PRODUCTION OF LEthal INFECtion THAT RESEMBLES FATAL HUMAN DISEASE BY INTRanasal INOCULATION OF MACaques WITH JAPANESE ENCEPHALITIS VIRUS

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Abstract. Twelve rhesus macaques (Macaca mulatta) challenged intranasally with a wild-type Japanese encephalitis virus (JEV) developed clinical signs 11–14 days later. Tissues from the cerebral cortex, cerebellum, brainstem, thalamus, meninges, and all levels of the spinal cord were stained for JEV antigen with hyperimmune mouse ascitic fluid and streptavidin–alkaline phosphatase; immunofluorescent staining was also done on frozen sections. Viral antigen was found in all cell layers of the cerebellum, the gray matter of the thalamus and brainstem, and the ventral horn of all levels of the spinal cord. Staining was limited to neurons and their processes. Histopathologic changes were limited to the nervous system and characterized by nonsuppurative meningoencephalitis. These results were comparable with those of previous studies done with human autopsy tissues. Intranasal inoculation of rhesus monkeys with JEV was effective in producing clinical disease comparable with natural disease in humans and may serve as a model to evaluate protective efficacy of candidate JEV vaccines.

Japanese encephalitis (JE), which is endemic to much of eastern and Southeast Asia, is the most common arthropod-borne human encephalitis in the world, accounting for more than 35,000 cases and 10,000 deaths each year. In endemic areas, the highest age-specific attack rates occur in children 3–6 years of age. Infection with JE virus (JEV) may be asymptomatic or manifest as a mild febrile illness, aseptic meningitis, or classic severe meningoencephalitis. The case fatality rate is approximately 25%, with 50% having neuropsychiatric sequelae and 25% recovering fully. Long-term sequelae in survivors include weakness, ataxia, tremors, athetoid movements, paralysis, memory loss, and abnormal emotional behavior. The principle vector for JEV in Thailand is the mosquito Culex tritaeniorynchus, which is difficult to control.

The current highly purified Biken (Research Foundation of Microbial Diseases of Osaka University, Osaka, Japan) vaccine (Nakayama-Yoken strain) is efficacious, but there are some concerns about safety, cost, and necessity for booster doses. Randomized, controlled efficacy trials of second-generation vaccines will be difficult to perform. Clinical attack rates are low and according to conventional vaccine testing principles, since an efficacious first generation vaccine is available, testing using a placebo control in humans might be considered unethical. Using a proven vaccine such as the Biken vaccine with 91% efficacy as the control vaccine would require an unreasonably large study population to assess efficacy. An alternative approach would include demonstration of safety and immunogenicity in volunteers and protective efficacy against lethal challenge in a nonhuman primate model. Ideally, the model should closely mirror natural disease in humans. As part of an effort to evaluate second-generation JEV vaccines, a challenge model in rhesus macaques was developed involving intranasal instillation of a defined dose of a highly characterized JEV. We reasoned that the more closely our model of experimental JE in rhesus monkeys matches human disease, the more relevant the model will be for evaluating protective efficacy of vaccines. Therefore, when encephalitis was observed in inoculated animals, tissues from all levels of the central nervous system (CNS) were examined for infectious virus, virus antigen, and histopathology. Findings were correlated and compared with those previously reported in human disease.

MATERIALS AND METHODS

Inoculation. Twelve healthy, young adult rhesus monkeys (Macaca mulatta) of both sexes, weighing 2–8 kg, were used. All monkeys lacked neutralizing antibodies to JEV and dengue virus types 1–4. The JEV challenge virus was isolated from the brain of a six-year-old Thai boy who died of encephalitis in Kamphaeng Phet, Thailand in 1983. The virus, originally isolated in AP-61 cells and passaged once in C6/36 cells, was designated KE-93 (AP61-2, C6-1); it was inoculated into two monkeys (DA 379 and DA 349). The remaining 10 monkeys were inoculated with an isolate made from the brain of monkey DA 349 that was subsequently passaged twice in suckling mice to obtain a higher titered, and possibly more virulent, inoculum than possible in mosquito cell culture. This virus, which was selected as the challenge virus for future studies, was designated KE-93 (AP61-2, C6-1, Mm-1, SM-2). The virus dose inoculated is described in Table 1. Monkeys were inoculated via the intranasal route as follows. Animals were sedated with ketamine hydrochloride and the virus suspension (0.5 ml) was instilled slowly in each nostril via a syringe after which the animal remained supine for 5 min.

Tissue preparation and staining. Inoculated animals were monitored closely for signs of encephalitis. When animals developed stupor or coma (11–14 days postinoculation), they were anesthetized with ketamine and killed with a lethal dose of barbiturate. In all 12 animals, a diagnosis of JE was made by isolation of JEV from brain tissue; in all cases the diagnosis was confirmed by detection of IgM to JEV in serum or cerebrospinal fluid or an increase in hemagglutination-inhibiting antibody to JEV. Sections of the CNS were taken from frontal lobe, thalamus, brainstem, cerebellum, and cerebral meninges; spinal cord sections were...
taken from the cranial and caudal cervical cord, the thoracic cord, and the lumbar cord, as well as from the spinal meninges. Olfactory bulbs for antigen staining were recovered from five of the 12 monkeys. In addition, specimens were taken from non-neural tissues such as liver, spleen, thymus, axillary lymph node, myocardium, adrenal glands, bone marrow, skin, and lung. Tissues were cut into small cross sections and immersion-fixed in 10% neutral-buffered formalin for at least 72 hr. They were further trimmed and routinely processed and stained with hematoxylin and eosin for light microscopic evaluation. For viral antigen determination, tissues were rapidly fixed in Millonig’s formalin for 2 hr, irradiated in a microwave oven (model Carousal II; Sharp Electronics Corp., Mahwah, NJ) at power level 9 for 2 min, and then embedded in paraffin. Viral antigen was detected using the immunoalkaline phosphatase method of Hall and others18 with some modifications. Briefly, the staining procedure included the following steps: 1) deparaffinization in absolute alcohol and water, 2) immersion for 3 min in a 0.05% solution of protease VIII (Sigma, St. Louis, MO), 3) application for 30 min of naphthol AS-B1 phosphate and hexazotized diazonium 4’-amino-2’, 5’-diethoxybenzanilide (HistoMark Blue; Kirkegaard & Perry Laboratories, Gaithersburg, MD) to label endogenous alkaline phosphatase blue, 4) blocking for 30 min with 2% normal horse serum and 2% bovine serum albumin (BSA), 5) incubation overnight with primary antibody (polyclonal mouse antibody raised against the Nakayama strain of JEV) at a 1:1,500 dilution in phosphate-buffered saline (PBS), pH 7.4, containing 40% rhesus serum, 6) incubation for 30 min with streptavidin-alkaline phosphatase (Gibco-BRL, Gaithersburg, MD) at a 1:50 dilution in PBS, 7) application for 30 min of AS-B1 phosphate, 8) application for 30 min of the chromogenic substrate, 9) counterstaining with Mayer’s hematoxylin, and 10) covering with Immuno-mount (Shandon, Inc., Pittsburgh, PA). All steps were done at 37°C except the primary antibody, which was incubated at 4°C. Antigen-positive cells and tissues were identified by the presence of a scarlet red precipitate; staining was graded as + to +++++ (- = no infected neurons; + = 1–5 positive neurons/low power field; +++++ = diffuse, 11–20 neurons involved/low power field; ND = not done.

### RESULTS

All infected monkeys exhibited similar signs of neurologic disease that appeared approximately 7–10 days postinoculation. Early signs included localized mild muscle tremors and fasciculations, usually on the head and arms. The tremors steadily progressed and became more pronounced. The animals became anorexic and lethargic following initial signs and lapsed into coma.

There were no gross pathologic findings in any monkeys. Histopathologic changes were limited to the nervous system, with similar findings present in all affected monkeys in varying degrees. Central nervous tissue changes were characterized by a nonsuppurative meningoencephalitis extending deep within the brain as expansive mononuclear cell perivascular cuffing. Hypercellularity in the parenchyma indicated a microglial response that occurred with greater fre-
Figure 1. Immunoalkaline phosphatase staining of Japanese encephalitis virus (JEV) antigen counterstained with hematoxylin in rhesus monkeys inoculated intranasally. A, diffuse infection of neurons in the thalamus (magnification × 40, bar = 80 μm). B, antigen-positive neurons in thalamus (magnification × 100, bar = 30 μm). C, JEV antigen in the cerebellum. GL = granular layer; PCL = Purkinje cell layer; ML = molecular layer; W = white matter (magnification × 40, bar = 80 μm). D, JEV antigen in a single heavily infected Purkinje cell (magnification × 200, bar = 16 μm). E, infected olfactory bulb (magnification × 200, bar = 16 μm). F, ventral horn of caudal cervical spinal cord (magnification × 200, bar = 16 μm). G, negative control, uninfected cerebellum (magnification × 100, bar = 30 μm). H, JEV antigen in cerebellum detected with indirect immunofluorescent staining (magnification × 100, bar = 30 μm).
quency in the outer layers of the cerebral cortex, the thalamic region, and the gray matter of the spinal cord. Scattered neurons in the cerebrum, the cerebellum (Purkinje cells), and the spinal cord were occasionally dark and shrunken with a central dense magenta center, suggesting degenerative changes. Isolated and scattered necrosis was present and appeared to be from the microglial cell response. Within the gray matter of the spinal cord, occasional acute hemorrhage was present. Other tissue changes were either relatively common findings in the rhesus monkey or were incidental findings, both with little significance.

The distribution of the viral antigen in the CNS is shown in Table 1. In all monkeys, JEV antigen was present in the CNS tissues examined, especially in the gray matter, as cytoplasmic staining of neurons. Neurons of the thalamus, brainstem, and cerebellum were intensely stained (Figure 1D). Staining of neurons was either diffuse (+ + +) or discrete (+); the discrete pattern was seen mostly in the cerebral cortex. No staining was seen in the meninges or vascular endothelium. In the cerebellum, antigen was found in all three layers: granular, molecular, and the intervening Purkinje cells. Antigen was found in neurons of the olfactory bulb in two (DA 314 and DA 322) of five monkeys, but was not as prominent as elsewhere (Figure 1E). Infected neurons and neuronal processes were also seen at all levels of the spinal cord, involving primarily the ventral horn. No antigen was found in the cerebral or spinal meninges. Glial cells did not appear to be infected. There was no staining in the non-neural tissues examined. No staining was seen in negative controls.

There was no apparent difference in staining intensity and distribution of infected cells in monkeys that received the original virus and the virus that was further passaged in monkey DA 349 and suckling mice. The staining pattern and intensity was also the same with different challenge dose and day of killing.

**DISCUSSION**

Japanese encephalitis in rhesus monkeys is almost identical to that of humans. In naive monkeys given an intranasal challenge of JEV, the response was between that of humans. In naive monkeys given an intranasal challenge of JEV, the response was between that of humans and monkeys. Neurons of the thalamus, brainstem, and cerebellum were intensely stained (Figure 1D). Staining of neurons was either diffuse (+ + +) or discrete (+); the discrete pattern was seen mostly in the cerebral cortex. No staining was seen in the meninges or vascular endothelium. In the cerebellum, antigen was found in all three layers: granular, molecular, and the intervening Purkinje cells. Antigen was found in neurons of the olfactory bulb in two (DA 314 and DA 322) of five monkeys, but was not as prominent as elsewhere (Figure 1E). Infected neurons and neuronal processes were also seen at all levels of the spinal cord, involving primarily the ventral horn. No antigen was found in the cerebral or spinal meninges. Glial cells did not appear to be infected. There was no staining in the non-neural tissues examined. No staining was seen in negative controls.

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**DISCUSSION**

Japanese encephalitis in rhesus monkeys is almost identical to that of humans. In naive monkeys given an intranasal challenge of JEV, the response was between that of a peripheral subcutaneous or intradermal challenge, in which monkeys generally do not become ill, and a direct CNS injection, in which paralysis sets in early. Pathology was limited to the nervous system and was similar in all animals. The primary tissue change was a multifocal-to-diffuse, minimal-to-moderate nonsuppurative meningoencephalitis with parenchymal mononuclear perivascular cuffing. A generalized microglial response occurred with minimal discernible neuronal changes. No brain edema was observed. In summary, as in those human cases in which death occurs rapidly after the onset of coma, the degree of neuronal injury was minimal. Nevertheless, critical brain functions had been deranged.

Although immunocytochemical studies of human autopsy material have been used to map the tissue distribution of viral antigen in fatal cases, little is known about the antigen distribution pattern in monkeys. In our study with rhesus macaques, the heaviest staining was observed in the cerebellum, thalamus, and brainstem, explaining the observed muscle tremors, fasciculations, ataxia, depression, and profound coma. Antigen was localized to neurons, in some cases filling the neuronal processes. Its presence in the dendritic processes and/or axons suggests a trans-cellular spread of virus to distant neurons. As in human cases of JE, we found that the extent of infection inferred by immunocytochemical staining of viral antigen was wider than can be estimated from the extent of inflammatory cell reactions. The distribution of JE virus in the brains of macaques experimentally inoculated with JEV duplicates that previously reported in human disease. In both, the virus distribution in the CNS was disseminated, with the greatest intensity in the thalamus and brain stem. A large number of infected Purkinje and small granule cells were observed in the cerebellum of the macaques, similar to what has been described for humans by Desai and others. However, Johnson and others found no infection of Purkinje cells. The cerebral cortex was less involved in the macaques but considerable antigen was localized in all levels of their spinal cords, especially the ventral horn. Although there have been no published reports of antigen staining of the spinal cord in humans, histopathologic studies revealed extensive involvement of the anterior horns. Infection with JEV in both macaques and humans is predominantly an infection of neurons. Antigen was not seen in meningeal or glial cells. Experimental flavivirus infections have not usually involved vascular endothelium and none was found in ours; the small amounts of antigen occasionally found in human cases may represent phagocytosis. There are reports of olfactory bulb infection in some human cases; olfactory bulb involvement was found in two of five monkeys examined. The widespread pattern of lesions, all with a similar progression inferred by the degree of staining, suggests that virus was disseminated hematogenously throughout the nervous system. This must have occurred by transport of virus across the blood-brain barrier, which is also seen with the accepted mouse model of disease. In summary, the observed findings support the concept that following intranasal inoculation, virus entered the blood stream and seeded the CNS. This is the possible pathogenesis of JE in humans as well and is a critical point because it implies that the protective response required from a vaccine is the ability to diminish or prevent viremia sufficiently to prevent CNS infection.

Intranasal inoculation of macaques with JEV produced lethal infections that resembled fatal human disease in the distribution of infected cells as well as in histopathology. This suggests that intranasally infected macaques may be a suitable model for testing second-generation JEV vaccine candidates.

Acknowledgments: We gratefully acknowledge the expert laboratory work of Aree Na-Nongkai (deceased) and Pranee Hansukjariya, and are indebted to Dr. Gary Marit and Dr. Ronald Rosenberg for critical review of the manuscript.

Financial support: This study was supported by Pasteur-Mérieux-Connaught Laboratories, Inc. and the U.S. Army Medical Research, Development, and Materiel Command.

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