

## Specific tropism of Japanese encephalitis virus for developing neurons in primary rat brain culture

### Brief Report

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**Summary.** Among all the neural cells in fetal rat brain culture developing neurons showed the highest rate of infection by Japanese encephalitis virus (JEV). JEV specifically bound to these cells as measured by immuno-staining. These results indicate that developing neurons are the major target of JEV, and that the initial specific binding of virus to these cells may be one of the reasons for the neurotropism of JEV.

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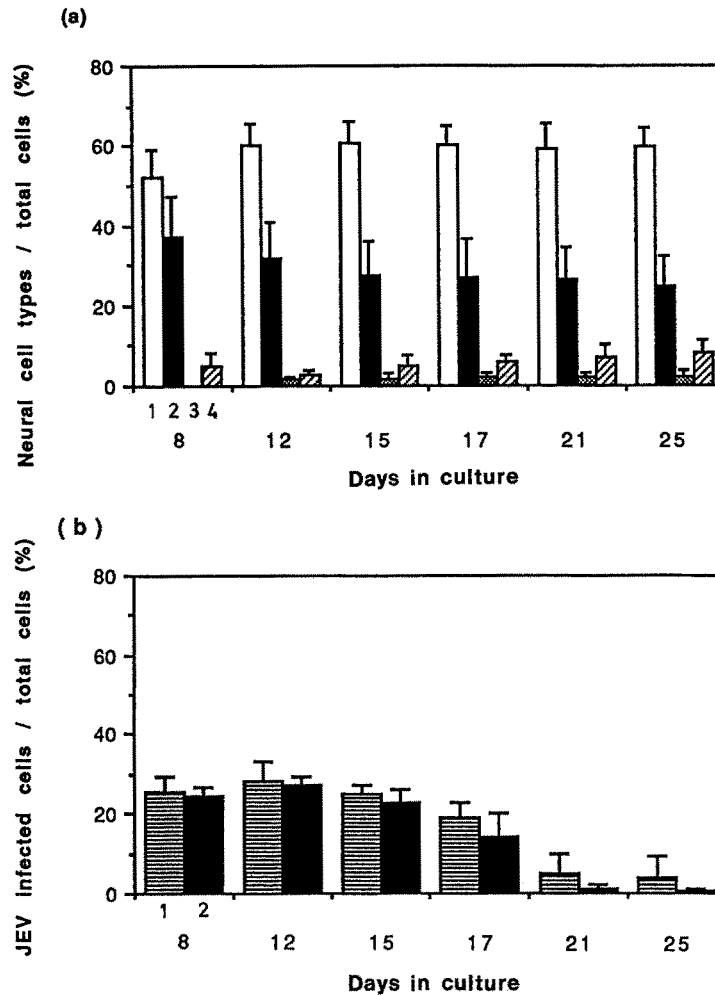
Japanese encephalitis virus (JEV) is a prototypic member of the family *Flaviviridae*; the virus causes severe, even lethal encephalitis in humans. Pathological studies of Japanese encephalitis have shown that the virus specifically infects neurons in the brain [3, 5]. However, this neurotropism has not been well characterized. Previously, we have shown that JEV specifically infects developing neurons in the suckling rat [12]. Interactions between host and virus influencing the outcome of infection are dynamic and complex. Usually, neural cell lines, such as neuroblastoma or glioma cell lines, have been used for studying the neurotropism of JEV [2] and other viruses [17]. The characteristics of original neural cells can, in general, be very different from those of cell lines, especially in the case of neurons. Recently it has been reported that primary brain culture are useful for studying the neurotropism of some viruses, for example, coronaviruses [13] and human immunodeficiency virus [10, 16]. In order to investigate the neurotropism of JEV, we adapted a procedure for the

culture of neural cells from rat fetus brain. Here we demonstrate that developing neurons in primary brain culture can be specifically infected with JEV.

Primary cultures of neural cells were prepared by a modification of the method of Muramoto et al. [11]. Cerebral cortices were dissected from 14 day embryonic rats. After digestion of the tissue with 0.15 unit/ml papain in 0.1 M phosphate buffered saline (pH 7.4) (PBS) for 15 min at 37°C, the dissociated cells were plated on poly-L-lysine coated chamber slides (8-well, NUNC) at a density of  $1-2 \times 10^5$  cells/well. Cultures were maintained by changing half the medium 2 times per week. We used Eagle's MEM containing 5% fetal bovine serum (FBS), 5% horse serum, 0.5 µg/ml insulin and 0.2% (w/v) glucose. For study, cultured cells were fixed with 4% paraformaldehyde in PBS. As markers to identify each neural cell, anti-microtubule-associated protein 2 monoclonal antibody (MAb) (MAP2; Amersham) and anti-neurofilament antibody (Ab) (NF; InRo Biomedtek) were used for neurons; antiglial fibrillary acidic protein Ab (GFAP; Dako) for astrocytes; anti-galactocerebroside Ab (GC; Chemicon) for oligodendrocytes; anti-rat monocyte/macrophage MAb (ED 1; Serotec) for macrophages/microglia; and anti-fibronectin Ab (Chemicon) for fibroblasts [14, 15]. After treating cultures with the first MAb or rabbit polyclonal Ab, they were treated with biotinylated sheep anti-mouse IgG or donkey anti-rabbit IgG and then with streptoavidin-conjugated to Texas Red (Amersham). Stained cultures were observed with a fluorescence microscope (Zeiss Axioplan). The average number of cells per culture chamber ranged from  $1.5$  to  $2.0 \times 10^5$  cells.

Neural cells developed dramatically over the first week. Significantly, type 1-like astrocytes, which were GFAP-positive and fibroblast-like in form, grew up and covered almost all the bottom of the chambers. At 8–25 days in culture, 52–60% of the cells in cultures were astrocytes (Fig. 1 a). On these cultures, MAP2-positive cells (25–37%) showed characteristic features of neurons; they had extending cell processes and increasing nuclear size (Fig. 2 a, b, d, and e). It has been reported that the differentiation of neuronal cells in primary brain culture closely resembles that in intact rat brain: morphological characteristics, synapse formation and electrophysiology resemble intact brain for at least the first 7 weeks of culture [4, 11]. We confirmed neuronal maturation in these cultures (data not shown). After 2 weeks in culture, GC-positive oligodendrocytes, which were not detected for about 8 days in culture, developed gradually to comprise 2% of all cells (Fig. 1 a). The cultures usually contained 2–8% ED 1-positive microglia or macrophages. Sometimes 1–2% fibroblasts (stained specifically with anti-fibronectin) contaminated cultures. These brain cultures also contained a small population of other neural cells not identified with specific markers.

After identification, the susceptibility of neural cells to JEV was determined. After 8 to 25 days in culture, cells were infected with JEV (JaGAr-01 strain) at a multiplicity of infection (m.o.i.) of 1. Two days after infection, cells were fixed and stained with rabbit or mouse anti-JEV serum followed by FITC-labelled anti-rabbit IgG or anti-mouse IgG (Amersham). Similar results were

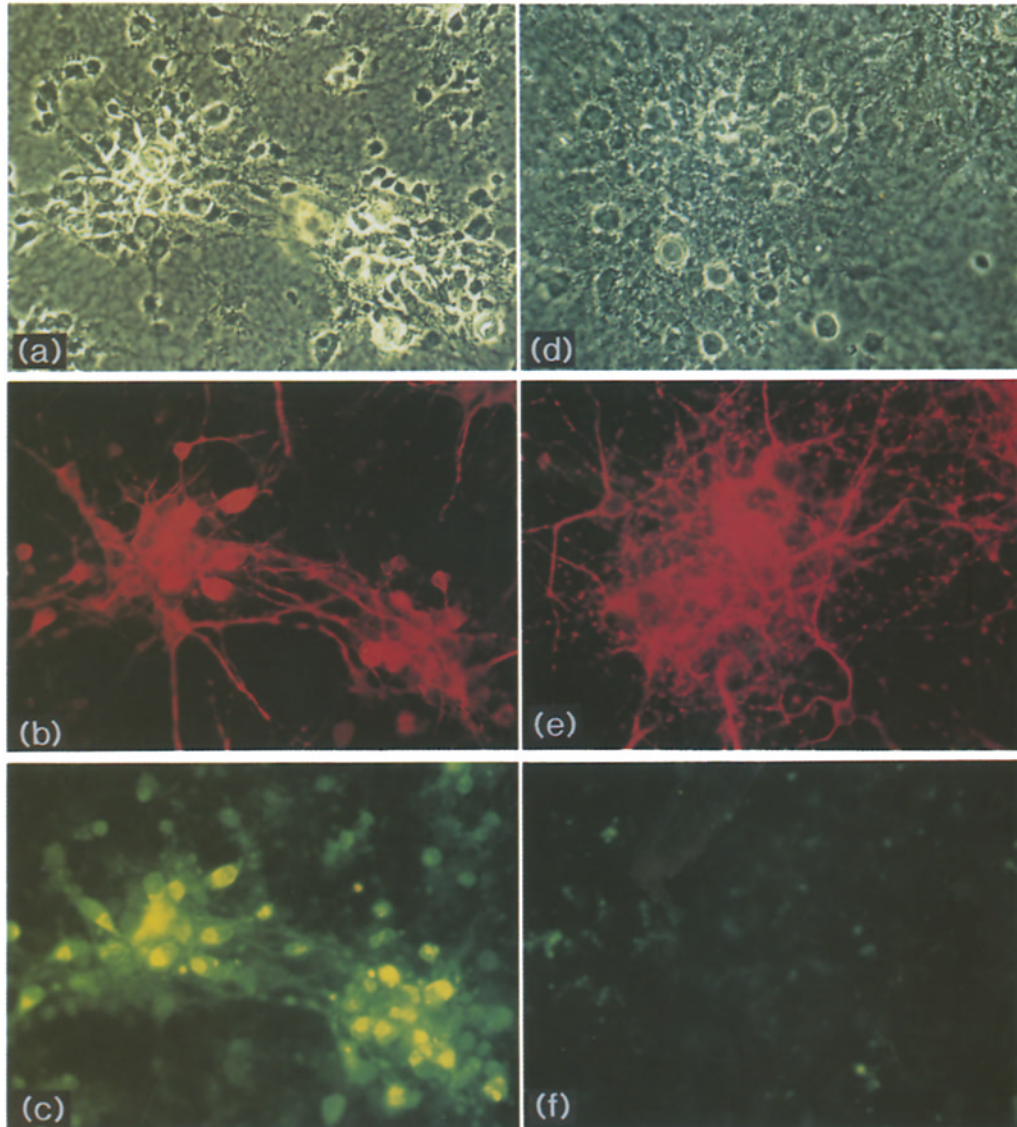


**Fig. 1.** **a** Percentage of cell types in 8–25 day brain culture: astrocytes (1), neurons (2), oligodendrocytes (3), microglia/macrophage (4). **b** Percentage of JEV-infected total neural cells (1), and JEV-infected neurons (2) at 8–25 in culture. Cells stained with specific marker and/or anti-JEV were counted in 10 fields at predetermined stage coordinates. The total area counted was 20 mm<sup>2</sup>. Each value is the mean of two wells from three separate experiments. Error bars indicate the standard deviation

obtained when cultures were fixed three days after infection, but staining was less distinct because of cytopathic effects. Rabbit anti-JEV serum was prepared from rabbits immunized by two intravenous inoculations of 10<sup>9</sup> PFU of JEV (JaGAR-01 strain). Mouse anti-JEV serum was prepared from BALB/C mice (8 weeks old) which had been given two to three intraperitoneal injections of formalin inactivated JEV vaccine (JaGAR-01 strain, Chiba-Kessei).

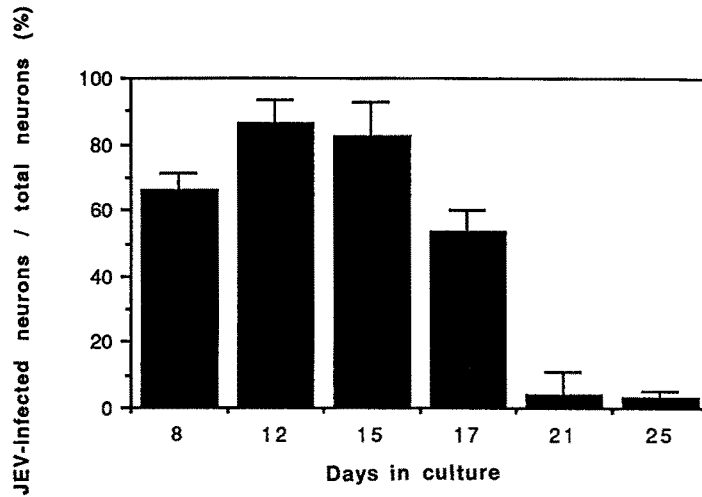
Cells were double-stained with neural cell specific antibodies, as described above, as well as with anti-JEV antibodies.

As shown in Fig. 1 b, the percentage of JEV-positive cells was 25% in 8 day

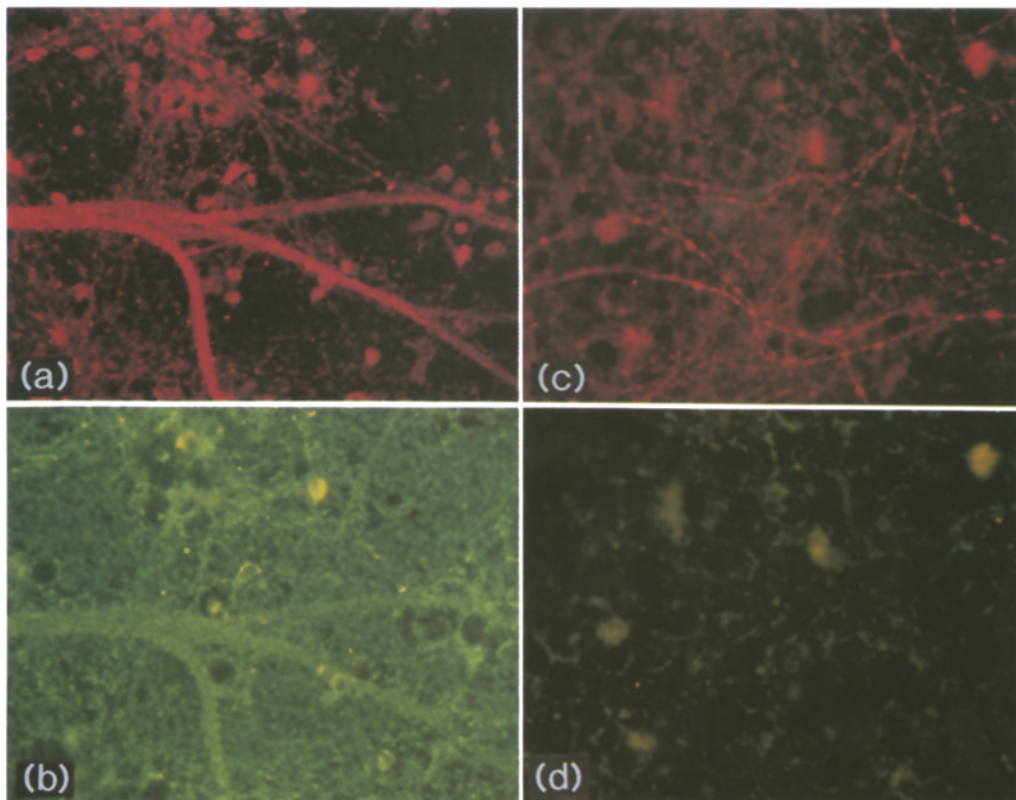


**Fig. 2.** JEV infection of primary rat brain culture. At 12 (**a–c**) and 25 (**d–f**) days in culture, brain cells were infected with JEV. After 2 days, cells were fixed and stained immunocytochemically. **a** and **d** Phase contrast; **b** and **e** anti-MAP2; **c** and **f** anti-JEV

cultures, 28% in 12 day cultures, and then decreased gradually in 17 day cultures. From 8 to 15 days in culture, most JEV-susceptible cells were MAP2-positive neurons (90–96%, Figs. 1 b and 2 b, c). A small number of astrocytes and other neural cells were also infected with JEV. To eliminate the possibility that JEV could not access the underlayer of astrocytes, primary brain cultures were passaged at a lower cell density ( $10^4$  cells/well) and examined for JEV-infection: results were similar. After 15 days in culture, JEV-positive cells were dramatically decreased in number (Fig. 1 b), whereas the decrease in numbers of neurons



**Fig. 3.** JEV-infected neurons per total neurons. Double stained cells with anti-JEV and anti-MAP2 were counted as described for Fig. 2. The percentage of double positive cells per MAP 2-positive neurons was calculated. Each value is the mean of 2–3 wells from three separate experiments. Error bars indicate the standard deviation



**Fig. 4.** Strong binding of JEV to NF-positive neurons at 12 days in culture (**a** and **b**) and low binding to other neural cells at 25 days in culture (**c** and **d**). After cultures had been maintained an appropriate time, JEV was added to the brain cells at a high m.o.i. and stained immunocytochemically. **a** and **c** Anti-NF; **b** and **d** JEV and anti-JEV

was not as great (Fig. 1 a). Figure 3 shows that more than 66% of MAP2-positive neurons were stained specifically with anti-JEV antibody after 8 to 15 days in culture. These cells had the characteristic morphology of immature neurons. We could not observe morphological differences between JEV-infected and non-infected neurons. By examining uptake of bromo-deoxyuridine (data not shown) we confirmed that neuronal mitogenesis had already finished at 8 days in culture. This indicated that JEV-susceptibility is not related to cell proliferation. After 21 days in culture, most neurons, which were electrophysiologically active (data not shown) and morphologically mature, were not infected (Fig. 2 e and f). A low percentage of some other cells were infected at this time (Fig. 1 b). At this stage, we could not determine the cell types of most JEV-infected cells by specific markers, but from their morphology they appeared to be glial progenitor cells. Fibronectin-positive fibroblasts, which were sometimes present due to contaminating pia mater, were not infected with JEV. A small population of GC-positive oligodendrocytes and GFAP-positive astrocytes were infected with JEV after 17 days in culture. These results confirm that JEV specifically infects immature developing neurons in rat primary brain culture, just as is the case in vivo [12].

To investigate the basis of the neurotropism of JEV, virus binding to neural cells was examined. After cultures had been maintained for an appropriate time, a high concentration of JEV (m.o.i. 500) (in PBS containing 3% FBS and 0.1% sodium azide) was added. After incubation for 1 h at 4 °C, cells were treated with mouse anti-JEV antibody, and stained immunocytochemically as described above. To identify the cell types [6], the JEV-bound cells were treated with acetone at 0 °C for 2 min, and then stained with specific antibodies. As shown in Fig. 4 a and b, JEV bound strongly to NF-positive neurons, especially neuronal processes of cells at 8–15 days in culture. Because these neuronal processes stained with anti-NF (dendrite, axon-positive) but not with anti-MAP2 (dendrite-positive, axon-negative) [1], they were suspected of being axons (data not shown). Some binding to other neural cells, mainly astrocytes, was found, but this was minimal. To determine the specificity of binding, 10<sup>8</sup> PFU of JEV was pretreated with 100 µg of purified anti-E MAbs [7–9]; binding of virus to cells was specifically inhibited by some anti-E MAbs, such as 301; hemagglutination inhibition (HI) MAb, such as 503; neutralizing (N) MAb, but not by other anti-E MAbs, such as 204 and 504; and other non-HI and non-N MAbs. After 25 days in culture (Fig. 4 c and d), we observed little or no JEV-binding to NF-positive neurons and their processes, and only little binding to other neural cells.

These observations indicate that JEV-binding to neurons is specific and important for infection, and that one of the reasons for the neurotropism of JEV may be the initial specific binding of virus to the cell surface. Specific molecules to which JEV binds strongly might be expressed on the surface of neurons only at certain stages of development. Further investigation is required

to understand the initial interaction between JEV and its host cells. Rat brain culture should be a useful tool for studying the neurotropism of JEV.

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