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Anita Desai · S. K. Shankar · V. Ravi · A. Chandramuki
M. Gourie-Devi

Japanese encephalitis virus antigen in the human brain and its topographic distribution

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Abstract This study reports the pathological findings and the distribution of viral antigen in the brains of 13 confirmed and autopsied cases of Japanese encephalitis (JE) in correlation with other virus-specific immunological parameters measured in the cerebrospinal fluid (CSF) antemortem. Japanese encephalitis virus (JEV)-specific antibodies were detected in the CSF of 10 of 13 patients, JEV antigen was detected in the CSF of 7 of 13 and JEV-specific immune complexes were detected in the CSF of 3 of 11 patients. Viral antigen was localised immunocytochemically in the brain tissue of 11 of 13 cases, indicating, that viral antigen could not be cleared from the tissues by the antibody. The topographic distribution of the tissue-associated antigen in the thalamus, hippocampus, substantia nigra and medulla oblongata explain the evolution of post JE sequelae.

Key words Japanese encephalitis virus · Antigen · Brain · Immune response · Cerebrospinal fluid

Introduction

Japanese encephalitis (JE) is one of the common causes of acute viral encephalitis in India. The disease is endemic in Southern India, the target population being children below the age of 15 years [6]. The mortality in this acute encephalitic illness varies from 20% to 40% and generally averages at 30% in various parts of India. Among the survivors neurological morbidity has been noted in over 50% of cases [5]. The histopathological features of JE were initially described in Japanese cases [12, 18]. Later, Mukherjee and Biswas [13] from North Eastern India, Shankar et al. [17] from South India and Johnson et al. [7] from Thailand studied the pathological changes and noted some variations in the degree of involvement and the type of the lesions in the brain. There is, however, very little information of the immunobiology of the viral antigen in the brain and its relation to the presence of virus-specific antibody in the cerebrospinal fluid (CSF). Johnson et al. [7] using an immunocytochemical approach studied the brains of seven children who succumbed to JE in Northern Thailand. They noted greater involvement and presence of viral antigen in the neurons of thalamus and brain stem, and progressive clearance of viral antigen with duration of illness of 6 days or more.

The present study was undertaken to correlate the pathological changes and the distribution of viral antigen in the brains of patients who succumbed to JE, with the presence of JE-specific antibody, antigen and immune complexes in the CSF, to gain a better understanding of the evolution of the encephalitic process.

Materials and methods

Patients

The 13 patients included in this study were among the 129 patients admitted to the neurological services of the National Institute of Mental Health and Neuro Sciences, Bangalore, South India, during the JEV epidemic season (October to December) of two consecutive years, 1990 and 1991. Clinically, all the patients presented with symptoms of acute encephalitis. The aetiology was confirmed by the detection of JEV- specific antibodies and/or viral antigen in the CSF. Amongst the 129 confirmed cases, 88 patients recovered completely (48/88) or had variable neurological sequelae (40/88), while 41 patients succumbed to the illness. Consent for post-mortem study of the brain was obtained in 13 cases, and was performed within 12 h of death.

A. Desai · V. Ravi (✉) · A. Chandramuki
Department of Neurovirology,
National Institute of Mental Health and Neuro Sciences,
Bangalore 560029, India
Tel.: 91-080-642121, ext. 321; Fax 91-080-6631830

S. K. Shankar
Department of Neuropathology,
National Institute of Mental Health and Neuro Sciences,
Bangalore 560029, India

M. Gourie-Devi
Department of Neurology,
National Institute of Mental Health and Neuro Sciences,
Bangalore 560029, India

Detection of virus specific antibodies

CSF samples were tested for the presence of virus-specific IgM (MAC) and IgG (GAC) antibodies using a capture ELISA described by Burke et al. [1] with minor modifications [14]. The results of MAC and GAC ELISA were expressed as units [1]. A CSF sample containing > 100 IgM units was considered positive and diagnostic of recent JEV infection. A sample with > 10 GAC ELISA units was considered positive for JEV-specific IgG antibodies. Neutralising antibodies to JEV in the CSF were detected using a microneutralisation test [2].

Detection of JEV antigen

Soluble antigen in the CSF was detected by the reverse passive haemagglutination assay (RPHA) [15], while the presence of cell-associated JEV antigen in the CSF mononuclear cells was detected by an immunofluorescence method [3, 11].

Detection of virus-specific immune complexes

JEV-specific immune complexes in the CSF were detected using a monoclonal antibody-based ELISA. Flavivirus-cross-reactive clones (mAb 109, 203, 204 and 301) and JEV-specific (clone 112) monoclonal antibodies [9] were diluted to 10 μg/ml and the mixture containing equal amounts of monoclonal antibody was coated onto ELISA plates (100 μl/well) for 16 h at 4°C. After blocking the non-specific binding sites with 3% defatted milk powder in PBS, 100 μl of CSF (1 : 10 dilution) was added to the plates, in duplicate, and allowed to react for 2 h at room temperature. The plates were washed and reacted with goat anti-human immunoglobulins conjugated to peroxidase (Sigma, USA) for 1 h at room temperature. After washing three times, the colour was developed using orthophenyline diamine dihydrochloride (Sigma) and H_2O_2 as the substrate. The absorbance (A) of the reaction product was read in an ELISA reader (Dynatech MR 700). The CSF sample with an A value greater than the cut-off (mean + 3 SD), established from control CSF samples obtained from 40 individuals undergoing spinal anaesthesia for minor surgical problems with no obvious signs or symptoms of neurological illness, was considered positive for immune complexes. This procedure was used only as a qualitative assay to detect the presence/absence of JEV immune complexes in the CSF.

Pathological study of brains

Post-mortem examination of the brains was performed within 12 h after death. Representative sections from different areas of the formalin-fixed brain were processed for histological study. The sections were stained with haematoxylin-eosin and luxol-fast blue for myelin.

Immunoperoxidase stain

Paraffin sections (6-8 μm thick) were collected on polylysine-coated slides, from hippocampus, thalamus, temporal cortex, midbrain at the level of superior colliculus, medulla oblongata and cerebellum with dentate nucleus. Based on the earlier pathological studies carried out at our Institute during the 1979 epidemic [17], these six areas of the brain were frequently found to show the pathological lesions of JE and, hence, were selected for tissue localization of the viral antigen by immunohistochemistry. Paraffin sections of normal brain collected at autopsy from two children (6 and 14 years) who had died of road traffic accidents, and not known to have any infective (including viral) or neurological illness, were used as negative tissue controls. The endogenous peroxidase in the tissue section was quenched with 0.3% H_2O_2 in methanol and the non-specific immune binding sites were blocked with 3% solution of defatted milk powder in PBS. Ammonium sulphate-precipitated human convalescent anti-JEV immunoglobulin was the source of primary antibody. The specificity of this antibody was confirmed by comparing with a reference JEV-specific hyperimmune mouse ascitic fluid (obtained from the National Institute of Virology, Pune, India). The sections were incubated in primary antibody (1 : 100), at 4°C overnight. After three 5-min rinses in PBS, the sections were incubated for 1 h in biotinylated goat anti-human IgG, diluted 1 : 1000 (Vector laboratories, USA). After further washing, the sections were incubated in freshly prepared, 1 : 1000 dilution of streptavidin-peroxidase complex (Dakopats, USA) for 1 h. All the dilutions of the primary and secondary antibody and streptavidin were made in 50 mM PBS containing 0.05% Tween-20 (PBS-T) and 1% defatted milk powder. After washing with PBS-T the immune reaction was visualized by reacting the sections with diaminobenzidine tetrahydrochloride (50 mg/100 ml in TRIS-HCl buffer, pH 7.5) and H_2O_2 (10 μl/10 ml). The sections were counterstained with Mayer's haematoxylin. Some of the sections were simultaneously stained with the reference antibody, mouse JEV-specific hyperimmune ascitic fluid, at 1 : 100 dilution. In addition, as a negative control, brain sections from the JE cases were also processed identically, omitting the primary antibody and incubating with an unrelated antibody. The density distribution of labelled cells in the sections from different areas of the brain were graded semiquantitatively into four groups.

Results

The 13 patients in this study were in the age group of 4–57 years (mean 16.8 years). The duration of the clinical illness at the time of hospital admission and initial CSF sampling varied from 6–12 days in 11 cases, this information was not available for 2 cases (Table 1). The clinical features were essentially stereotyped, with fever, headache as the initial symptom, followed by seizures and features of cerebral oedema. The level of consciousness declined progressively in 3 patients (cases 5, 8 and 13) and rapidly in 7 (cases 3, 4, 6, 7, 9, 11 and 12) with ultimate respiratory arrest. Death occurred between 8–19 days after the onset of initial clinical symptoms and within hours to 14 days after the hospital admission. One confirmed case of JE (case 10, Table 1) developed bacterial meningitis during the stay in the hospital and succumbed to it after 60 days.

The JEV-specific IgM antibodies of various levels were detected in 10 cases, while IgG was found only in 3 instances (Table 1). The virus-specific neutralising antibodies were present in the CSF in 9 cases, all of them also having IgM antibody. The free or cell-bound JEV antigen was found either by RPHA or immunofluorescence in 7 cases. In cases 3 and 8, the soluble antigen could be demonstrated in CSF by RPHA, while viral-specific IgM or neutralising antibodies were not detectable. JEV-specific immune complexes were detected in cases 2, 10 and 12, in addition to the antibodies and/or circulating free antigen. The viral antigen could be demonstrated immunocytochemically in 11 cases (Table 1). In 2 instances (cases 2 and 10), where tissue-bound antigen could not be detected in the brain immunocytochemically, free antigen and immune complexes could be found in CSF.

Table 1 Correlation of immunological findings in the CSF and JEV antigen localization in the brain (*CSF* cerebrospinal fluid, *JEV* Japanese encephalitis virus, *Ag* antigen, *ND* not done, *NK* not known, *JEV-IC* JEV immune complexes, *JEV-IgM* IgM antibodies to JEV, *JEV-IgG* IgG antibodies to JEV, *Nt AB* neutralising antibodies, + presence of parameter tested, – absence of parameter tested)

Case no.	Day of sampling	Immunological findings in the CSF					JEV-AG in brain	Days of illness
		JEV-IgM (ELISA units)	JEV-IgG (ELISA units)	Nt-AB (titres)	JEV-AG	JEV-IC		
1	6	171	–	20	+	–	+	8
2	NK	4	–	–	+	+	–	NK
3	6	10	–	–	+	ND	+	6
4	8	595	–	40	–	–	+	8
5	12	100	ND	10	–	ND	+	12
6	4	789	ND	40	–	–	+	4
7	3	359	422	–	–	–	+	6
8	3	10	–	–	+	–	+	11
9	3	190	–	10	–	–	+	6
10	NK	779	1380	320	+	+	–	60[a]
11	4	906	15	40	–	–	+	6
12	3	607	ND	40	+	+	+	5
13	5	748	ND	10	+	–	+	19

[a] The exact date of onset of illness in this patient was not known. However, he stayed in the hospital for more than 2 months before he succumbed to the infection

Table 2 Regional distribution of JEV antigen in the human brain ($n = 13$) + 1–5 cells positive for JEV antigen, ++ 5–10 immunolabelled cells, +++ 10–20 immunolabelled cells, ++++ more than 20 cells / high power field positive for JEV antigen

Case no.	Hippocampus	Temporal cortex	Thalamus	Midbrain	Medulla	Cerebellum
1	++++	++++	++++	++++	++++	+/–
2	–	–	–	–	–	–
3	–	–	–	–	+	+[a]
4	++++	++	++	++	++++	++++
5	++	++	–	–	–	–
6	+++	+++	++	–	–	–
7	++	++	+	–	–	–
8	+	+	++	+	–	++++[a]
9	+	+	+	NA	NA	NA
10	–	–	–	–	–	–
11	++++	++++	++++	++++	++	+++
12	++++	++++	++	++++	++	+++
13	–	–	–	–	+++	–
	9	9	8	5	6	6

[a] In these two cases antigen was localized only in the granule cells of the cerebellum and Purkinje cells in the remaining

Pathological findings in the brain

Grossly, the brains revealed congestion and variable degree of oedema with cerebellar tonsillar and hippocampal uncal herniation. Serial coronal slicing of the brain showed features of oedema with narrowing of ventricles and grey matter congestion. In 5 of the 13 (38.4%) cases (nos. 3, 9, 10, 12 and 13, Table 1) multiple intraparenchymal cysticercal cysts associated with a variable degree of host reactions were also observed. One patient (case 10), during the course of hospital stay, developed pneumococcal meningitis and succumbed to it. The brain, in this case revealed haziness of meninges with purulent exudate along the superolateral surface. Histological examination revealed variable meningeal inflammation, perivascular lymphocytic cuffing and microglial proliferation, both diffuse and focal, forming gliomesenchymal nodules. The focal gliomesenchymal nodules had discrete aggregates of microglial cells and lymphocytes around degenerating or dead neurons. The gliomesenchymal nodules were seen fairly consistently in the hippocampus, temporal cortex, thalamic nuclei, inferior olivary complex in medulla oblongata, substantia nigra in midbrain, reticular nuclei along the midline of brain stem and dentate nucleus and the molecular layer in the cerebellum. In an occasional case, the microglial response was also seen in the white matter, with focal demyelination. Relatively acellular, round-to-oval necrolytic zones, another characteristic pathological lesion in JE, were observed rather infrequently. These necrolytic lesions were observed in thalamus, hippocampus, cerebral cortex and the molecular layer of the cerebellum. Astroglial reaction was conspicuously absent. One of the patients (case 10) survived for 60 days after the initial onset of illness. In this case, the brain revealed only sparce and diffuse microglial response in medulla oblongata and cerebellum, in addition to characteristic features of acute pyogenic meningitis.

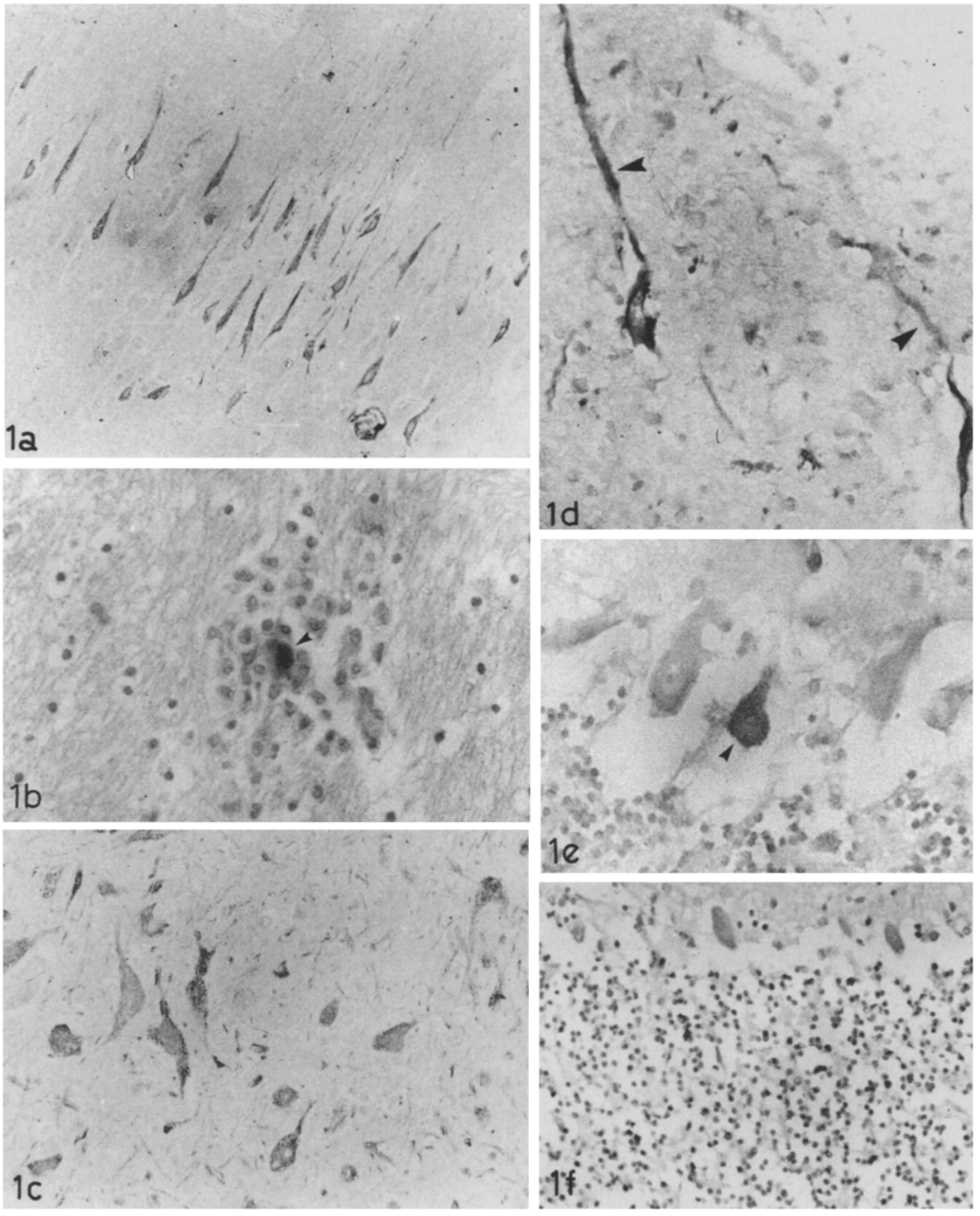

Fig. 1a Hippocampal pyramidal neurons in CA1 area showing the intracytoplasmic Japanese encephalitis virus antigen; × 60. **b** A microglial nodule around an antigen-bearing degenerating neuron in the thalamus. The microglial cells are devoid of the viral antigen; × 300. **c** Neurons of the substantia nigra in mid brain containing large amounts of viral antigen. They show spread of antigen along the dendrites and the axons in the neuropil; × 240. **d** Large neurons of the hypoglossal nucleus in the medulla oblangata shows spread of JEV antigen along the axon *(arrow)* from the cell soma; × 240 . **e** Purkinje cell in the cerebellum staining positive for JEV antigen *(arrow)*. Note the absence of antigen in the adjoining Purkinje cells and the small granule cells, × 320. **f** Small neurons of the granule cell layer in cerebellum showing the presence of viral antigen in the cytoplasm. The Purkinje cells are not labelled; × 200

Immunocytochemistry studies (Table 2)

JE virus-specific antigen could be localised in the neurons of the affected areas. The hippocampus, the adjoining temporal cortex and the thalamus often showed a high density of antigen-bearing neurons, followed by midbrain, medulla oblongata and cerebellum. In the hippocampus, large pyramidal neurons of the Ammon's horn (Fig. 1a) and in subiculum and entorhinal cortex, the superficial layers of the neurons contained the viral antigen, while in the temporal cortex, the antigen-positive neurons were found in layers 4–6. Many of these antigen-bearing neurons were well preserved, with a distinct nucleus and nucleolus, and a microglial reaction in their vicinity was conspicuously absent. An occasional immunolabelled degenerating neuron was surrounded by a microglial aggregate, but these microglial cells were devoid of the antigen (Fig. 1b). In midbrain, the neurons of substantia nigra (Fig. 1c) and the oculomotor nucleus showed a large amount of viral antigen. In medulla oblongata, the neurons of inferior olivary nucleus, hypoglossal and vagal nuclei had intense immunolabelling, while the neurons of spinal tract of trigeminal and vestibulococclear nucleus were lightly stained. The spread of the antigen along the axons and dendrites was conspicuous in the neurons of the vagus, hypoglossal (Fig. 1d) nuclei. In the cerebellum, the Purkinje cells (Fig. 1e) and small granule cells (Fig. 1f) were immunolabelled for the viral antigen in three cases, in the absence of microglial reaction or neuronophagia. On the other hand, microglial reaction and neuronophagia were prominent in the cerebellar nuclei, followed by inferior olivary nucleus; many of the microglia being positive for the phagocytosed viral antigen. The endothelial cells lining some of the small blood vessels in the thalamus and brain stem contained the viral antigen. The ependyma lining the ventricles were immunolabelled in four cases and the subependymal astrocytes in two. However, the viral antigen could not be detected in the astrocytes and oligodendroglia of the grey and white matter and in the leptomeninges.

Discussion

The clinical and the immunological parameters, like the presence of JEV-specific IgM antibody and antigen in CSF, in the patients under study established the diagnosis of JE. The histological features noted in the brain during the course of illness were essentially similar to the earlier descriptions [7, 12, 17]. However, some of the pathological findings of this study are in variance to those reported by Johnson et al. [7], but similar to those reported by Kimoto et al. [8] based on autopsy/biopsy studies of JE cases. The study from Thailand by Johnson et al. [7] included cases who succumbed to the illness by 3–9 days after the onset, thus representing the acute-phase pathological response. The present study had patients surviving 4–60 days, and Kimoto et al. [8] had patients who succumbed to the illness beyond 23 days (23–151 days of illness), thus representing the subacute and chronic phases of the encephalitic process. Of the 13 patients in this study 10 had IgM class of antibody in CSF, including the patient who survived for 60 days after the diagnosis, suggesting a recent and persisting viral infection. JE-specific neutralising antibodies were present in 9 cases and in 4 of these, viral antigen was also detected in the CSF. JE-specific immune complexes were found in 3 of these cases (cases 2, 10 and 12). The presence of the antigen in CSF, along with relatively high titres of IgM and neutralising antibodies, indicates that these immunoglobulins are not capable of offering protection in the presence of an overwhelming antigen load. The patient who survived for more than 60 days (case 10) had high titres of antibody, antigen and immune complexes, although apparently he succumbed to bacterial meningitis. In addition, he also had neurocysticercosis. In this study, 38.4% (5 out of 13) patients had intracerebral cysticercal cysts, with a variable degree of the host reaction. In the earlier autopsy study carried out at this centre during the 1979 JE epidemic [17], association with neurocysticercosis was also observed in 32.3% (11 of 34) cases. In both instances with the co-existence of neurocysticercosis, the JE-related pathological lesions were found to be more diffuse and florid. Hence, this was considered to be a factor influencing the clinical outcome. Similar observations were made by the Chinese workers [10]. In this study, the viral antigen could be immunocytochemically localised in 11 of 13 confirmed cases of JE. Similar to the findings of Johnson et al. [7], the viral antigen was found in thalamus (8 of 11 cases) and midbrain (5 of 11 cases). In addition, a significant degree of antigen was localised in hippocampus and the temporal cortex. Curiously, in these areas, the topographic distribution of the viral antigen-bearing neurons correspond to the zones involved in some of the neurodegenerative diseases like Alzheimers's disease, in which a cholinergic neuronal defect is implicated in pathogenesis. It will be of great interest to investigate the cases of dementia in this geographic locale, to look for the contribution of JE-related pathological lesions producing cognitive deficits as the long-term sequelae. The infection and destruction of the reticular and other neurons in the brain stem and the neurons in thalamus account for the deep coma and respiratory failure. The involvement of striatum and the substantia nigra is consistent with frequent tremors seen during the acute phase, and parkinsonian features as the long-term sequelae [5, 6]. The density distribution of the antigen-bearing neurons and the pathological lesions were at variance with the degree of inflammatory reaction and did not show a temporal correlation in the evolution. The antigen continued to be located in the neurons even beyond the 10th day of illness, contrary to the observations of Johnson et al. [7]. During the 1965–67 epidemic of JE in Japan, Kimoto et al. [8] could localise the viral antigen in pons by immunofluorescence, even at 151 days after the onset of illness. This feature highlights the fact that the virus could persist in tissues for long periods. The phenomenon of JE viral persistence in the human nervous system

has been reported earlier from this laboratory [16]. This contention is further supported by the presence of many antigen-bearing, yet well-preserved neurons, with no associated microglial reaction and neuronophagia at different sites of the brain, including cerebellum. In this study, many antigen-containing Purkinje cells and granule cells without microglial response have been observed. The involvement of the cranial nerve nuclei and the reticular neurons in the medulla oblongata was rather frequent, many of them having viral antigen in the soma. Johnson et al. [7] in their study noted an absence of viral antigen in cerebellar Purkinje cells and a rare occurrence in the neurons of medulla oblongata. The presence of the viral antigen both in the long dendritic arborisation and the axons, as observed in the substantia nigra, cerebellum and medulla oblongata, suggest a transcellular spread of virus to distant but functionally related neurons. The astroglia appear to occasionally ingest the virus by phagocytosis and, thus have a limited role in the spread of the disease. This intraneuronal antigen localisation and the absence of neuropil staining at different times, suggests that an extracellular spread of JE virus to distant areas may not occur. The intracellular localisation of the viral antigen probably prevents access to the viral-specific antibodies, and, thus, may not be effective in neutralising the virus to offer protection. This idea is supported by the finding of high titre of IgM and neutralising antibodies in CSF, and the large number of antigen-bearing neurons in many areas of the brain, even in fatal cases. The presence of viral antigen and the JEV-specific immune complexes in CSF, and failure to find antigen in tissue immunocytochemically in two cases in the present study, could be a sampling error. The viral clearance could be taking place only following neuronal death and neuronophagia by the macrophage system and, thus, sensitizing the immune system. The presence of small amounts of the viral antigen in occasional vascular endothelial cells suggests phagocytosis and accessary function of antigen presentation similar to microglia. The involvement of the critical neuronal targets and the resultant secondary effect, rather than the failure of the viral-specific immunity, appear to determine the clinical outcome. Recent studies from our laboratory have shown a positive correlation between the presence of autoantibodies to host neurofilaments and mortality [4].

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