

AD-A269 082

2

ne for reviewing instructions, searching existing data sources, nts regarding this burden estimate or any other aspect of this rate for Information Operations and Reports, 1215 Jefferson on Project (0704-0188), Washington, DC 20503.

E AND DATES COVERED

COVERED
WR-210-92

5. FUNDING NUMBERS

6. AUTHOR(S)

Tatsuo Hase, Doria R Dubois, Peter L. Summers,
Mary Beth Downs, Michael A. Ussery

**8. PERFORMING ORGANIZATION
REPORT NUMBER**

Walter Reed Army Institute of Research
Washington, D.C. 20307-5100

**10. SPONSORING / MONITORING
AGENCY REPORT NUMBER**

U.S. Army Medical Research & Development Command,
Ft. Detrick, MD 21702-5100

NTIS CRA&I

DTIC TAB

Unannounced

Justification

11. SUPPLEMENTARY NOTES

By

Distribution /

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release:
distribution unlimited.

12b. DISTRIBUTION CODE	
Availability Codes	

Dist

	Avail	and/or Special
--	-------	-------------------

13. ABSTRACT (Maximum 200 words)

The replication rates and pathogenicities of the SA14 parent and SA14-14-2 vaccine strains of Japanese encephalitis (JE) virus in neurons of the mouse brain following intracerebral inoculation were compared. All the mice inoculated with the SA14 parent strain died within one week postinoculation (p.i.), whereas all the mice inoculated with the SA14-14-2 vaccine strain survived without showing any signs of central nervous system (CNS) involvement. The virus titers of the mouse brains inoculated with the SA14 strain reached progressively higher levels until day 5 when the animals died. On the other hand, the virus titers of the mouse brains inoculated with the SA14-14-2 strains persisted at low levels for several days and could not be detected after 10 days. In a routine electron microscopical study, a majority of neurons in the mouse brains inoculated with the SA14 strains contained virions and showed characteristic cytopathological changes in connection with viral replication. In the brains inoculated with the SA14-14-2, however, we failed to find neurons containing virions or showing characteristic cytopathological changes. In the alkaline phosphatase immunostaining of paraffin-embedded sections, a majority of neurons in the brains of mice inoculated with the

14. SUBJECT TERMS

Japanese encephalitis virus, mouse intracerebral inoculation,
pathogenicity, SA14 parent strain, SA14-14-2 vaccine strain.

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT
 UNCLASSIFIED

18. SECURITY CLASSIFICATION OF THIS PAGE

19. SECURITY CLASSIFICATION OF ABSTRACT

20. LIMITATION OF ABSTRACT

Comparison of replication rates and pathogenicities between the SA 14 parent and SA 14-14-2 vaccine strains of Japanese encephalitis virus in mouse brain neurons

T. Hase¹, D. R. Dubois², P. L. Summers², M. B. Downs³, and M. A. Ussery⁴

Departments of ¹ Ultrastructural Pathology and ² Biologics Research,
Walter Reed Army Institute of Research, Washington, D.C.

³ Department of Pathology, US Army Medical Research
Institute of Infectious Diseases, Ft. Detrick, Maryland

⁴ Division of Antiviral Drug Products, Food and Drug Administration,
Rockville, Maryland, U.S.A.

Accepted September 2, 1992

Summary. The replication rates and pathogenicities of the SA 14 parent and SA 14-14-2 vaccine strains of Japanese encephalitis (JE) virus in neurons of the mouse brain following intracerebral inoculation were compared. All the mice inoculated with the SA 14 parent strain died within one week postinoculation (p.i.), whereas all the mice inoculated with the SA 14-14-2 vaccine strains survived without showing any signs of central nervous system (CNS) involvement. The virus titers of the mouse brains inoculated with the SA 14 strain reached progressively higher levels until day 5 when the animals died. On the other hand, the virus titers of the mouse brains inoculated with the SA 14-14-2 strain persisted at low levels for several days and could not be detected after 10 days. In the routine electron microscopical study, a majority of neurons in the mouse brains inoculated with the SA 14 strain contained virions and showed characteristic cytopathological changes in connection with viral replication. In the brains inoculated with the SA 14-14-2 strain, however, we failed to find neurons containing virions or showing characteristic cytopathological changes. In the alkaline phosphatase immunostaining of paraffin-embedded sections, a majority of neurons in the brains of mice inoculated with the SA 14 strain stained positively on day 5 p.i., but only a small number of neurons in scattered small foci stained positively in the brains inoculated with the SA 14-14-2 strain. The immunogold staining of Vibratome sections also revealed the identical patterns; moreover, electron microscopical examination of the immunopositive foci of the brain inoculated with the vaccine strain revealed neurons that contained virions in dilated cisternae of rough endoplasmic reticulum (RER), in-

dicating that the SA 14-14-2 strain also replicated, albeit poorly, in neurons. The present results showed that upon intracerebral inoculation into mice the SA 14 parent strain of JE virus grew vigorously in a large number of neurons, killing the animals, while the SA 14-14-2 vaccine strain grew poorly only in a small number of neurons without causing mortality. Possible mechanisms involved in the alteration of pathogenicity between the SA 14 parent virus and the SA 14-14-2 vaccine virus are discussed.

Introduction

The SA 14 neurovirulent strain of JE virus was originally isolated from mosquitoes, and the SA 14-14-2 attenuated strain was subsequently derived from the SA 14 parent strain by serial passages in primary hamster kidney cells for vaccine purposes in the People's Republic of China [25, 26]. The SA 14-14-2 vaccine strain was further modified at Walter Reed Army Institute of Research by passages through primary canine kidney (PCK) cells that were pretested for the absence of adventitious agents to satisfy the World Health Organization standards set for human vaccines [4]. Upon intracerebral inoculation, the SA 14-14-2 vaccine strain does not cause death in mice older than 2 weeks of age, whereas the parent SA 14 strain causes 100% mortality within one week [4]. Although it has been well established that, unlike the SA 14 parent strain, the SA 14-14-2 vaccine strain lacks neurovirulence in adult mice [4], mechanisms involved in the disappearance of neurovirulence in the vaccine strain are not well understood.

In recent years, the growth pattern of JE virus in host cells has become increasingly clear. After attachment to the cell surface, JE virus enters host cells by directly penetrating through the plasma membrane and uncoats [7, 8, 12]. JE virus genome released into the cytoplasm replicates genomic RNA and translates viral proteins on the endoplasmic reticulum membranes and discharges the viral products into the rough endoplasmic reticulum (RER) cisternae for viral assembly [9]. The progeny virions assembled in the cisternae are released extracellularly through the host-cell secretory channel via the Golgi apparatus by secretory-type exocytosis [6, 10, 17]. Likewise, in the infection of mouse brain neurons, JE virus has been shown to replicate selectively in the neuronal secretory system including RER and the Golgi apparatus, causing characteristic ultrastructural changes of these cytoplasmic organelles [10, 11].

In this study, we compared the replication rates and the cytopathogenicities of the SA 14 parent and SA 14-14-2 vaccine strains of JE virus in the mouse brain in an attempt to elucidate the mechanisms involved in the alteration of neurovirulence between the two strains.

Materials and methods

Virus

The SA 14 and SA 14-14-2 strains of JE virus were supplied by Dr. Yu Yong Xin (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China). The

SA 14 parent strain was derived from mouse brain passages of the original mosquito isolate, followed by three PCK cell-culture passages. The passage history of the SA 14-14-2 vaccine strain was reported in detail by Eckels et al. [4].

Mouse infection

Mice (ICR strain, 5–6 weeks old) were inoculated into the right hemisphere of the brain with 0.03 ml (4.3×10^4 PFU) of the SA 14 or the SA 14-14-2 strain of JE virus.

Virus titration of mouse brain

For the titration of daily viral growth in mouse brains following the intracerebral inoculation, two mice inoculated with either the SA 14 strain or the SA 14-14-2 strain were sacrificed daily until all the animals died in the case of SA 14 strain inoculation and for 21 days p.i. in the case of SA 14-14-2 strain inoculation. The brains from the two mice were pooled and weighed; a 20% brain homogenate was made with Eagle's minimal essential medium containing 50% fetal bovine serum (EMEM/FBS). For the virus titration of the right and left hemispheres, 20% brain homogenates were made separately from the right and left hemispheres in the same manner. The brain homogenates were titrated for JE virus by a plaque assay [3].

Electron microscopy

For the electron microscopical study, 3 mice were inoculated with the SA 14 or the SA 14-14-2 strains and 2 control mice were inoculated with the same amount of virus-free EMEM/FBS. They were sacrificed on day 5 p.i. For the collection of mouse brain samples, the infected and control mice were anesthetized with ether and perfused by injecting first 20 ml of cold phosphate-buffered saline (PBS) and then 20 ml of 4F1G [20] into the left ventricle under mild pressure and draining them from a cut in the right atrium. The perfused animals were placed in a refrigerator at 4°C for 30 min. The brains were excised, and tissue samples measuring about 1 mm³ were taken from the cerebral cortex, the basal ganglia-thalamic region, the cerebellar cortex, and the medulla oblongata. The tissue samples were further fixed in 4F1G fixative at 4°C overnight, washed in 0.1 M cacodylate buffer (pH 7.4), postfixed in 1% cacodylate-buffered osmium tetroxide, dehydrated, and embedded in Polybed 812 (Polysciences Inc., Warrington, PA). Thin sections were placed on uncoated grids, stained with uranyl acetate and lead citrate, and examined with a Zeiss SM 109 electron microscope.

Immunocytochemistry

For immunostaining, two mice that were inoculated with the SA 14 or SA 14-14-2 strain, or virus-free EMEM/FBS were sacrificed on day 5 p.i. They were perfused with 20 ml of cold PBS, followed with 20 ml of cold fixative (4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) in the same manner as detailed in the preceding section. The mouse brains were cut into six coronal slices. The slices were either dehydrated and embedded in paraffin or cut into 40 µm sections using a Vibratome 1000 (Technical Products International, Inc., St Louis, MO). For all immunocytochemical studies, hyper-immune mouse ascites fluid (HMAF) directed against the Nakayama strain of JE virus was used as the primary antibody, and non-immune mouse ascites fluid (NMAF) was used as a control. The paraffin-sections of the mouse brains were stained by the alkaline phosphatase-labeled streptavidin-biotin technique [5]. The 5 µm thick sections were mounted on glass slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA), deparaffinized, rehydrated, immersed in a 0.05% solution of protease VIII (pH 7.8, Subtilisin, Sigma, St. Louis,

MO) at 37°C for 3 min and washed in distilled water. The slides were then placed in a solution of 1.5% normal horse serum in PBS (pH 7.4) for 20 min and incubated with HMAF or NMAF diluted 1:250 in PBS at 4°C overnight, followed by 30 min incubation at room temperature with biotinylated horse antimouse IgG (Vector Laboratories, Burlingame, CA) and then with alkaline phosphatase-labeled streptavidin (Kirkegaard and Perry Labs, Gaithersburg, MD). The slides were washed in 0.1 M Tris buffer solution and incubated with the phosphate ester of 6-bromo-2-hydroxy-3-naphthoic acid as the chromogenic substrate and levamisole to inhibit endogenous alkaline phosphatase according to the manufacturer's instruction (HistoMark Red, Kirkegaard and Perry). The slides were counterstained with Mayer's hematoxylin. To locate infected areas in the brains inoculated with the vaccine strain, the immunogold technique [2] was applied. The brain slices were cut into serial sections of 40 µm thick by a Vibratome. Two Vibratome serial sections were incubated with a 1:1000 dilution of HMAF or NMAF in BSA-Tris buffer (BTT; 20 mM Tris, 0.9% NaCl, 0.1% bovine serum albumin, 20 mM NaN₃, pH 8.2) for 2 h at room temperature. After rinsing in BTT, all the sections were incubated with goat anti-mouse IgG conjugated to 5 nm colloidal gold (Auoprobe EM, Amersham Life Science Products, Arlington Heights, IL) diluted 1:40 in BTT as the secondary antibody. One of each set of the serial sections was silver enhanced for light microscopy by IntenSE M (Amersham Life Science Products, Arlington Heights, IL) for 30 min, counterstained with Mayer's hematoxylin, dehydrated, and mounted on glass microscope slides. These sections were used to locate areas of positive staining under the light microscope. The remaining sections were enhanced with IntenSE M for 5 min, dehydrated, and flat embedded in Polybed 812. Areas corresponding to positively stained areas in the sections prepared for light microscopy were cut out and mounted on blank epoxy blocks for thin sectioning. The thin sections were post-stained with uranyl acetate and lead citrate, and examined with a JEOL 1200 EX electron microscope.

Results

Virus replication in mouse brains

All the mice inoculated intracerebrally with the SA 14 strain of JE virus died with a mean survival time of 5 days; on the other hand, all the mice inoculated with the SA 14-14-2 strain survived without showing any apparent sickness. As shown in Table 1, the virus titers of the brains inoculated with the SA 14 strain rose daily to progressively higher levels until death of the animals at day 5. In contrast, the virus titers of the brains inoculated with the SA 14-14-2 strain remained at a relatively low level for several days following the inoculation and could not be detected after 10 days. Since it was not clear whether the low virus titers of the SA 14-14-2 strain-inoculated brains represented replicated virus in brain neurons or residual virus from the inoculum, we separately titrated the right and left hemispheres of the brains 5 days after the inoculation with the SA 14 or SA 14-14-2 strain into the right hemisphere to see whether the right and left hemispheres showed different virus titers. As shown in Table 2, the titers of the right and left hemispheres of the SA 14 strain-inoculated brains were almost equally high, and the titers of the right and left hemispheres of the SA 14-14-2 strain-inoculated brains were almost equally low.

Table 1. JE virus titers (PFU/ml homogenate) in the cerebrum of mouse brains following IC inoculation of the SA 14 and SA 14-14-2 strains

Day	SA 14	SA 14-14-2
1	1.8 ± 10^5	8.0×10^3
2	4.1 ± 10^5	5.0×10^3
3	3.6 ± 10^7	1.2×10^4
4	1.0 ± 10^9	1.4×10^4
5	2.1 ± 10^8	9.5×10^3
6		4.5×10^3
7		5.0×10^2
8		3.0×10^1
9		1.0×10^1
10		5.0×10^0

Table 2. JE virus titers (PFU/ml homogenate) in the right and left hemispheres of the cerebrum 5 days after IC inoculation of the SA 14 and SA 14-14-2 strains into the right hemisphere

	SA 14	SA 14-14-2
Right hemisphere	1.5×10^7	1.5×10^3
Left hemisphere	2.5×10^7	5.0×10^2
Whole	8.0×10^6	5.0×10^3

Transmission electron microscopy

In the mouse brains inoculated with the SA 14 strain of JE virus, a majority of neurons showed characteristic cytoplasmic changes involving primarily the cellular secretory system (Fig. 1). The cytoplasmic changes consisted of clumping of RER whose cystically dilated cisternae contained multiple virions and characteristic ER vesicles and fragmentation and vacuolation of the Golgi apparatus whose saccules contained virions. Mitochondria were irregular in size and showed varying degrees of swelling and dissolution. The perikaryon of infected neurons showed rarefaction as the result of loss of the cytoplasmic membranous organelles and other cytoplasmic components such as free ribosomes and polyosomes, neurofilaments, microtubules, and secretory vesicles.

In the mouse brains inoculated with the SA 14-14-2 strain, all the neurons examined showed a normal-looking cellular secretory system (Fig. 2). Neurons showing characteristic ultrastructural changes related to JE virus replication were not found in routine electron microscopical examination. RER in the perikaryon appeared normal, showing no clumping and cisternal dilation (Fig. 2). Neither virions nor ER vesicles were found in the RER cisternae. The Golgi apparatus showed a normal, delicate membrane structure; no virions were found in the Golgi saccules. The rest of the cytoplasmic components appeared normal. Occasionally, small particles of the size of JE virions were found within cytoplasmic vesicles (Fig. 2); however, they did not show distinct ultrastructural characteristics of JE virions with clearly recognizable nucleocapsids surrounded by distinct viral membrane envelopes and, therefore, could not be confirmed as JE virions morphologically.



Fig. 1. A neuron in the cortex of a mouse brain inoculated with the SA 14 strain of JE virus, 5 days p.i. RER (*R*) shows clumping and cystic degeneration. Multiple virions (arrow) and ER vesicles (arrowhead) are seen in the cystically dilated RER cisternae. The Golgi apparatus (*G*) shows vesiculation and vacuolation. *M* Mitochondrion; *N* nucleus. Bar: 200 nm

Immunohistochemistry

Representative histological pictures of the cerebral cortices of the mouse brains inoculated with the SA 14 or SA 14-14-2 strain stained by the alkaline phosphatase method are shown in Fig. 3. A majority of neurons in the mouse brain inoculated with the SA 14 strain stained positively (Fig. 3 a). On the other hand, only a small number of neurons in scattered small foci stained positively in the



Fig. 2. A neuron in the cortex of a mouse brain inoculated with the SA 14-14-2 strain of JE virus, 5 days p.i. RER (*R*), the Golgi apparatus (*G*), and the cytoplasm show a normal appearance. An arrow points to a particle within a vesicle, that is of the same size as a virion but does not show distinct ultrastructural characteristics of a virion. *M* Mitochondrion; *N* nucleus. Bar: 200 nm

mouse brains inoculated with the SA 14-14-2 strain (Fig. 3 b, c). No neurons stained positively in the control brains (Fig. 3 d). Immunogold staining of vibratome-sections of the brains inoculated with the SA 14 strain or the SA 14-14-2 strain produced staining patterns identical to those of paraffin-embedded sections stained by alkaline phosphatase (Fig. 4 a, b). Electron microscopical examination of the positively stained foci in the brains inoculated with the

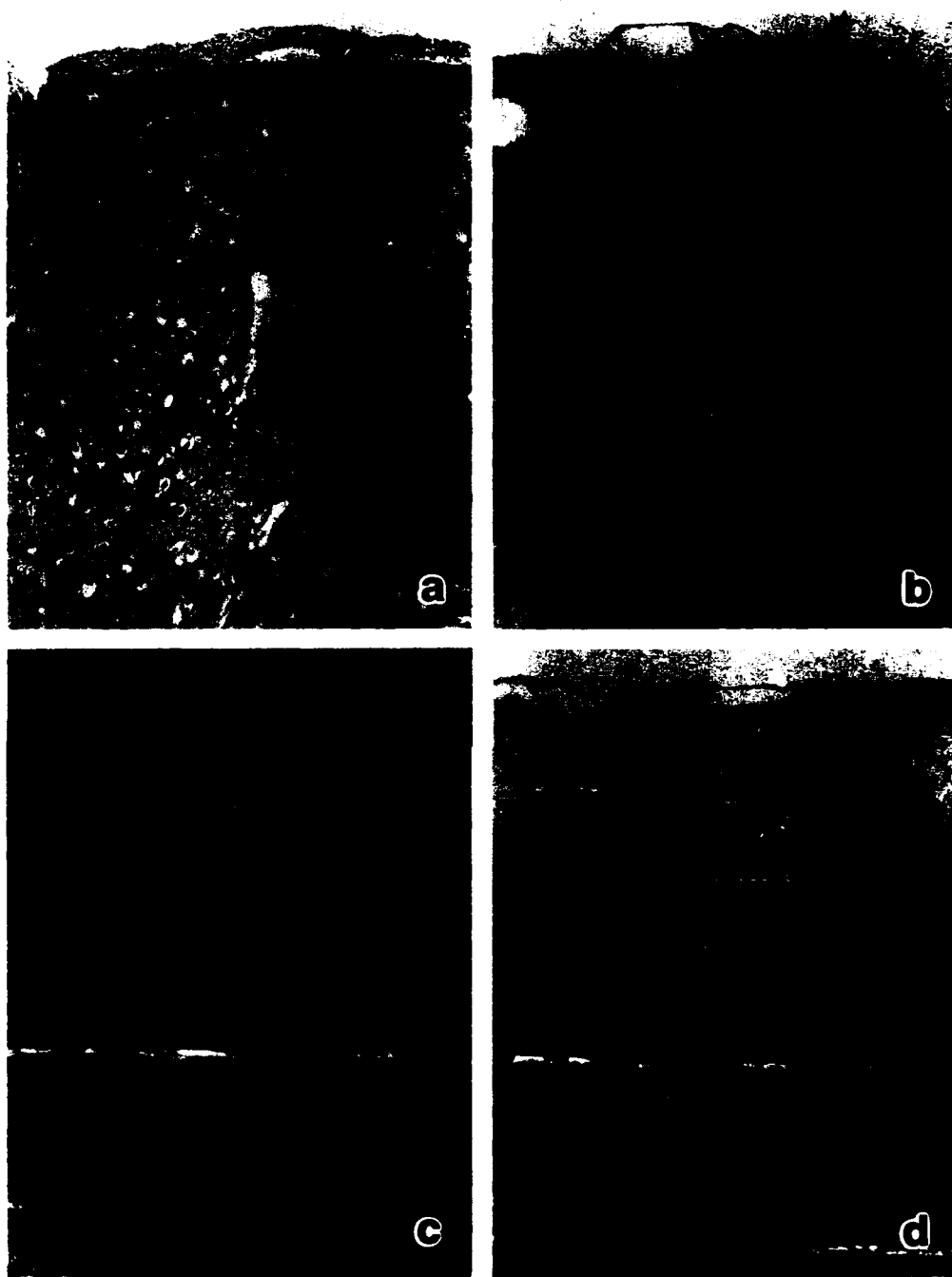


Fig. 3. Alkaline phosphatase staining of cerebral cortices of mouse brains, 5 days p.i. **a** A brain inoculated with the SA 14 strain. **b** and **c** A brain inoculated with the SA 14-14-2 strain. **d** A control brain. Mag. $\times 99$

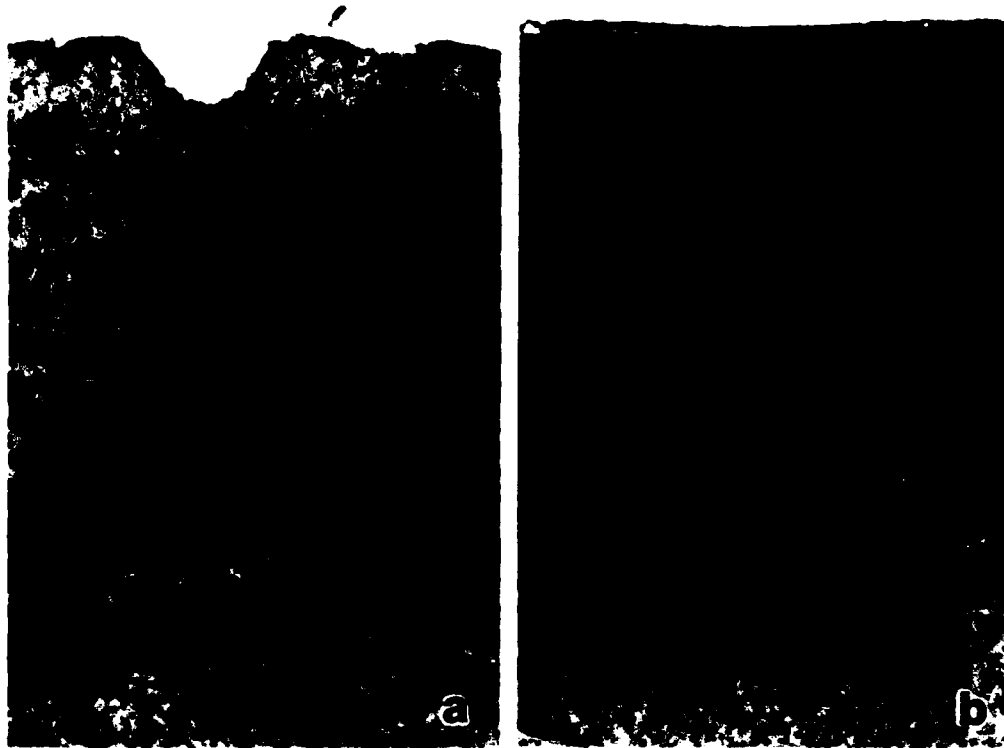


Fig. 4. Immunogold staining of cerebral cortices of mouse brains, 5 days p.i. **a** A brain inoculated with the SA 14 strain. **b** A brain inoculated with the SA 14-14-2 strain. Mag. $\times 75$

SA 14-14-2 strain revealed neurons that contained virions in dilated RER cisternae and Golgi saccules (Fig. 5a). Thus, the immunogold technique served well for locating infected neurons in the mouse brains inoculated with the vaccine strain for electron microscopical examination. Generally, neurons infected with the vaccine strain showed less prominent fragmentation and clumping of RER than neurons infected with the parent strain. Only very few immunogold particles were found around RER in neurons infected with the vaccine strain as well as those infected with the parent strain, indicating that immunoreagents penetrated rather poorly into the cytoplasm in the pre-embedding immunogold technique used in this study (Fig. 5b, c).

Discussion

Upon intracerebral inoculation into adult mice, the SA 14 parent strain of JE virus caused death to the animals within 1 week while the SA 14-14-2 vaccine strain did not cause recognizable morbidity or mortality. The viral titration study showed that the SA 14 strain grew vigorously in the mouse brain, producing a high titer of virus until the time of death of the animals, whereas the SA 14-14-2 strain grew poorly, producing a low titer of virus that disappeared

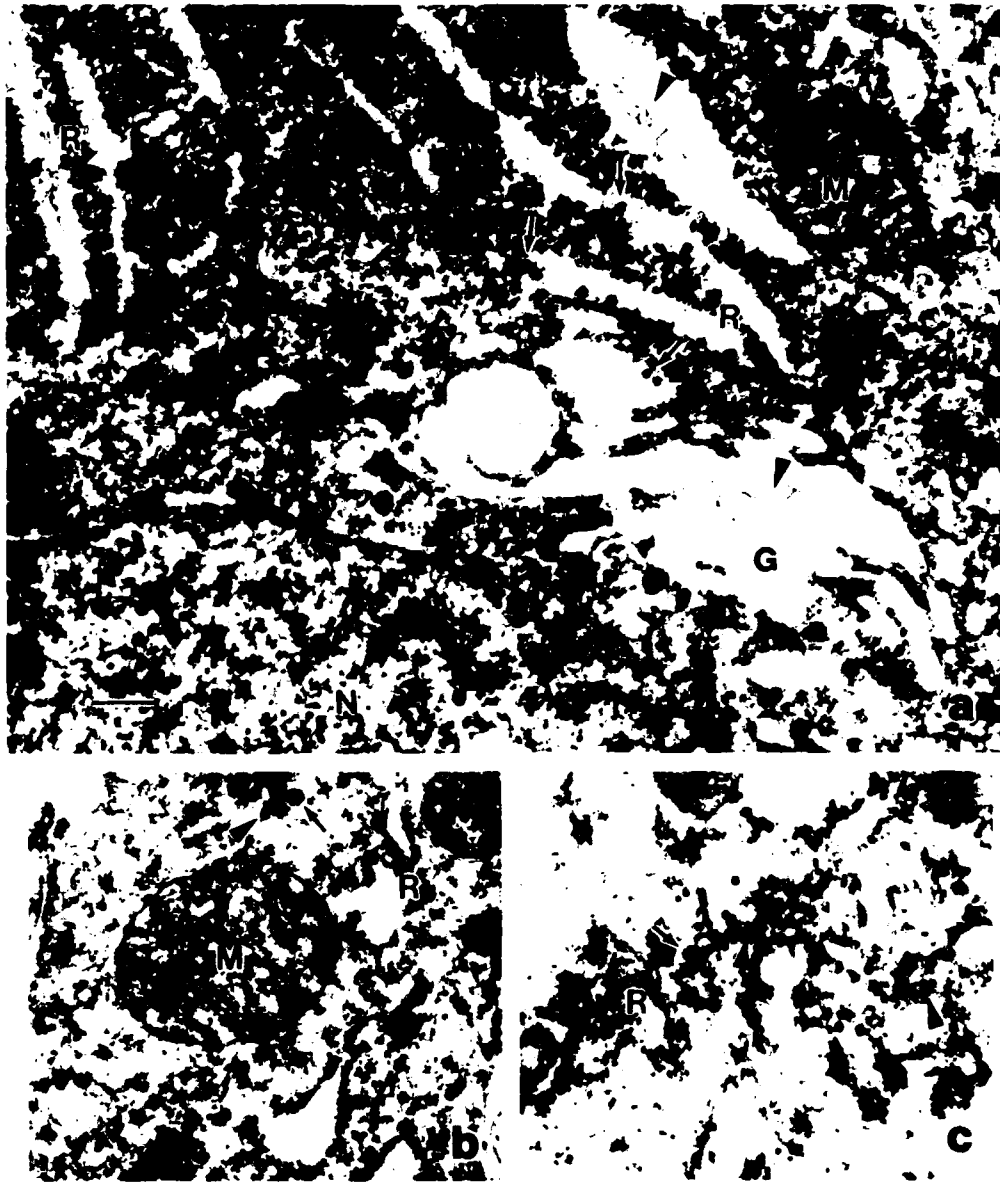


Fig. 5. A neuron in an immunopositive focus of a mouse brain inoculated with the SA 14-14-2 strain, 5 days p.i. Bar: 200 nm. **a** The cytoplasm exhibiting RER (*R*) and the Golgi apparatus (*G*) whose variably dilated cisternae and saccules contain virions (arrow) and filamentous material (arrowhead). An empty arrow points to a coated vesicle containing a virion and coming off from the Golgi apparatus. *M* Mitochondrion; *N* nucleus. **b** and **c** Immunogold particles (arrowhead) scattered around RER (*R*). Arrows point to virions. *M* Mitochondrion

after 10 days. Hall et al. [5] have recently reported that flavivirus antigens are demonstrable in formalin-fixed, paraffin-embedded tissues by the immunohistochemical method. The alkaline phosphatase immunostaining of paraffin-embedded sections and the immunogold staining of vibratome sections demonstrated that a majority of neurons stained positively in the brains inoculated with the SA 14 strain, but only a small number of neurons in scattered foci stained positively in the brains inoculated with the SA 14-14-2 strain. The ultrastructural study demonstrated that the SA 14 strain caused the characteristic cytopathological changes in connection with JE virus replication in a majority of neurons as described elsewhere [10, 11]. On the other hand, the SA 14-14-2 strain caused less prominent cytopathological changes in a small number of neurons that could be located by the immunogold technique. Thus, the results of both the virus titration and morphological observation indicated that, following intracerebral inoculation into mice, the SA 14 parent strain of JE virus replicated vigorously and spread rapidly to a majority of neurons in the mouse brain, while the SA 14-14-2 strain replicated poorly and infected only a small number of neurons in scattered small foci before disappearing.

Since JE virus replicates by trans-type maturation [6, 9], it is believed that the viral growth in host cells occurs in the following sequence: (a) viral entry and uncoating at the cell surface, (b) viral replication at smooth endoplasmic reticulum (SER) and viral translation on RER, (c) viral assembly and maturation in the host-cell secretory channel, and (d) viral release into the extracellular space by secretory-type exocytosis. Therefore, it seems reasonable to believe that poor viral replication results from either partial impairment or complete blockage of one or more of the above processes. In this respect, point-mutations of certain nucleotides in the virus genome that controls viral entry, replication, assembly, and release are thought to be ultimately responsible. Nitayaphan et al. [21] have compared the nucleotide sequences of the genomes of the SA 14 and SA 14-14-2 strains and found 45 nucleotide differences resulting in 15 amino acid substitutions that involved both the structural and non-structural proteins. Among the structural proteins, a single nucleotide change in the C gene altered a single amino acid; a single silent nucleotide change occurred in the prM but did not alter the M protein; and 7 nucleotide changes in the E gene altered 5 amino acids. The rest of the nucleotide changes occurred in the genes encoding the non-structural proteins and in the non-coding regions. The amino acid change in the C protein may affect the viral assembly [21]. The E protein is known to contain biologically active sites and to play an important role in viral entry [1, 14, 15, 18, 22, 23]; presently, this protein is a major target in recombinant vaccine production [16, 19, 24]. Holzman et al. [13] have reported that a single amino acid substitution in the envelope protein E of tick-borne encephalitis virus led to attenuation in a mouse model. The nucleotide changes in the non-structural proteins may also affect the viral RNA replication, viral protein synthesis, and viral assembly. Therefore, the presence of multiple mutation points involving the structural and non-structural proteins between the

genomes of the SA 14 and the SA 14-14-2 strains may cause alterations at multiple viral growth processes between the two strains.

The chemical nature of viral ligands and neuronal receptors involved in JE virus entry into neurons is not well defined at present. Nonetheless, JE virus entry into neurogenic cultured cells seems to be dependent on expression of specific surface receptors by these cells [12]. In this respect, the absence of wide-spread infection among neurons of the brains inoculated with the SA 14-14-2 strain might suggest inefficient entry of virions into neurons. At the same time, since our serial dilution study indicated that intracerebral inoculation of 1 PFU of the SA 14 parent virus was sufficient to kill a mouse (unpubl. data), the low level of infection that occurred in the mouse brains inoculated with the SA 14-14-2 strain suggested that the replication and maturation of the vaccine virus within neurons were impaired in comparison with those of the parent virus. Consequently, the present study seemed to indicate that the difference in pathogenicity between the neurovirulent SA 14 parent strain and the non-neurovirulent SA 14-14-2 vaccine strain of JE virus results from alterations in both viral entry and intracellular growth.

References

1. Cecilia D, Gadakari DA, Kedarnath N, Ghosh SN (1988) Epitope mapping of Japanese encephalitis virus envelope protein using monoclonal antibodies against an Indian strain. *J Gen Virol* 69: 2741-2747
2. Downs MB, White JD (1989) Localization of viral antigens: immunogold labeling and silver enhancement of Vibratome tissue sections. *EMSA Bull* 19: 74-77
3. Eckels KH, Brandt WE, Harrison VR, McCown JM, Russell PK (1976) Isolation of a temperature-sensitive dengue-2 virus under conditions suitable for vaccine development. *Infect Immun* 14: 1221-1227
4. Eckels KH, Yu YX, Dubois DR, Marchette NJ, Trent DW, Johnson AJ (1988) Japanese encephalitis virus live-attenuated vaccine, Chinese strain SA 14-14-2; adaptation to primary canine kidney cell cultures and preparation of a vaccine for human use. *Vaccine* 6: 513-518
5. Hall WC, Crowell TP, Watts DM, Barros VLR, Kruger H, Pinheiro F, Peters CJ (1991) Demonstration of yellow fever and dengue antigens in formalin-fixed paraffin-embedded human liver by immunohistochemical analysis. *Am J Trop Med Hyg* 45: 408-417
6. Hase T, Summers PL, Eckels KH, Baze WB (1987) Maturation process of Japanese encephalitis virus in cultured mosquito cells in vitro and mouse brain cells in vivo. *Arch Virol* 96: 135-151
7. Hase T, Summers PL, Eckels KH (1989) Flavivirus entry into cultured mosquito cells and human peripheral blood monocytes. *Arch Virol* 104: 129-143
8. Hase T, Summers PL, Cohen WH (1989) A comparative study of entry modes into C6/36 cells by Semliki Forest and Japanese encephalitis virus. *Arch Virol* 108: 101-114
9. Hase T, Summers PL, Eckels KH, Putnak JR (1980) Morphogenesis of flaviviruses. In: Harris JR (ed) *Virally infected cells*. Plenum, New York, pp 275-305 (Subcellular biochemistry, vol 15)
10. Hase T, Summers PL, Dubois DR (1990) Ultrastructural changes of mouse brain neurons infected with Japanese encephalitis virus. *Int J Exp Pathol* 71: 493-505

11. Hase T, Dubois DR, Summers PL (1990) Comparative study of mouse brains infected with Japanese encephalitis virus by intracerebral or intraperitoneal inoculation. *Int J Exp Pathol* 71: 857-869
12. Hase T, Summers PL, Ray P (1990) Entry and replication of Japanese encephalitis virus in cultured neurogenic cells. *J Virol Methods* 30: 205-214
13. Holzmann H, Heinz FX, Mandl CW, Guirakhoo F, Kunz C (1990) Single amino acid substitution in envelope protein E of tickborne encephalitis virus leads to attenuation in the mouse model. *J Virol* 64: 5156-5159
14. Kimura-Kuroda J, Yasui K (1983) Topographical analysis of antigenic determinants on envelope glycoprotein V3 (E) of Japanese encephalitis virus, using monoclonal antibodies. *J Virol* 45: 124-132
15. Kimura-Kuroda J, Yasui K (1986) Antigenic comparison of envelope protein E between Japanese encephalitis virus and some other flaviviruses using monoclonal antibodies. *J Gen Virol* 67: 2663-2672
16. Konishi E, Pincus S, Fonseca BAL, Shope RE, Paoletti E, Mason PW (1991) Comparison of protective immunity elicited by recombinant vaccinia viruses that synthesize E or NS1 of Japanese encephalitis virus. *Virology* 185: 401-410
17. Leary K, Blair CD (1980) Sequential events in the morphogenesis of Japanese encephalitis virus. *J Ultrastruct Res* 72: 123-129
18. Mason PW, Dalrymple JM, Gentry MK, McCown JM, Hoke CH, Burke DS, Fournier MJ, Mason TL (1989) Molecular characterization of a neutralizing domain of the Japanese encephalitis virus structural glycoprotein. *J Gen Virol* 70: 2037-2049
19. Mason PW, Pincus S, Fournier MJ, Mason OL, Shope RE, Paoletti E (1991) Japanese encephalitis virus-vaccinia recombinants produce particulate forms of the structural membrane proteins and induce high levels of protection against lethal JEV infection. *Virology* 180: 294-305
20. McDowell EM, Trump BF (1976) Histopathologic fixatives suitable for diagnostic and electron microscopy. *Arch Pathol Lab Med* 100: 405-414
21. Nitayaphan S, Grant JA, Chang GJ, Trent DW (1990) Nucleotide sequence of the virulent SA-14 strain of Japanese encephalitis virus and its attenuated vaccine derivative, SA-14-14-2. *Virology* 177: 541-552
22. Summers PL, Cohen WH, Ruiz MM, Hase T, Eckels KH (1989) Flavivirus can mediate fusion from without in *Aedes albopictus* mosquito cell cultures. *Virus Res* 12: 383-392
23. Takegami T, Miyamoto H, Nakamura H, Yasui K (1982) Biological activities of the structural proteins of Japanese encephalitis virus. *Acta Virol* 26: 312-320
24. Yasuda A, Kimura-Kuroda J, Ogimoto M, Miyamoto M, Sata T, Sato T, Takamura C, Kurata T, Kojima A, Yasui K (1990) Induction of protective immunity in animals vaccinated with recombinant vaccinia viruses that express preM and E glycoproteins of Japanese encephalitis virus. *J Virol* 64: 2788-2795
25. Yu YX, Wu PF, Ao J, Liu LH, Li HM (1981) Selection of a better immunogenic and highly attenuated live vaccine virus strain of Japanese encephalitis. I. Some biological characteristics of SA 14-14-2 mutant. *Chin J Microbiol Immunol* 1: 77-83
26. Yu YX, Zahng GM, Guo YP, Ao J, Li HM (1988) Safety of a live attenuated Japanese encephalitis virus vaccine (SA 14-14-2) for children. *Am J Trop Med Hyg* 39: 214-217

Authors' address: Dr. T. Hase, Department of Ultrastructural Pathology, Division of Pathology, Walter Reed Army Institute of Research, Washington, DC 20307-5100, USA.

Received April 24, 1992