Flavivirus encephalitis—pathological aspects of mouse and other animal models

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Abstract. Encephalitic flaviviruses are important arthropod-borne pathogens of humans and other animals. In particular, the recent emergence of the West Nile virus (WNV) and Japanese encephalitis virus (JEV) in new geographic areas has caused a considerable public health alert and international concern. Among the experimental *in vivo* models of WNV and JEV infection, mice and other laboratory rodents are the most thoroughly studied and well-characterized systems, having provided data that are important for understanding the infectious process in humans. Macaca monkeys have also been used as a model for WNV and JEV infection, mainly for the evaluation of vaccine efficacy, whereas a limited number of published studies address pathomorphology. These animal models develop encephalitis with many similarities to the human disease; however, the histological events that occur during infection, especially in peripheral tissues, have not been fully characterized.

Keywords: Experimental Animal Models; Flavivirus; Histopathology; Japanese Encephalitis Virus; Mice; Mosquito-Borne Encephalitis; West Nile virus.

The genus Flavivirus comprises more than 70 viruses, many of which are arthropod-borne human pathogens.[59] The genome of flaviviruses is a single-stranded RNA of approximately 11 kb. Based on antigenic and genetic characteristics, arthropod-borne flaviviruses have been classified in four groups: the Japanese encephalitis virus (JEV) complex, the tick-borne encephalitis (TBE) virus complex, the Dengue virus (DENV), and the yellow fever virus (YFV).[4,10,20,44,57,63] The JEV and TBE complexes are neurovirulent and can cause encephalitis in humans. In contrast, YFV and DENV are more viscerotropic and can cause hemorrhagic fever.[40] Among the mosquito-borne flaviviruses, current major global concerns include DENV, JEV, West Nile virus (WNV), and YFV.[60] JEV and WNV, which belong to the JEV complex, are serologically crossreactive with each other.[64,115]

Development of flaviviral encephalitis in infected hosts has been thought to occur mainly via hematogenous spread of the virus to the central nervous system (CNS).[13,79] After injection of virus-containing saliva from mosquito, the virus replicates in local tissues and regional lymph nodes. This results in a primary viremia and subsequent infection of extraneural tissues (e.g., the reticuloendothelial system) where further replication leads to a more sustained secondary viremia, which causes CNS infection. The exact mechanism underlying the crossing of the blood-brain barrier by

flaviviruses during natural infection remains uncertain.

While a variety of animal models of JEV and WNV exists, the rodent models are the most thoroughly characterized of these and are thus the focus of our review. A considerable body of knowledge concerning the pathogenesis of flaviviral encephalitis has been generated from experimental infection studies using rodent models (reviewed in [14,78]). However, the pathomorphological changes that occur in those models during infection have not been fully characterized.

Pathological changes in mice infected with WNV

WNV is maintained in an enzootic cycle between mosquitoes and wild birds, but it can also infect humans, horses and other vertebrate animals.[43] Human infection may result in a febrile illness that can progress to lethal encephalitis with cognitive dysfunction and poliomyelitis with flaccid paralysis.[12,88,101] WNV infection was recognized previously as endemic to Africa, Europe, and Asia;[76] however, WNV outbreaks occurred in Romania, Russia, Israel, and the Americas in the 1990s with fatal encephalitis as a significant feature.[19,60] Seroprevalence studies of the outbreaks suggest that approximately 20% of infected individuals develop clinical manifestations characterized as WNV fever.[88,122] About one in 150 people infected with WNV develop a neuroinvasive disease.[89,123] The

mortality rate following neuroinvasive infection is approximately 10%.[81,88] Pathologic evaluation of human cases with WNV encephalitis revealed that they exhibit perivascular lymphocytic infiltrates, microglial nodules, loss of neurons, and viral antigens predominantly within the brainstem and anterior horn of the spinal cord, although the cerebral cortex, cerebellum, and posterior horn of the spinal cord are also involved.[39] The WNV antigen was detected in the cytoplasm of neurons and neural processes.[39] In immunosuppressed individuals, infection can disseminate throughout the CNS.[54]

Animals that have been used to model experimental WNV infection include mouse, rat[28], hamster,[117,127] monkey,[37,91,94] horse,[8] pig,[116] dog,[3] and cat.[3] Among them, mouse models are the most intensively studied and have fostered a significant understanding of WNV pathogenesis, as previouslyreviewed.[22,96] After intradermal inoculation with WNV, initial viral replication is thought to occur in Langerhans dendritic cells of the skin.[6,50] The infected Langerhans cells migrate to draining lymph nodes[9,49] and the virus is considered to enter the bloodstream through the lymphatic and thoracic ducts.[62] This primary viremia disseminates the virus to peripheral tissues, including the spleen, where further viral replication occurs.[23,108] It is conceivable that most of the viral particles in viremic blood are free and exist in the plasma.[61] By the end

of the first week after infection, WNV is largely cleared from the serum and peripheral organs, and infection of the CNS is observed.[105] Mice that succumb to infection develop CNS pathology similar to that observed in human WNV cases.[29,85] Mouse lesions described in the literature include necrosis of neurons, perivascular mononuclear infiltrates composed mainly of macrophages and lymphocytes, glial/microglial nodules, neuronal satellitosis, and neuronophagia in the brainstem, cerebral cortex, hippocampus, cerebellum and spinal cord.[13,31,106]

Many studies performed recently on the pathogenesis of North American WNV isolates used the C57BL/6 mouse strain and genetically engineered immunocompromised mice in the C57BL/6 background. C3H/HeN mice showed higher morbidity and mortality than C57BL/6 mice after infection with the recombinant virus derived from a 2000 New York isolate, although viral tropism, viral load, and kinetics did not differ substantially between these strains.[6] Our unpublished observations from 6-week-old C3H mice that received footpad inoculation of 1,000 plaque-forming units (p.f.u.) of a NY99-6922 strain-derived plaque isolate revealed that infected mice developed neuronal necrosis (Fig. 1) with infiltration of reactive microglia, neuronophagia, and perivascular infiltration of macrophage/microglial cells and lymphocytes (Fig. 2) into the gray matter of the CNS from day 9 postinfection (p.i.).

These lesions were distributed preferentially in the brainstem, cerebral cortex, caudate putamen, thalamus, and spinal cord; however, the hippocampus and cerebellum were also involved in many cases. Mild meningitis was also seen. Small hemorrhagic foci were occasionally observed. These histological findings are consistent with those previously reported by Garcia-Tapia et al.[31] using 8-week-old C57BL/6 mice that were inoculated in the footpad with 100 p.f.u. of a 2002 Missouri isolate.

Immunohistochemistry (IHC) revealed that viral antigens were present in the CNS, at first in a few neurons of the ventral horn of the lumbar spinal cord on day 7 p.i. (Fig. 3). Infected neurons were located on the ipsilateral side of inoculation, which is consistent with the findings of previous studies[97,120] and suggests that transneural spread of the virus via the sciatic nerve occurs. Foci of viral antigen were then seen in multiple regions including the brainstem, cerebral cortex, caudate putamen, and cervical spinal cord (Fig. 4). Viral antigens were mainly detected in the cytoplasm of neurons and neuronal processes (Fig. 5), although a few cells that were morphologically suggestive of astrocytes were also positive. Neuronophagia was common (Figs. 6; 7). In heavily infected foci, proliferation of microglia was prominent and many macrophage/microglial cells contained viral antigens (Fig. 8). These macrophage/microglial cells also accumulated in perivascular areas, which was a

morphological indication of the involvement of microglial cells in viral clearance and/or antigen presentation (Fig. 9). Vascular endothelial cells in the infected foci sometimes showed positivity for WNV antigens and, in most cases, they were associated with the infiltration of virus antigen-containing macrophages/microglia. Diffuse, severe inflammation and antigen distribution occurred in a small number of mice.

Several laboratories have analyzed the mode of lymphocyte recruitment into the CNS of WNV-infected mice. CD45 (which is common leukocyte antigen)-positive ($CD45^+$) leukocytes were detected in the inflamed parenchyma of the CNS of WNV-infected mice.[106] Trafficking of $CD8^+$ cells to the brain is more predominant than that of $CD4^+$ T cells after WNV infection in mice.[105,107,121] In response to WNV infection, neurons secrete the CXCL10 chemokine, which in turn recruits effector $CD8^+$ T cells via an interaction with the CXCR3 chemokine receptor.[53] The CCR5 chemokine receptor of leukocytes participates in the promotion of leukocyte recruitment into the brain of WNV-infected mice and plays a crucial role in host survival.[36] Other chemokines, such as CCL5 (RANTES), CCL3 (MIP-1α), and CCL4 (MIP-1β), are also upregulated in the brains of WNV-infected mice.[103]

The pathological changes that occur in peripheral tissues infected with WNV remain incompletely characterized. In human cases, WNV has been detected in renal

and splenic tissues using cell culture and reverse transcriptase polymerase chain reaction;[85] in renal tubular epithelium of an AIDS patient using IHC;[39] in testis of a transplant recipient using electron microscopy;[110] and in macrophages, intravascular mononuclear cells, and vascular endothelia of the skin, stomach, lung, liver, kidney, spleen, and bone marrow using IHC.[86] Systemic distribution of WNV in severely immunocompromised transplant recipients has been reported, in whom the epithelial cells of the lung, pancreas, thyroid, intestine, stomach, esophagus, bile duct, skin, prostate, and testes were assessed as positive for viral antigen using IHC.[1] In mice, the WNV antigen has been detected immunohistochemically in macrophages and fibrocytes of the skin, in a few macrophages of the spleen, and in some renal tubular cells with no signs of inflammation.[31] Our unpublished observations from C3H mice that received footpad inoculation of WNV, as described above, revealed that viral antigens are rarely present in the squamous epithelial cells of the footpad inoculation site (Fig. 10). This finding seems to be consistent with that of a previous report in which the skin, duodenum, and pancreas of C3H mice were identified as novel sites of viral replication.[6] Reportedly, monocytes and a subset of lymphocytes are able to support the productive replication of WNV after infection.[32] However, we did not detect specific WNV immunoreactivity in the spleen and lymph nodes of infected mice, both of which

are major organs of extraneural viral replication,[6,108] using IHC on paraffin-embedded sections. It may be that the level of viral replication in each mononuclear cell is below the threshold of detection of the conventional immunohistochemical technique. Significant non-CNS lesions associated with viral antigens were observed frequently in the digestive tract of C3H mice from day 7 p.i., in which the upper small intestine and sometimes the stomach were abnormally dilated and filled with a greenish watery content (Fig. 11). Villi of the upper small intestine of WNV-infected mice were markedly shortened (Fig. 12; 13). Degeneration and necrosis of myenteric ganglia cells with WNV immunoreactivity were a consistent feature (Fig. 14). The myenteric plexi of the upper small intestine were most severely affected, whereas ganglia cells in other regions were also positive for WNV (Fig. 15). The lesions observed in the intestines of WNV-infected C3H mice are not features that are relevant in human WNV cases. As reported previously,[31] thymic atrophy was common in moribund mice.

In surviving immunocompetent mice, WNV cleared from all tissues within two to three weeks after infection. Innate immunity, humoral immunity, and T-cell-mediated immunity are all involved in viral clearance and in the protection of mice against WNV infection, as previouslyreviewed.[96] Among them, humoral IgM response is the most critical for averting lethal outcomes in acute WNV infection.[23,24] In rodent models,

persistent infection has been reported in the brains of $CD4^+$ T cell-deficient, $CD8^+$ T cell-deficient, or perforin-deficient mice, and in the brain and kidney of infected hamsters.[105,107,109,117,127] Persistent infection in WNV-infected immunosuppressed patients has also been reported, in whom viremia was detected for more than 60 days.[5]

Wild-type mice are resistant to flavivirus infection, whereas laboratory mouse strains exhibit varying degrees of susceptibility to flaviviruses.[17,98] The susceptibility of inbred mouse strains to WNV has been mapped to a mutation in the 2′-5′-oligoadenylate synthase 1b (*OAS1b*) gene, which results in the expression of a truncated OAS isoform.[65,87] The *OAS1b* gene is a member of an IFN-regulated gene family that is involved in the degradation of viral RNA. Knocking in of the *OAS1b* resistance allele into a susceptible mouse strain generated mice with the flavivirus resistance phenotype.[100] Although the detailed mechanisms of how *OAS* gene alleles affect WNV pathogenesis remain uncertain, pathologists should keep in mind this genetic phenomenon that renders inbred mice highly susceptible to flavivirus infection when compared with other animal models.

Pathological changes in other animal models of WNV

Hamsters inoculated intraperitoneally with a NY isolate of WNV develop viremia and

subsequent meningoencephalitis.[127] Infectious viral particles were cultured from the brains of convalescent hamsters up to 53 days after the initial infection, which indicates the establishment of a persistent viral infection.[127] In hamsters that were infected chronically with WNV, the antigen is found in macrophages and vascular endothelial cells within renal interstitia.[117]

Studies that describe the histopathological changes that occur in WNV-infected monkeys are limited. Pogodina et al.[91] reported that intracerebral inoculation of rhesus monkeys with the African, Asian, and European isolates of WNV results in the establishment of persistent viral infection in the CNS and in other organs (i.e., spleen, lymph nodes, and kidney) up to 167 days after the initial infection. Infected monkeys that experienced encephalitis or febrile or asymptomatic infection exhibit a protracted pathological process in their CNS. The histopathological findings in the acute stage of WNV-induced fatal encephalitis in monkeys include marked perivascular and diffuse inflammatory infiltrates, neuronal degeneration and necrosis, and glial reactions in the gray matter, especially in the cerebral cortex, subcortical ganglia, thalamus, medulla oblongata, midbrain nuclei, cerebellum, and spinal cord. A protracted pathological process was observed in the CNS of monkeys that were examined at various points of convalescence or in animals with asymptomatic infection. Lesions were found mainly in

the cerebellum, brainstem, and anterior horns of the spinal cord. Loss of cerebellar Purkinje cells and spinal motoneurons was characteristic. Fresh foci of inflammation with neuronal death and formation of neuronophagic nodules were observed up to 2 months after inoculation, simultaneously with older lesions that exhibited a reparative glial reaction. Replacement of the dead nerve cells by astrocytes became prominent at 167 days after infection. Monkeys that were challenged intracerebrally with the NY99 strain of WNV exhibit a lethal outcome, which is preceded by fever and tremors that progress to ataxia and spasticity.[2] Generally, intradermal or subcutaneous infection of monkeys with WNV does not cause clinical disease, as it does in humans,[90,94] although a low level of viremia develops postinfection, which can be monitored via serial blood collections.[58,90] The prevention or inhibition of the development of viremia by prior vaccination is a demonstration of the protective efficacy of the vaccine, regardless of clinical presentation.[58,90] A case of severe nonsuppurative meningoencephalitis due to naturally acquired infection with WNV was reported in a Barbary macaque (*Macaca sylvanus*) that was housed in a zoo.[84]

Wild birds infected with WNV develop prolonged high levels of viremia, thus acting as the primary amplifying hosts. The WNV strains that circulate in North America and the related strains from Israel are considered more virulent for birds than

those that were previously isolated in Africa, Asia, and Europe.[55] WNV reportedly infects all major organ systems and a wide variety of individual cell types in wild birds;[113] thus, it yields pathological features that are different from those that are present in human WNV cases. The most prominent lesions observed in wild birds (27 birds representing eight orders and 14 species) during the 1999 WNV outbreak in New York city were gross hemorrhage of the brain, splenomegaly, meningoencephalitis, and myocarditis.[113] The WNV antigen was detected most frequently in the kidney, heart, and brain of these animals, but was also found in the spleen, liver, adrenal gland, intestine, pancreas, lung, and ovary. Infected cells included neurons and glial cells of the brain, spinal cord, and peripheral ganglia; myocardial fibers; macrophages and blood monocytes; renal tubular epithelium; adrenal cortical cells; pancreatic acinar cells and islet cells; intestinal crypt epithelium; fibroblasts; and smooth-muscle cells.[113] High mortality associated with WNV is particularly prominent among American crows and other American corvids.[55,80] The most common histological findings reported in naturally infected American crows were multifocal necrosis of the spleen and bone marrow.[126] The WNV antigen was detected consistently in the heart and kidney of these animals, but was also found in the bone marrow, duodenum, proventriculus, liver, lung, spleen, pancreas, and brain.[126] Monocytes/macrophages appear to be important targets

for virus replication and to contribute to the systemic dissemination of the virus.[113,124] WNV is not likely to cause significant disease in domestic chickens (*Gallus gallus*), whereas chickens that were inoculated subcutaneously with the NY strain of WNV developed relatively low levels of viremia, myocardial necrosis, nephritis, and pneumonitis.[102]

Mouse models of JEV infection

Japanese encephalitis is a major viral encephalitis in Southeast Asian countries, with 30,000–50,000 cases reported annually. The disease affects mostly children, but all age groups are affected in areas where the virus was recently introduced.[119] Although the ratio of apparent to unapparent infection may be different in different populations, it is about one in 300 in rural Asia.[119] Case fatality rates range from 5 to 40%, and most survivors who develop severe disease have neurological and/or psychiatric sequelae.[40] Pigs and birds serve as effective amplifying hosts in the transmission cycle of JEV.[7]

Patients who succumb to Japanese encephalitis usually die within one week of hospital admission for deepening coma and respiratory arrest.[48] Pathological studies of Japanese encephalitis in children[48] report moderate meningeal inflammatory response, marked perivascular cuffing, infiltration of cells into the parenchyma, neuronophagia,

and glial nodule formation in the cerebral cortex, thalamus, and brainstem. Polymorphonuclear cells are present in moderate numbers in the meninges and perivascular cuffs. JEV antigens are localized in neurons using IHC, with no evidence of glial-cell infection. The highest concentration of infected neurons is found in the thalamus and brainstem. In perivascular cuffs, T cells are more predominant than B cells and macrophages. $CD8^+$ T cells are a minority among the T-cell population. The majority of the inflammatory cells infiltrating the parenchyma are macrophages. In a recent study of human cases, acellular necrotic foci ("punched-out" appearance), vascular endothelial cell damage, and prominent astrocytic activation were also seen. [33] A significant degree of antigen localization in the hippocampus, temporal cortex, cerebellum, in addition to the thalamus and brainstem, was also reported.[21] Flaccid paralysis is occasionally seen, and pathological studies that included the spinal cord reported extensive involvement of the anterior horn.[25,27,128] Conscious patients with polio-like acute flaccid paralysis have also been reported.[16,111,112]

Histological findings of the brain lesions in JEV-infected mice include neuronal degeneration and necrosis, neuronophagia, microglial proliferation forming glial nodules, mild perivascular hemorrhage, and perivascular cuffing.[33,40] Although the severity of the inflammation and the distribution of viral antigens are influenced by the

phenotype of the inoculated viruses and by the route of inoculation, respectively, the histological changes occur mostly in the gray matter and are predominantly found in the cerebral cortex and hippocampus;[30,33,42] however, they are also found frequently in basal ganglia, thalamus, brainstem, and other regions.

In the pathogenicity of neurotropic viruses, the outcome of intracerebral inoculation reflects viral neurovirulence, whereas the outcome of peripheral inoculation (e.g., subcutaneous or intraperitoneal) reflects both neurovirulence and neuroinvasiveness.[118] Intraperitoneal inoculation is a preferential route of peripheral virus delivery in rodent models of JEV infection, versus subcutaneous inoculation (e.g., footpad), which mimics the bite of an infected mosquito vector. This is due in part to the observation that mice, especially older animals, are somewhat more resistant to JEV infection when the virus is administered subcutaneously,[46,71] although several studies have reported an absence of significant differences in mortality and infectivity among intraperitoneal, intravenous, subcutaneous, and intradermal inoculations.[38,70]

Suckling mice are highly susceptible to JEV, and their brains have been used for efficient virus propagation. JEV infection in infant mice results in fatality, either by intracerebral or peritoneal inoculation. Weanling mice are also susceptible to intracerebral JEV inoculation; however, there is a considerable variation in the

development of fatal encephalitis when the virus is administered via peripheral inoculation.[45,46] This variation stems from the titer of the virus, the viral strain used, and the age of the mice. In mice inoculated subcutaneously, the early phase of viral replication occurs in the peripheral tissues, which include the liver, spleen, heart and kidney, and is followed by the secondary phase, which occurs in the brain.[47] It remains unclear whether this scenario is applicable to all cases that develop CNS infection after peripheral inoculations, i.e., JEV sometimes establishes CNS infection without conclusive evidence of prior viral proliferation in the peripheral tissues. In the hamster model of the St. Louis encephalitis virus, which is a flavivirus antigenically related to both JEV and WNV, the possibility of the involvement of the olfactory pathway in natural infection has been proposed because of the observation of initial CNS involvement of the olfactory bulbs after intraperitoneal infection.[72] However, the role of this pathway in JEV neuroinvasion remains unknown. Infectious viral particles are usually detected in the brain as early as five days after intraperitoneal inoculation.

Intraperitoneal inoculation of the low-neuroinvasive JEV strain SA-14 generates a more marked inflammatory response than intracerebral inoculation[42] and intranasal inoculation of this strain does not lead to fatality,[11] suggesting the route of inoculation may influence the pathological phenotype. Mice that received

intraperitoneal inoculation with the neuroinvasive strain JaTH160 have a prominent infiltration of T cells that are mainly $CD8^+$ and have the Th1/Tc1 phenotype in the brain.[30] Activation of microglial cells may play a significant role in the development of Japanese encephalitis in mice by producing proinflammatory mediators (i.e., inducible nitric oxide synthase, cyclooxygenase-2, monocyte chemotactic protein-1, interleukin-6, and tumor necrosis factor–alpha) and inducing neuronal death.[35] The protective role of inducible nitric oxide synthase during JEV infection has also been suggested, based on results from a mouse model.[99]

Peripheral organs, which include the lung, liver, spleen, heart, and kidney, are possible sites for viral replication after peripheral inoculation.[47] However, little is known about the pathological changes in these extraneural tissues during infection with JEV. After intraperitoneal inoculation, the non-neuroinvasive JEV strain 78668A replicates first in peritoneal macrophages, the viral antigen then appears in the splenic macrophages of the perifollicular region on day 3 p.i., and later in cells of the periarteriolar lymphoid sheath (PALS).[68] Productive infection occurs both in macrophages and in T cells. Morphologically, the spleen shows proliferative changes with an enlarged PALS, which is followed by the appearance of a prominent germinal center and an increase in the number of macrophages and polymorphonuclear

leukocytes in the perifollicular region.[68]

Transplacental JEV infection in swine causes fetal encephalitis, abortion, or stillbirth. Pregnant mice inoculated intraperitoneally with the 78668A strain transmit the virus to the fetus, which results in an increased incidence of abortion.[66] Pregnant mice infected with this strain for six months transmit the virus to fetuses, which is indicative of the establishment of latent infection in mice.[66] The virus can be reactivated from latently infected mice via cyclophosphamide treatment or via induction of pregnancy.[67] Latent infection in T cells of infected mice was demonstrated using the cocultivation technique.[69]

Other animal models of JEV

As described in the previous section, older mice are more resistant than younger mice to the lethal effects of JEV administered peripherally[46] and, to some extent, to the lethal effects of JEV administered intracerebrally.[38] However, the age-dependent resistance to intracerebral infection is more striking in rat models,[26,82] in which the resistance is closely associated with neuronal maturation. JEV specifically binds to and infects developing neurons among the neural cells in primary fetal rat brain cultures, which suggests that specific molecules that bind JEV strongly may be expressed on the surface

of neurons at certain stages of development.[51] Several studies have reported the possible involvement of host molecules, such as glycosaminoglycans,[114,125] a 57 kDa molecule derived from BHK-21 cells,[15] a 74 kDa molecule derived from Vero cells,[52] and a 74 kDa heat-shock cognate protein 70 derived from C6/36 cells,[95] in the attachment and/or entry of JEV into cells. A recent study demonstrated that the heat-shock protein 70 serves as a putative receptor for JEV in the mouse neuronal cell line Neuro2a.[18] The relationship, if any, between these molecules and the age-dependent resistance to infection remains unclear. It has also been reported that suckling rats that were intracerebrally inoculated with JEV developed Parkinson's disease 12 weeks after infection because of the selective depletion of tyrosine hydroxylase-positive neurons in the substantia nigra.[83] This experimental finding is consistent, to some extent, with the clinical findings that a fraction of patients with Japanese encephalitis may have lesions predominantly in the substantia nigra and manifest Parkinsonian features after recovery from acute encephalitic illness.[92]

Several review articles reported rabbits and guinea pigs develop asymptomatic infection regardless of the route of inoculation. Additionally, hamsters reportedly die after intracerebral or intranasal inoculation, but develop asymptomatic viremia after peripheral infection.[40,78]

The experimental intracerebral challenge of monkeys using JEV leads to uniformly lethal encephalitis.[74] Intradermal inoculation of JEV into rhesus monkeys does not cause fatal encephalitis, although the infected monkeys develop viremia.[75] In contrast, Taiwan macaques (*Macaca cyclopis*) that were infected experimentally with JEV via an intranasal route develop viremia followed by clinically evident encephalitis and death.[56] Harrington et al.[41] described the pathological features of encephalitis in rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fasicularis*) monkeys that were inoculated intranasally with JEV. The neuropathology of rhesus and cynomolgus macaques is similar. Diffuse meningeal and perivascular lymphocytic infiltrations were observed both in the brain and in the spinal cord. Neuronal degeneration, necrosis, and neuronophagia were most common in the spinal cord and medulla of these animals, but were also present in other CNS areas. Glial cell proliferation was pronounced in the gray matter of the spinal cord, brainstem, and thalamus. In contrast, the olfactory lobes were normal, with the exception of the presence of minimal meningeal lymphocytic infiltrates and occasional perivascular cuffing. Myint et al.[77] reported the distribution of viral antigens in the CNS of rhesus monkeys that were infected intranasally with JEV. The JEV antigen was found in the cytoplasm of neurons, but not in glial cells, meningeal cells, and vascular endothelium. The JEV antigen was disseminated

throughout the CNS, with greater intensity in the thalamus, brainstem, and cerebellum. The preferential localization of JEV antigens in the thalamus and brainstem was also reported in cases of human Japanese encephalitis.[21,48] Infection of Purkinje and granule cells of the cerebellum of monkeys with JEV is similar to what was described by Desai et al. for human cases;[21] in contrast, another study that was performed by Johnson et al. using human cases did not identify infection of Purkinje cells.[48] The widespread pattern of lesion and virus distribution that was observed in the CNS of the macaque models suggests the hematogenous spread of the virus after intranasal inoculation; however, the possibility of the transneural spread of the virus via the olfactory neurons, the trigeminal nerve, and other nerves that are involved in the development of the CNS lesions should also be considered. The JEV strain that exhibits relatively low neurovilurence and neuroinvasiveness reportedly does not cause encephalitis in monkeys via intranasal challenge; however, intracerebral delivery of this strain causes lethal encephalitis.[74] The rhesus macaque has been proposed as a model that may be used for the evaluation of vaccines[73,93] and of antiviral treatments.[34,41]

Summary

Studies of rodent models have provided a body of information about flaviviral

pathogenesis, which includes the identification of genetic determinants and of immune reactions that influence disease outcome. Recent developments in the generation and characterization of genetically engineered mice have rendered mouse models a more powerful tool for the dissection of flaviviral pathogenesis. Although intradermal or subcutaneous inoculation mimic the natural route of viral inoculation and subsequent systemic dissemination, a wide variety of viral and host factors influence the course and outcome of infection. The elucidation of the molecular mechanisms that underlie the influence of host age on disease outcome remains one of the important issues of this field of research. Regarding viral neuroinvasion, the exact mechanisms via which viruses cross the blood–brain barrier warrant additional investigation. Intracerebral inoculation into suckling mice or suckling hamsters is a more sensitive system for the purpose of infectivity assays and virus isolation. WNV and JEV are strongly neuronotropic, both in rodents and in humans, and infection can cause nonsuppurative polioencephalitis or polioencephalomyelitis. Basic histological findings in the CNS lesions observed in WNV-infected mice are similar to those described in JEV-infected and other encephalitogenic flavivirus-infected mice; hence, it is difficult to differentiate these lesions microscopically. A precise diagnosis should be based on specific serological tests or via the isolation and identification of the virus involved in each case.

The neuropathology of macaques that were inoculated intranasally with JEV resembles that of humans with encephalitis that results from naturally acquired infection, whereas intradermal or subcutaneous inoculation of WNV and JEV into macaques generally results in asymptomatic infection. In view of the close genetic and immunological relationships between non-human primates and humans, the macaque models provide useful information that can be particularly useful for the evaluation of vaccines and antiviral therapies.

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References

1 Armah HB, Wang G, Omalu BI, Tesh RB, Gyure KA, Chute DJ, Smith RD, Dulai P, Vinters HV, Kleinschmidt-DeMasters BK, Wiley CA: Systemic distribution of West Nile virus infection: postmortem immunohistochemical study of six cases. Brain Pathol **17:** 354-362, 2007

2 Arroyo J, Miller C, Catalan J, Myers GA, Ratterree MS, Trent DW, Monath TP: ChimeriVax-West Nile virus live-attenuated vaccine: preclinical evaluation of safety, immunogenicity, and efficacy. J Virol **78:** 12497-12507, 2004

3 Austgen LE, Bowen RA, Bunning ML, Davis BS, Mitchell CJ, Chang GJ: Experimental infection of cats and dogs with West Nile virus. Emerg Infect Dis **10:** 82-86, 2004

4 Blok J, McWilliam SM, Butler HC, Gibbs AJ, Weiller G, Herring BL, Hemsley AC, Aaskov JG, Yoksan S, Bhamarapravati N: Comparison of a dengue-2 virus and its candidate vaccine derivative: sequence relationships with the flaviviruses and other viruses. Virology **187:** 573-590, 1992

5 Brenner W, Storch G, Buller R, Vij R, Devine S, DiPersio J: West Nile Virus encephalopathy in an allogeneic stem cell transplant recipient: use of

quantitative PCR for diagnosis and assessment of viral clearance. Bone Marrow Transplant **36:** 369-370, 2005

6 Brown AN, Kent KA, Bennett CJ, Bernard KA: Tissue tropism and neuroinvasion of West Nile virus do not differ for two mouse strains with different survival rates. Virology **368:** 422-430, 2007

7 Buescher EL, Scherer WF: Ecologic studies of Japanese encephalitis virus in Japan. IX. Epidemiologic correlations and conclusions. Am J Trop Med Hyg **8:** 719-722, 1959

8 Bunning ML, Bowen RA, Cropp CB, Sullivan KG, Davis BS, Komar N, Godsey MS, Baker D, Hettler DL, Holmes DA, Biggerstaff BJ, Mitchell CJ: Experimental infection of horses with West Nile virus. Emerg Infect Dis **8:** 380-386, 2002

9 Byrne SN, Halliday GM, Johnston LJ, King NJ: Interleukin-1beta but not tumor necrosis factor is involved in West Nile virus-induced Langerhans cell migration from the skin in C57BL/6 mice. J Invest Dermatol **117:** 702-709, 2001

10 Calisher CH, Karabatsos N, Dalrymple JM, Shope RE, Porterfield JS, Westaway EG, Brandt WE: Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. J Gen Virol **70 (Pt 1):** 37-43,

1989

11 Cao JX, Ni H, Wills MR, Campbell GA, Sil BK, Ryman KD, Kitchen I, Barrett AD: Passage of Japanese encephalitis virus in HeLa cells results in attenuation of virulence in mice. J Gen Virol **76 (Pt 11):** 2757-2764, 1995

12 Ceausu E, Erscoiu S, Calistru P, Ispas D, Dorobat O, Homos M, Barbulescu C, Cojocaru I, Simion CV, Cristea C, Oprea C, Dumitrescu C, Duiculescu D, Marcu I, Mociornita C, Stoicev T, Zolotusca I, Calomfirescu C, Rusu R, Hodrea R, Geamai S, Paun L: Clinical manifestations in the West Nile virus outbreak. Rom J Virol **48:** 3-11, 1997

13 Chambers TJ, Diamond MS: Pathogenesis of flavivirus encephalitis. Adv Virus Res **60:** 273-342, 2003

14 Charlier N, Leyssen P, De Clercq E, Neyts J: Rodent models for the study of therapy against flavivirus infections. Antiviral Res **63:** 67-77, 2004

15 Chen LK, Lin YL, Liao CL, Lin CG, Huang YL, Yeh CT, Lai SC, Jan JT, Chin C: Generation and characterization of organ-tropism mutants of Japanese encephalitis virus in vivo and in vitro. Virology **223:** 79-88, 1996

16 Chung CC, Lee SS, Chen YS, Tsai HC, Wann SR, Kao CH, Liu YC: Acute flaccid paralysis as an unusual presenting symptom of Japanese encephalitis: a

case report and review of the literature. Infection **35:** 30-32, 2007

17 Darnell MB, Koprowski H, Lagerspetz K: Genetically determined resistance to infection with group B arboviruses. I. Distribution of the resistance gene among various mouse populations and characteristics of gene expression in vivo. J Infect Dis **129:** 240-247, 1974

18 Das S, Laxminarayana SV, Chandra N, Ravi V, Desai A: Heat shock protein 70 on Neuro2a cells is a putative receptor for Japanese encephalitis virus. Virology **385:** 47-57, 2009

19 Dauphin G, Zientara S, Zeller H, Murgue B: West Nile: worldwide current situation in animals and humans. Comp Immunol Microbiol Infect Dis **27:** 343-355, 2004

20 De Madrid AT, Porterfield JS: The flaviviruses (group B arboviruses): a cross-neutralization study. J Gen Virol **23:** 91-96, 1974

21 Desai A, Shankar SK, Ravi V, Chandramuki A, Gourie-Devi M: Japanese encephalitis virus antigen in the human brain and its topographic distribution. Acta Neuropathol **89:** 368-373, 1995

22 Diamond MS: Virus and host determinants of West Nile virus pathogenesis. PLoS Pathog **5:** e1000452, 2009

23 Diamond MS, Shrestha B, Marri A, Mahan D, Engle M: B cells and antibody play critical roles in the immediate defense of disseminated infection by West Nile encephalitis virus. J Virol **77:** 2578-2586, 2003

24 Diamond MS, Sitati EM, Friend LD, Higgs S, Shrestha B, Engle M: A critical role for induced IgM in the protection against West Nile virus infection. J Exp Med **198:** 1853-1862, 2003

25 Dickerson RB, Newton JR, Hansen JE: Diagnosis and immediate prognosis of Japanese B encephalitis; observations based on more than 200 patients with detailed analysis of 65 serologically confirmed cases. Am J Med **12:** 277-288, 1952

26 Duffy CE: Japanese B encephalitis in the rat. Proc Soc Exp Biol Med **76:** 566-569, 1951

27 Edgren DC, Palladino VS, Arnold A: Japanese B and mumps encephalitis: a clinicopathological report of simultaneous outbreaks on the island of Guam. Am J Trop Med Hyg **7:** 471-480, 1958

28 Eldadah AH, Nathanson N: Pathogenesis of West Nile Virus encepahlitis in mice and rats. II. Virus multiplication, evolution of immunofluorescence, and development of histological lesions in the brain. Am J Epidemiol **86:** 776-790,

1967

29 Fratkin JD, Leis AA, Stokic DS, Slavinski SA, Geiss RW: Spinal cord neuropathology in human West Nile virus infection. Arch Pathol Lab Med **128:** 533-537, 2004

30 Fujii Y, Kitaura K, Nakamichi K, Takasaki T, Suzuki R, Kurane I: Accumulation of T-cells with selected T-cell receptors in the brains of Japanese encephalitis virus-infected mice. Jpn J Infect Dis **61:** 40-48, 2008

31 Garcia-Tapia D, Hassett DE, Mitchell WJ, Jr., Johnson GC, Kleiboeker SB: West Nile virus encephalitis: sequential histopathological and immunological events in a murine model of infection. J Neurovirol **13:** 130-138, 2007

32 Garcia-Tapia D, Loiacono CM, Kleiboeker SB: Replication of West Nile virus in equine peripheral blood mononuclear cells. Vet Immunol Immunopathol **110:** 229-244, 2006

33 German AC, Myint KS, Mai NT, Pomeroy I, Phu NH, Tzartos J, Winter P, Collett J, Farrar J, Barrett A, Kipar A, Esiri MM, Solomon T: A preliminary neuropathological study of Japanese encephalitis in humans and a mouse model. Trans R Soc Trop Med Hyg **100:** 1135-1145, 2006

34 Ghosh SN, Goverdhan MK, Sathe PS, Chelliah SC, Naik SV, Godbole PV,

Banerjee K: Protective effect of 6-MFA, a fungal interferon inducer against Japanese encephalitis virus in bonnet macaques. Indian J Med Res **91:** 408-413, 1990

35 Ghoshal A, Das S, Ghosh S, Mishra MK, Sharma V, Koli P, Sen E, Basu A: Proinflammatory mediators released by activated microglia induces neuronal death in Japanese encephalitis. Glia **55:** 483-496, 2007

36 Glass WG, Lim JK, Cholera R, Pletnev AG, Gao JL, Murphy PM: Chemokine receptor CCR5 promotes leukocyte trafficking to the brain and survival in West Nile virus infection. J Exp Med **202:** 1087-1098, 2005

37 Goverdhan MK, Kulkarni AB, Gupta AK, Tupe CD, Rodrigues JJ: Two-way cross-protection between West Nile and Japanese encephalitis viruses in bonnet macaques. Acta Virol **36:** 277-283, 1992

38 Grossberg SE, Scherer WF: 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, 1966

39 Guarner J, Shieh WJ, Hunter S, Paddock CD, Morken T, Campbell GL, Marfin AA, Zaki SR: Clinicopathologic study and laboratory diagnosis of 23 cases with West Nile virus encephalomyelitis. Hum Pathol **35:** 983-990, 2004

40 Gubler D, Kuno G, Markoff L: Flaviviruses. *In*: Fields Virology, eds. Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman BStraus SE, 5th ed., pp. 1153-1252. Lippincott Williams and Wilkins, Philadelphia, 2007

41 Harrington DG, Hilmas DE, Elwell MR, Whitmire RE, Stephen EL: Intranasal infection of monkeys with Japanese encephalitis virus: clinical response and treatment with a nuclease-resistant derivative of poly (I).poly (C). Am J Trop Med Hyg **26:** 1191-1198, 1977

42 Hase T, Dubois DR, Summers PL: Comparative study of mouse brains infected with Japanese encephalitis virus by intracerebral or intraperitoneal inoculation. Int J Exp Pathol **71:** 857-869, 1990

43 Hayes EB, Komar N, Nasci RS, Montgomery SP, O'Leary DR, Campbell GL: Epidemiology and transmission dynamics of West Nile virus disease. Emerg Infect Dis **11:** 1167-1173, 2005

44 Heinz FX: Epitope mapping of flavivirus glycoproteins. Adv Virus Res **31:** 103-168, 1986

45 Huang CH: Studies of virus factors as causes of inapparent infection in Japanese B encephalitis: virus strains, viraemia, stability to heat and infective dosage. Acta Virol **1:** 36-45, 1957

46 Huang CH: Studies on host factors in inapparent infection with Japanese B encephalitis; influence of age, nutrition and luminalinduced sleep on the course of infection in mice. Acta Virol **1:** 83-91, 1957

47 Huang CH, Wong C: Relation of the Peripheral Multiplication of Japanese B Encephalitis Virus to the Pathogenesis of the Infection in Mice. Acta Virol **7:** 322-330, 1963

48 Johnson RT, Burke DS, Elwell M, Leake CJ, Nisalak A, Hoke CH, Lorsomrudee W: Japanese encephalitis: immunocytochemical studies of viral antigen and inflammatory cells in fatal cases. Ann Neurol **18:** 567-573, 1985

49 Johnston LJ, Halliday GM, King NJ: Langerhans cells migrate to local lymph nodes following cutaneous infection with an arbovirus. J Invest Dermatol **114:** 560-568, 2000

50 Johnston LJ, Halliday GM, King NJ: Phenotypic changes in Langerhans' cells after infection with arboviruses: a role in the immune response to epidermally acquired viral infection? J Virol **70:** 4761-4766, 1996

51 Kimura-Kuroda J, Ichikawa M, Ogata A, Nagashima K, Yasui K: Specific tropism of Japanese encephalitis virus for developing neurons in primary rat brain culture. Arch Virol **130:** 477-484, 1993

52 Kimura T, Kimura-Kuroda J, Nagashima K, Yasui K: Analysis of virus-cell binding characteristics on the determination of Japanese encephalitis virus susceptibility. Arch Virol **139:** 239-251, 1994

53 Klein RS, Lin E, Zhang B, Luster AD, Tollett J, Samuel MA, Engle M, Diamond MS: Neuronal CXCL10 directs CD8+ T-cell recruitment and control of West Nile virus encephalitis. J Virol **79:** 11457-11466, 2005

54 Kleinschmidt-DeMasters BK, Marder BA, Levi ME, Laird SP, McNutt JT, Escott EJ, Everson GT, Tyler KL: Naturally acquired West Nile virus encephalomyelitis in transplant recipients: clinical, laboratory, diagnostic, and neuropathological features. Arch Neurol **61:** 1210-1220, 2004

55 Komar N, Langevin S, Hinten S, Nemeth N, Edwards E, Hettler D, Davis B, Bowen R, Bunning M: Experimental infection of North American birds with the New York 1999 strain of West Nile virus. Emerg Infect Dis **9:** 311-322, 2003

56 Lee GC, Grayston JT, Wang SP: Protective studies in mice and monkeys with an inactivated Japanese encephalitis virus vaccine grown in hamster diploid cell culture. Proc Soc Exp Biol Med **125:** 803-808, 1967

57 Lewis JA, Chang GJ, Lanciotti RS, Kinney RM, Mayer LW, Trent DW: Phylogenetic relationships of dengue-2 viruses. Virology **197:** 216-224, 1993

58 Lieberman MM, Nerurkar VR, Luo H, Cropp B, Carrion R, Jr., de la Garza M, Coller BA, Clements D, Ogata S, Wong T, Martyak T, Weeks-Levy C: Immunogenicity and protective efficacy of a recombinant subunit West Nile virus vaccine in rhesus monkeys. Clin Vaccine Immunol **16:** 1332-1337, 2009

59 Lindenbach BD, Thiel H-J, Rice CM: Flaviviridae: The Viruses and Their Replication. *In*: Fields Virology, eds. Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman BStraus SE, 5th ed., pp. 1101-1152. Lippincott Williams and Wilkins, Philadelphia, 2007

60 Mackenzie JS, Gubler DJ, Petersen LR: Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat Med **10:** S98-109, 2004

61 Malkova D: Role of free cells in the lymph and blood vessels during viraemia in animals experimentally infected with tick-borne encephalitis virus. II. Virus bound in vivo to the cellular blood component in mice. Acta Virol **11:** 317-320, 1967

62 Malkova D: The role of the lymphatic system in experimental infection with tick-borne encephalitis. I. The tick-borne encephalitis virus in the lymph and blood of experimentally infected sheep. Acta Virol **4:** 233-240, 1960

63 Mandl CW, Holzmann H, Kunz C, Heinz FX: Complete genomic sequence of Powassan virus: evaluation of genetic elements in tick-borne versus mosquito-borne flaviviruses. Virology **194:** 173-184, 1993

64 Martin DA, Muth DA, Brown T, Johnson AJ, Karabatsos N, Roehrig JT: Standardization of immunoglobulin M capture enzyme-linked immunosorbent assays for routine diagnosis of arboviral infections. J Clin Microbiol **38:** 1823-1826, 2000

65 Mashimo T, Lucas M, Simon-Chazottes D, Frenkiel MP, Montagutelli X, Ceccaldi PE, Deubel V, Guenet JL, Despres P: A nonsense mutation in the gene encoding 2'-5'-oligoadenylate synthetase/L1 isoform is associated with West Nile virus susceptibility in laboratory mice. Proc Natl Acad Sci U S A **99:** 11311-11316, 2002

66 Mathur A, Arora KL, Chaturvedi UC: Transplacental Japanese encephalitis virus (JEV) infection in mice during consecutive pregnancies. J Gen Virol **59:** 213-217, 1982

67 Mathur A, Arora KL, Rawat S, Chaturvedi UC: Persistence, latency and reactivation of Japanese encephalitis virus infection in mice. J Gen Virol **67 (Pt 2):** 381-385, 1986

68 Mathur A, Bharadwaj M, Kulshreshtha R, Rawat S, Jain A, Chaturvedi UC: Immunopathological study of spleen during Japanese encephalitis virus infection in mice. Br J Exp Pathol **69:** 423-432, 1988

69 Mathur A, Kulshreshtha R, Chaturvedi UC: Evidence for latency of Japanese encephalitis virus in T lymphocytes. J Gen Virol **70 (Pt 2):** 461-465, 1989

70 Miura K, Goto N, Suzuki H, Fujisaki Y: Strain difference of mouse in susceptibility to Japanese encephalitis virus infection. Jikken Dobutsu **37:** 365-373, 1988

71 Miura T: A new method for a potency test of Japanese encephalitis vaccine. Direct challenge method on suckling mice by subcutaneous inoculation. Bull World Health Organ **43:** 553-557, 1970

72 Monath TP, Cropp CB, Harrison AK: Mode of entry of a neurotropic arbovirus into the central nervous system. Reinvestigation of an old controversy. Lab Invest **48:** 399-410, 1983

73 Monath TP, Levenbook I, Soike K, Zhang ZX, Ratterree M, Draper K, Barrett AD, Nichols R, Weltzin R, Arroyo J, Guirakhoo F: Chimeric yellow fever virus 17D-Japanese encephalitis virus vaccine: dose-response effectiveness and extended safety testing in rhesus monkeys. J Virol **74:** 1742-1751, 2000

74 Monath TP, Soike K, Levenbook I, Zhang ZX, Arroyo J, Delagrave S, Myers G, Barrett AD, Shope RE, Ratterree M, Chambers TJ, Guirakhoo F: Recombinant, chimaeric live, attenuated vaccine (ChimeriVax) incorporating the envelope genes of Japanese encephalitis (SA14-14-2) virus and the capsid and nonstructural genes of yellow fever (17D) virus is safe, immunogenic and protective in non-human primates. Vaccine **17:** 1869-1882, 1999

75 Morris JA, O'Connor JR, Smadel JE: Infection and immunity patterns in monkeys injected with viruses of Russian spring-summer and Japanese encephalitis. Am J Hyg **62:** 327-341, 1955

76 Murgue B, Zeller H, Deubel V: The ecology and epidemiology of West Nile virus in Africa, Europe and Asia. Curr Top Microbiol Immunol **267:** 195-221, 2002

77 Myint KS, Raengsakulrach B, Young GD, Gettayacamin M, Ferguson LM, Innis BL, Hoke CH, Jr., Vaughn DW: Production of lethal infection that resembles fatal human disease by intranasal inoculation of macaques with Japanese encephalitis virus. Am J Trop Med Hyg **60:** 338-342, 1999

78 Nalca A, Fellows PF, Whitehouse CA: Vaccines and animal models for arboviral encephalitides. Antiviral Res **60:** 153-174, 2003

79 Nathanson N: Pathogenesis. *In*: St. Louis Encephalitis, ed. P. MT, pp. 201-236. American Public Health Association, Washington, D.C., 1980

80 Nemeth NM, Beckett S, Edwards E, Klenk K, Komar N: Avian mortality surveillance for West Nile virus in Colorado. Am J Trop Med Hyg **76:** 431-437, 2007

81 O'Leary DR, Marfin AA, Montgomery SP, Kipp AM, Lehman JA, Biggerstaff BJ, Elko VL, Collins PD, Jones JE, Campbell GL: The epidemic of West Nile virus in the United States, 2002. Vector Borne Zoonotic Dis **4:** 61-70, 2004

82 Ogata A, Nagashima K, Hall WW, Ichikawa M, Kimura-Kuroda J, Yasui K: Japanese encephalitis virus neurotropism is dependent on the degree of neuronal maturity. J Virol **65:** 880-886, 1991

83 Ogata A, Tashiro K, Nukuzuma S, Nagashima K, Hall WW: A rat model of Parkinson's disease induced by Japanese encephalitis virus. J Neurovirol **3:** 141-147, 1997

84 Ølberg RA, Barker IK, Crawshaw GJ, Bertelsen MF, Drebot MA, Andonova M: West Nile virus encephalitis in a Barbary macaque (Macaca sylvanus). Emerg Infect Dis **10:** 712-714, 2004

85 Omalu BI, Shakir AA, Wang G, Lipkin WI, Wiley CA: Fatal fulminant

pan-meningo-polioencephalitis due to West Nile virus. Brain Pathol **13:** 465-472, 2003

86 Paddock CD, Nicholson WL, Bhatnagar J, Goldsmith CS, Greer PW, Hayes EB, Risko JA, Henderson C, Blackmore CG, Lanciotti RS, Campbell GL, Zaki SR: Fatal hemorrhagic fever caused by West Nile virus in the United States. Clin Infect Dis **42:** 1527-1535, 2006

87 Perelygin AA, Scherbik SV, Zhulin IB, Stockman BM, Li Y, Brinton MA: Positional cloning of the murine flavivirus resistance gene. Proc Natl Acad Sci U S A **99:** 9322-9327, 2002

88 Petersen LR, Marfin AA: West Nile virus: a primer for the clinician. Ann Intern Med **137:** 173-179, 2002

89 Petersen LR, Roehrig JT: West Nile virus: a reemerging global pathogen. Emerg Infect Dis **7:** 611-614, 2001

90 Pletnev AG, Claire MS, Elkins R, Speicher J, Murphy BR, Chanock RM: Molecularly engineered live-attenuated chimeric West Nile/dengue virus vaccines protect rhesus monkeys from West Nile virus. Virology **314:** 190-195, 2003

91 Pogodina VV, Frolova MP, Malenko GV, Fokina GI, Koreshkova GV, Kiseleva

LL, Bochkova NG, Ralph NM: Study on West Nile virus persistence in monkeys. Arch Virol **75:** 71-86, 1983

92 Pradhan S, Pandey N, Shashank S, Gupta RK, Mathur A: Parkinsonism due to predominant involvement of substantia nigra in Japanese encephalitis. Neurology **53:** 1781-1786, 1999

93 Raengsakulrach B, Nisalak A, Gettayacamin M, Thirawuth V, Young GD, Myint KS, Ferguson LM, Hoke CH, Jr., Innis BL, Vaughn DW: An intranasal challenge model for testing Japanese encephalitis vaccines in rhesus monkeys. Am J Trop Med Hyg **60:** 329-337, 1999

94 Ratterree MS, Gutierrez RA, Travassos da Rosa AP, Dille BJ, Beasley DW, Bohm RP, Desai SM, Didier PJ, Bikenmeyer LG, Dawson GJ, Leary TP, Schochetman G, Phillippi-Falkenstein K, Arroyo J, Barrett AD, Tesh RB: Experimental infection of rhesus macaques with West Nile virus: level and duration of viremia and kinetics of the antibody response after infection. J Infect Dis **189:** 669-676, 2004

95 Ren J, Ding T, Zhang W, Song J, Ma W: Does Japanese encephalitis virus share the same cellular receptor with other mosquito-borne flaviviruses on the C6/36 mosquito cells? Virol J **4:** 83, 2007

96 Samuel MA, Diamond MS: Pathogenesis of West Nile Virus infection: a balance between virulence, innate and adaptive immunity, and viral evasion. J Virol **80:** 9349-9360, 2006

97 Samuel MA, Wang H, Siddharthan V, Morrey JD, Diamond MS: Axonal transport mediates West Nile virus entry into the central nervous system and induces acute flaccid paralysis. Proc Natl Acad Sci U S A **104:** 17140-17145, 2007

98 Sangster MY, Heliams DB, MacKenzie JS, Shellam GR: Genetic studies of flavivirus resistance in inbred strains derived from wild mice: evidence for a new resistance allele at the flavivirus resistance locus (Flv). J Virol **67:** 340-347, 1993

99 Saxena SK, Mathur A, Srivastava RC: Induction of nitric oxide synthase during Japanese encephalitis virus infection: evidence of protective role. Arch Biochem Biophys **391:** 1-7, 2001

100 Scherbik SV, Kluetzman K, Perelygin AA, Brinton MA: Knock-in of the Oas1b(r) allele into a flavivirus-induced disease susceptible mouse generates the resistant phenotype. Virology **368:** 232-237, 2007

101 Sejvar JJ, Haddad MB, Tierney BC, Campbell GL, Marfin AA, Van Gerpen JA,

Fleischauer A, Leis AA, Stokic DS, Petersen LR: Neurologic manifestations and outcome of West Nile virus infection. Jama **290:** 511-515, 2003

102 Senne DA, Pedersen JC, Hutto DL, Taylor WD, Schmitt BJ, Panigrahy B: Pathogenicity of West Nile virus in chickens. Avian Dis **44:** 642-649, 2000

103 Shirato K, Kimura T, Mizutani T, Kariwa H, Takashima I: Different chemokine expression in lethal and non-lethal murine West Nile virus infection. J Med Virol **74:** 507-513, 2004

104 Shirato K, Miyoshi H, Goto A, Ako Y, Ueki T, Kariwa H, Takashima I: Viral envelope protein glycosylation is a molecular determinant of the neuroinvasiveness of the New York strain of West Nile virus. J Gen Virol **85:** 3637-3645, 2004

105 Shrestha B, Diamond MS: Role of CD8+ T cells in control of West Nile virus infection. J Virol **78:** 8312-8321, 2004

106 Shrestha B, Gottlieb D, Diamond MS: Infection and injury of neurons by West Nile encephalitis virus. J Virol **77:** 13203-13213, 2003

107 Shrestha B, Samuel MA, Diamond MS: CD8+ T cells require perforin to clear West Nile virus from infected neurons. J Virol **80:** 119-129, 2006

108 Shrestha B, Wang T, Samuel MA, Whitby K, Craft J, Fikrig E, Diamond MS:

Gamma interferon plays a crucial early antiviral role in protection against West Nile virus infection. J Virol **80:** 5338-5348, 2006

109 Sitati EM, Diamond MS: CD4+ T-cell responses are required for clearance of West Nile virus from the central nervous system. J Virol **80:** 12060-12069, 2006

110 Smith RD, Konoplev S, DeCourten-Myers G, Brown T: West Nile virus encephalitis with myositis and orchitis. Hum Pathol **35:** 254-258, 2004

111 Solomon T, Kneen R, Dung NM, Khanh VC, Thuy TT, Ha DQ, Day NP, Nisalak A, Vaughn DW, White NJ: Poliomyelitis-like illness due to Japanese encephalitis virus. Lancet **351:** 1094-1097, 1998

112 Solomon T, Vaughn DW: Pathogenesis and Clinical Features of Japanese Encephalitis and West Nile Virus Infections. *In*: Japanese Encephalitis and West Nile Viruses, eds. Mackenzie JS, Barrett ADTDeubel V, pp. 171-194. Springer-Verlag, Berlin, 2002

113 Steele KE, Linn MJ, Schoepp RJ, Komar N, Geisbert TW, Manduca RM, Calle PP, Raphael BL, Clippinger TL, Larsen T, Smith J, Lanciotti RS, Panella NA, McNamara TS: Pathology of fatal West Nile virus infections in native and exotic birds during the 1999 outbreak in New York City, New York. Vet Pathol **37:** 208-224, 2000

114 Su CM, Liao CL, Lee YL, Lin YL: Highly sulfated forms of heparin sulfate are involved in japanese encephalitis virus infection. Virology **286:** 206-215, 2001

115 Takasaki T, Yabe S, Nerome R, Ito M, Yamada K, Kurane I: Partial protective effect of inactivated Japanese encephalitis vaccine on lethal West Nile virus infection in mice. Vaccine **21:** 4514-4518, 2003

116 Teehee ML, Bunning ML, Stevens S, Bowen RA: Experimental infection of pigs with West Nile virus. Arch Virol **150:** 1249-1256, 2005

117 Tesh RB, Siirin M, Guzman H, Travassos da Rosa AP, Wu X, Duan T, Lei H, Nunes MR, Xiao SY: Persistent West Nile virus infection in the golden hamster: studies on its mechanism and possible implications for other flavivirus infections. J Infect Dis **192:** 287-295, 2005

118 Tyler KL, Gonzalez-Scarano F: Viral Diseases of the Central Nervous System: Acute Infection. *In*: Viral Pathogenesis, eds. Nathanson N, Ahmed R, Gonzalez-Scarano F, Griffin DE, Holmes KV, Murphy FARobinson HL, pp. 837-854. Lippincott-Raven, Philadelphia, 1997

119 Vaughn DW, Hoke CH, Jr.: The epidemiology of Japanese encephalitis: prospects for prevention. Epidemiol Rev **14:** 197-221, 1992

120 Wang H, Siddharthan V, Hall JO, Morrey JD: West Nile virus preferentially

transports along motor neuron axons after sciatic nerve injection of hamsters. J Neurovirol **15:** 293-299, 2009

121 Wang Y, Lobigs M, Lee E, Mullbacher A: CD8+ T cells mediate recovery and immunopathology in West Nile virus encephalitis. J Virol **77:** 13323-13334, 2003

122 Watson JT, Pertel PE, Jones RC, Siston AM, Paul WS, Austin CC, Gerber SI: Clinical characteristics and functional outcomes of West Nile Fever. Ann Intern Med **141:** 360-365, 2004

123 Weaver SC, Barrett AD: Transmission cycles, host range, evolution and emergence of arboviral disease. Nat Rev Microbiol **2:** 789-801, 2004

124 Weingartl HM, Neufeld JL, Copps J, Marszal P: Experimental West Nile virus infection in blue jays (Cyanocitta cristata) and crows (Corvus brachyrhynchos). Vet Pathol **41:** 362-370, 2004

125 Wu SC, Chiang JR, Lin CW: Novel cell adhesive glycosaminoglycan-binding proteins of Japanese encephalitis virus. Biomacromolecules **5:** 2160-2164, 2004

126 Wunschmann A, Shivers J, Carroll L, Bender J: Pathological and immunohistochemical findings in American crows (Corvus brachyrhynchos) naturally infected with West Nile virus. J Vet Diagn Invest **16:** 329-333, 2004

127 Xiao SY, Guzman H, Zhang H, Travassos da Rosa AP, Tesh RB: West Nile virus infection in the golden hamster (Mesocricetus auratus): a model for West Nile encephalitis. Emerg Infect Dis **7:** 714-721, 2001

128 Zimmerman HM: The Pathology of Japanese B Encephalitis. Am J Pathol **22:** 965, 1946

Figure legends

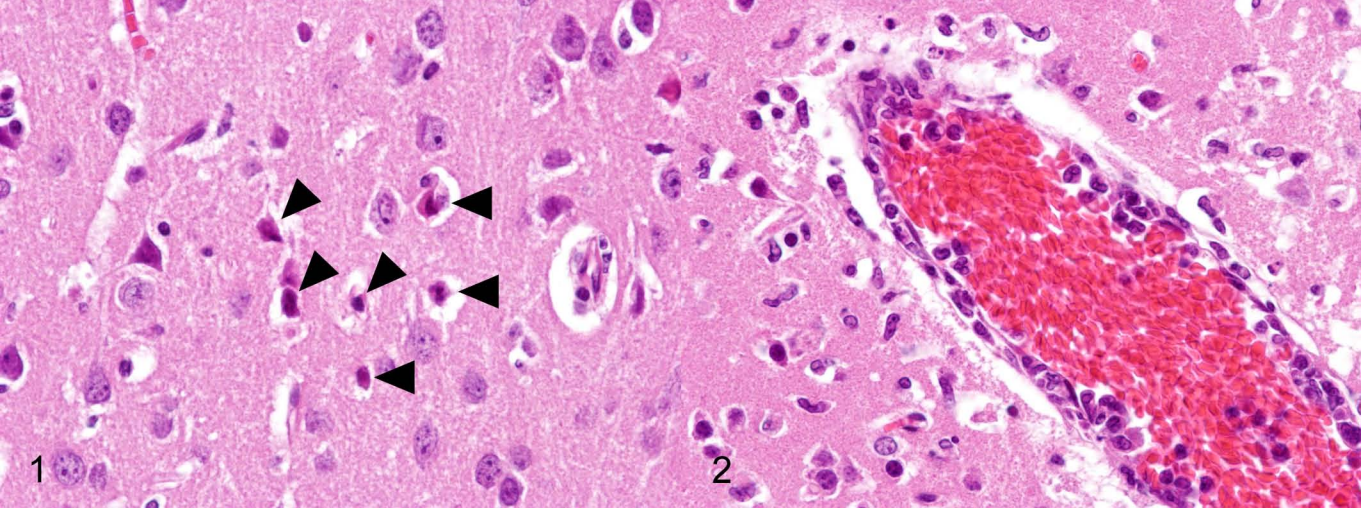
Fig. 1 and 2. Histopathology of the brains of WNV-infected mice. Six-week-old female C3H/HeN mice were infected subcutaneously by footpad injection with 1,000 plaque-forming units (p.f.u.) of the 6-LP strain, which was originally isolated from the NY99-6922 strain via plaque purification.[104] **Fig. 1.** Cerebral cortex at day 10 postinfection (p.i.). Necrotic neurons exhibited shrunken perikarya, deep eosinophilic cytoplasm, and pyknotic or karyorrhectic nuclei (arrowheads). Hematoxylin and Eosin (HE) stain. **Fig. 2.** Caudate putamen at day 9 p.i. Perivascular and vascular infiltration of mononuclear cells. HE stain.

Fig. 3-9. Distribution of viral antigens in the CNS of C3H/HeN mice infected with WNV as described in the legend of Fig. 1 and 2. IHC was performed using a rabbit anti-JEV serum that crossreacted with the structural proteins of WNV. Immunolabeled cells were visualized using 3,3'-diaminobenzidine tetrachloride and were counterstained with hematoxylin. **Fig. 3.** Lumbar spinal cord at day 7 p.i. A few viral antigen-positive neurons were observed in the ventral horn ipsilateral to the inoculation site. **Fig. 4.** Caudate putamen at day 10 p.i. The foci of WNV-positive cells (arrows) were scattered.

Fig. 5. Substantia nigra at day 9 p.i. WNV immunoreactivity was detected mainly in the cytoplasm of neurons. **Fig. 6.** (Substantia nigra at day 9 p.i.) and **Fig. 7.** (Caudate putamen at day 10 p.i.) depict neuronophagia. **Fig. 8.** Hippocampus at day 10 p.i. Prominent microgliosis was observed in and around heavily infected foci. These macrophage/microglial cells frequently showed immunoreactivity for WNV (arrowheads). **Fig. 9.** Caudate putamen at day 9 p.i. Perivascular accumulation of macrophages immunolabeled for the WNV antigen.

Fig. 10-15. Pathological changes in the peripheral tissues of C3H/HeN mice infected with WNV as described in the legend of Fig. 1 and 2. **Fig. 10.** Footpad skin of the left hind-limb (inoculation site) at day 9 p.i. A cluster of epidermal cells showed immunoreactivity for WNV. IHC for the WNV antigen. **Fig. 11.** Digestive tract of mock-infected (upper panel) and WNV-infected (lower panel) mice at day 9 p.i. The stomach and the upper two thirds of the small intestine of a WNV-infected mouse were distended. **Fig. 12.** Small intestine of a mock-infected mouse. HE stain. **Fig. 13.** Small intestine of a WNV-infected mouse at day 9 p.i. HE stain. Villi were markedly shortened. **Fig. 14.** Small intestine of a WNV-infected mouse at day 10 p.i. Neuronal depletion with infiltration of macrophages in a myenteric plexus. Some macrophages showed

WNV immunoreactivity. IHC for the WNV antigen. **Fig. 15.** Large intestine of a WNV-infected mouse at day 10 p.i. WNV-positive neurons in a myenteric plexus. IHC for the WNV antigen.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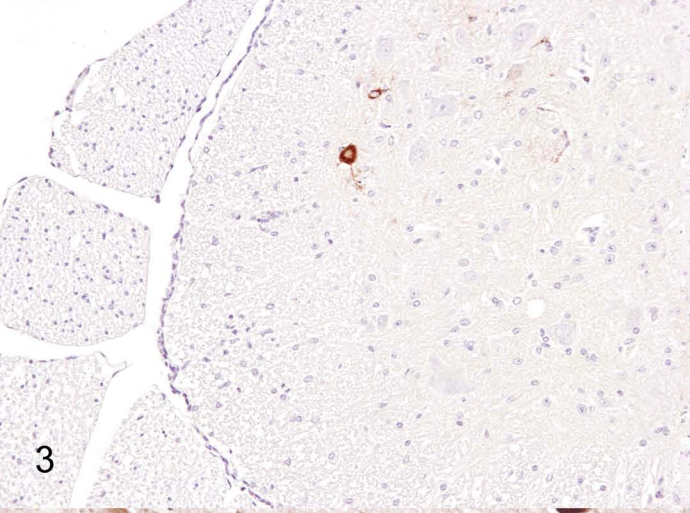
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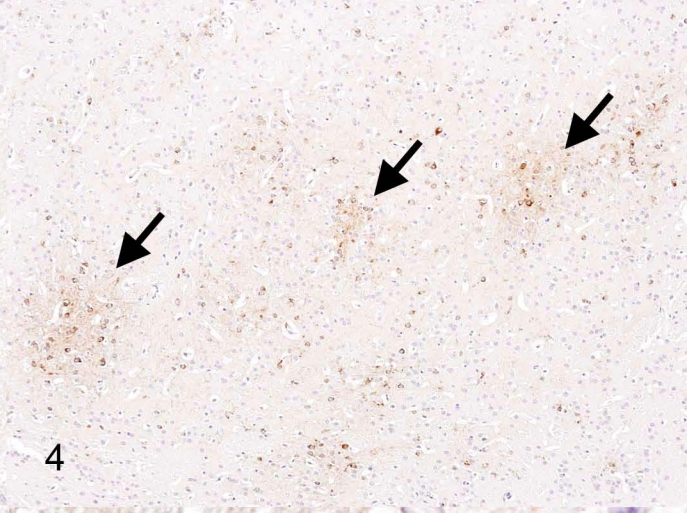
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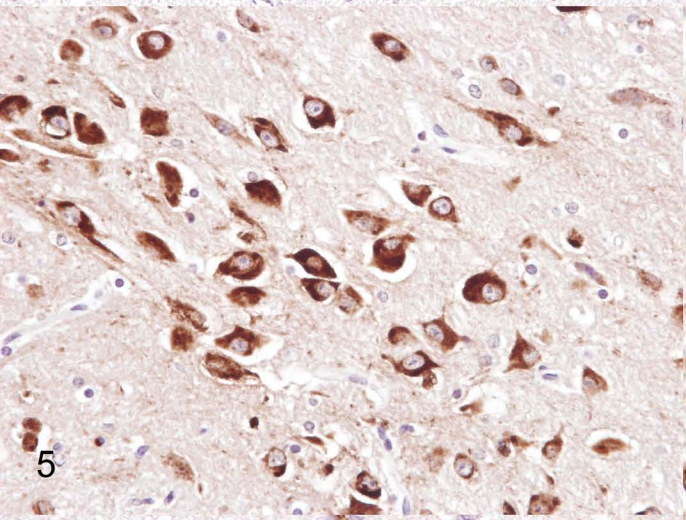
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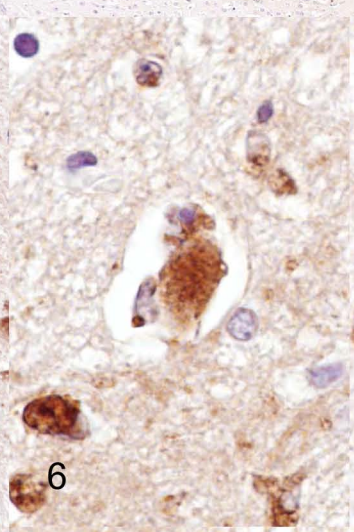
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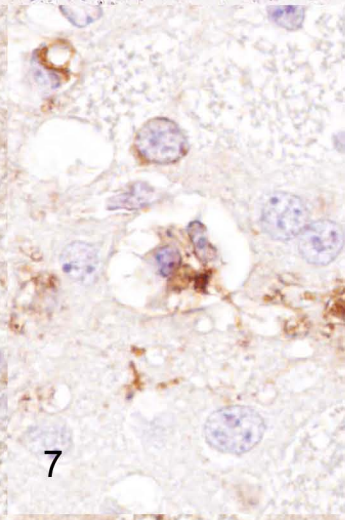
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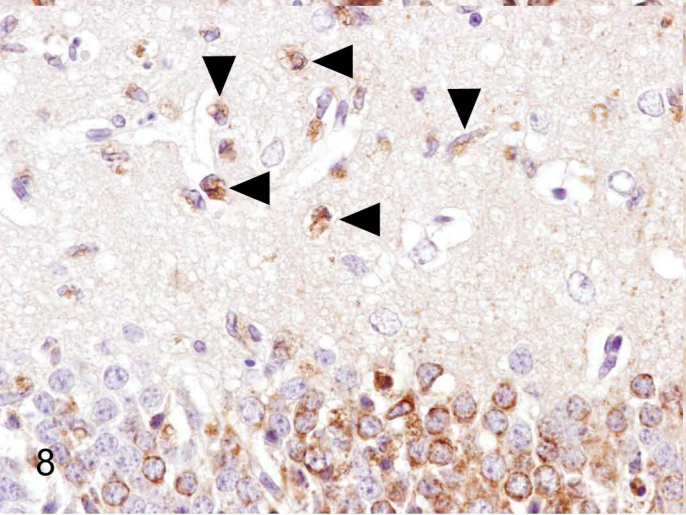
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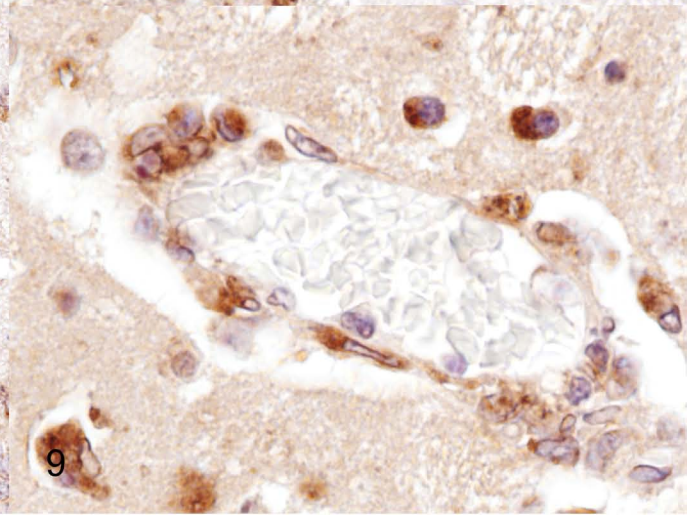
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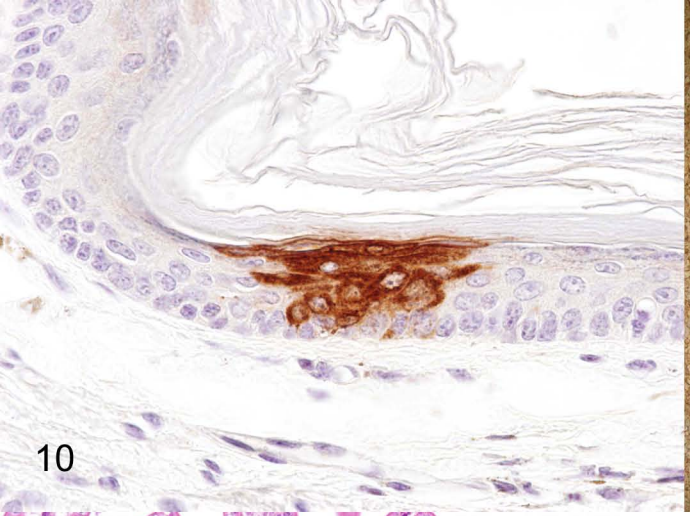

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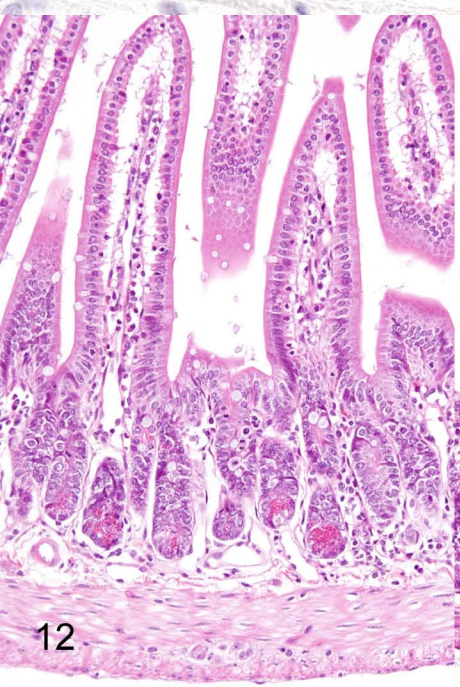

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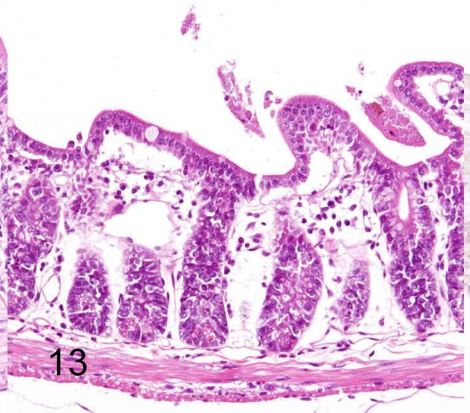

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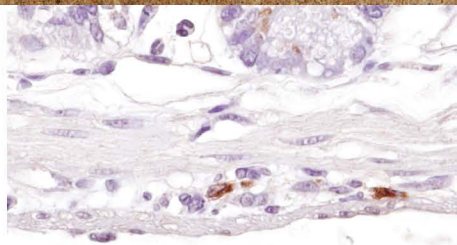

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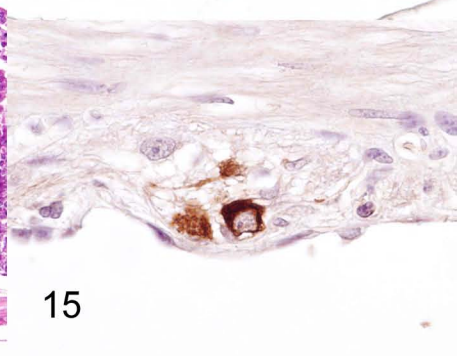

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