 days JEV antigen was present in the apical cytoplasmic portion of the neurons in the superficial areas of layer II (Fig. 3). Since the neuronal maturation of the motor cortex is known to proceed from the deeper layers (layers V and VI) to the upper layers (layers III and II), the results indicate that neurons in the motor cortex become resistant to JEV infection with maturation.

In the neuronal transplantation experiments, the transplanted neurons were recognized as being arranged differently compared with the original cortical neurons (Fig. 4a). These neurons were shown to be viable until 3 months after inoculation. Figure 4b shows transplanted neurons of animals that received transplants of embryonal neurons when they were 15 days old and were then infected with JEV 3 days later. The transplanted neurons could be well visualized by UV illumination. On immunohistochemical examination of the same area, only the transplanted neurons were seen to be infected with JEV (Fig. 4c). The host neurons around the transplanted cells remained uninfected. In contrast, when we examined the animals infected with JEV 9 days after transplantation, no viral antigen was detected in either donor neurons or host neurons. This showed that JEV only infected embryonal neurons soon after transplantation into the virus-resistant aged rat brain.

DISCUSSION

Neuropathological and immunohistochemical studies of the central nervous system in Japanese encephalitis patients have been previously reported (7, 9, 10, 13, 25). The experiments described in this report using JEV-infected rats demonstrated similar findings and showed that the distribution of virus antigen in rats was similar to that of humans, with the most severely affected lesions being in the thalamus, caudate nucleus, putamen, and substantia nigra.

The present studies also show that the age susceptibility of rats to JEV infection is related to the degree of neuronal maturity. To exclude a possible role of immunological factors in Japanese encephalitis, Fischer rats infected with JEV were sacrificed at 3 days after inoculation, as at this time they do not have a fully developed immune response (3). Viral antigen was demonstrated only in neurons, and no inflammatory changes were associated with neuronal infection. Accordingly, this *in vivo* experiment serves as a model to directly study the susceptibility of neurons to infection.

It is widely accepted that in the development of the cerebral cortex successively generated neurons in fetal life migrate outward, passing through their predecessors, and come to lie at a more superficial level (1, 8). This sequence of

events is known as the "inside-out" rule (8). Moreover, it is known that the maturation of cortical neurons and the attainment of synaptic connections progress rapidly during the first postnatal week and are mostly completed by the end of the second week of life (4, 15). The age-dependent cortical laminar alteration of JEV susceptibility noted in our study corresponded with the period of this neuronal maturation. In summary, our studies show that JEV seems to infect only immature neurons. The transplantation studies strongly support our findings, in that immature neurons transplanted into a host for which neuronal development was complete (12) were infected exclusively by JEV.

The finding that the neurons of the basal ganglia and deep cerebral nuclei remained susceptible to infection longer than those of the cerebral cortex was surprising. It is generally considered that their neuronal development and generation occurs earlier than those of the cortex (11, 17). This raises the possibility that factors other than maturation play a role in the susceptibility to infection in these neurons. One possibility is that virus receptors on these neurons may persist longer. Our experiments suggest that the alteration of neuronal surface molecules which may act as viral receptors changes with age. From these experiments, it can be concluded that the susceptibility of rat brain to JEV infection was closely associated with neuronal maturity. The findings appear to offer a novel explanation for viral tissue tropism and may provide a basis for studies whereby virus infection may be used to define the degree of neuronal maturation. Furthermore, this model offers a method for studying and identifying the receptors for JEV in neuronal tissues.

ACKNOWLEDGMENTS

This research was supported in part by The Suhara Memorial Foundation (Sapporo), grants from The Ministry of Education, Science, and Culture of Japan (01870024 and 01570261), W.H.O. grants for vaccine development, and The Special Grant-in-Aid for Promotion of Education and Science in Hokkaido University provided by the Ministry of Education, Science and Culture.

REFERENCES

1. Angevine, J. B., and R. L. Sudman. 1961. Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse. *Nature* (London) **192**:766-768.
2. Booss, J., and M. M. Esiri. 1986. Mosquito borne disease, p. 158-172. In J. Booss and M. M. Esiri (ed.), *Viral encephalitis. Pathology, diagnosis and management*. Blackwell Scientific Publications, London.
3. Casali, P., and G. Trinchieri. 1984. Natural killer cells in viral infection, p. 11-19. In A. L. Notkins and M. B. A. Oldstone (ed.), *Concepts in viral pathogenesis*. Springer-Verlag, New York.
4. Eayrs, J. T., and B. Goodhead. 1959. Postnatal development of the cerebral cortex in the rat. *J. Anat.* **93**:385-402.
5. Greve, J. M., G. Davis, A. M. Meyer, C. P. Forte, S. C. Yost, C. W. Marlor, M. E. Kamarck, and A. McClelland. 1989. The major human rhinovirus receptor is ICAM-1. *Cell* **56**:839-847.
6. Grossberg, S. E., and W. F. Scherer. 1966. The effect of host age, virus dose, and route of inoculation on inapparent infection in mice with Japanese encephalitis virus. *Proc. Soc. Exp. Biol. Med.* **123**:118-124.
7. Haymaker, W., and A. B. Sabin. 1947. Topographic distribution of lesions in central nervous system in Japanese B encephalitis. *Arch. Neurol. Psychiatry* **57**:673-692.
8. Ichikawa, M., and Y. Hirata. 1982. Morphology and distribution of postnatally generated glial cells in the somatosensory cortex of the rat: an autoradiographic and electron microscopic study. *Dev. Brain Res.* **4**:369-377.
9. Ishii, T., M. Matsushita, and S. Hamada. 1977. Characteristic residual neuropathological features of Japanese B encephalitis.

- Acta Neuropathol. **38**:181–186.
10. Iwasaki, Y., J.-X. Zhao, T. Yamamoto, and H. Konno. 1986. Immunohistochemical demonstration of viral antigens in Japanese encephalitis. *Acta Neuropathol.* **70**:79–81.
 11. Jacobson, M. 1978. *Developmental neurobiology*, 2nd ed. Plenum, New York.
 12. Jaeger, C. B., and R. D. Lund. 1981. Transplantation of embryonic occipital cortex to the brain of newborn rats: a golgi study of mature and developing transplants. *J. Comp. Neurol.* **200**: 213–230.
 13. Johnson, R. T., D. S. Burke, M. Elwell, C. J. Leake, A. Nisalak, C. H. Hoke, and W. Lersomrudee. 1985. Japanese encephalitis: immunocytochemical studies of viral antigen and inflammatory cells in fatal cases. *Ann. Neurol.* **18**:567–573.
 14. Kenney, S., V. Natarajan, D. Strike, G. Khoury, and N. P. Zalzman. 1984. JC virus enhancer-promoter active in human brain cells. *Science* **226**:1337–1339.
 15. Kristt, D. A. 1978. Neuronal differentiation in somatosensory cortex of the rat. 1. Relationship to synaptogenesis in the first postnatal week. *Brain Res.* **150**:467–486.
 16. Lents, T. L. 1988. Binding of viral attachment protein to host-cell receptor: the Achilles heel of infectious viruses. *Trends Pharmacol. Sci.* **9**:247–252.
 17. Lund, R. D. 1978. *Development and plasticity of the brain*. Oxford University Press, New York.
 18. Mendelsohn, C. L., E. Wimmer, and V. R. Racaniello. 1989. Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. *Cell* **56**:855–865.
 19. Nathanson, N. 1980. Pathogenesis, p. 201–236. *In* T. Monath (ed.), *St. Louis encephalitis*. American Public Health Association, Washington, D.C.
 20. O'Leary, J. L., M. G. Smith, and H. R. Reames. 1942. Influence of age on susceptibility of mice to St. Louis encephalitis virus and on the distribution of lesions. *J. Exp. Med.* **75**:233–247.
 21. Sabin, A. B. 1952. Nature of inherited resistance to viruses affecting the nervous system. *Proc. Natl. Acad. Sci. USA* **38**:540–546.
 22. Staunton, D. E., V. L. Merluzzi, R. Rothlein, R. Barton, S. D. Marlin, and T. A. Springer. 1989. A cell adhesion molecule, ICAM-1, is the major surface receptor for rhinoviruses. *Cell* **56**:849–853.
 23. Sumiyoshi, H., C. Mori, I. Fuke, K. Morita, S. Kuhara, J. Kondou, Y. Kikuchi, H. Nagamatsu, and A. Igarashi. 1987. Complete nucleotide sequence of the Japanese encephalitis virus genome RNA. *Virology* **161**:497–510.
 24. White, J. M., and D. R. Littman. 1989. Viral receptors of the immunoglobulin superfamily. *Cell* **56**:725–728.
 25. Zimmerman, H. M. 1945. The pathology of Japanese B encephalitis. *Am. J. Pathol.* **22**:965–991.