# GROWTH AND CYTOPATHIC EFFECT OF JAPANESE ENCEPHALITIS VIRUS IN ASTROCYTE-ENRICHED CELL CULTURES FROM NEONATAL MOUSE BRAINS

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**Summary.** – Neuron-free, astrocyte-enriched brain cell cultures from newborn mice could be infected with Japanese encephalitis virus (JEV) as evidenced by immunofluorescence (IF), viral replication and cytopathic effect (CPE). Virus-specific cytoplasmic fluorescence was detectable in astrocytes first after 18 hrs, released infectious progeny virus in the culture fluid after 24 hrs, and CPE after 11 days post infection (p.i.).

**Key words:** Japanese encephalitis virus; brain cell cultures; astrocytes; virus replication; cytopathic effect; immunofluorescence

## Introduction

Japanese encephalitis (JE) is a major public health problem in India. JEV produces encephalitis and death predominantly in the pediatric age group (Webb and Pereira, 1956; Carey *et al.*, 1969; Banerjee, 1975). It preferentially attacks neurons of the central nervous system (Miyake, 1964; Johnson *et al.*, 1985; Mukherji and Biswas, 1976; Ogata *et al.*, 1991). Infection of neurons in cortical areas and Purkinje cells is followed by their damage and loss (Miyake, 1964; Johnson *et al.*, 1985; Mukherji and Biswas, 1976; K. Banerjee, personal communication). The virus has been shown to multiply in primary cell cultures derived from mouse (Suri and Banerjee, 1987b) and rat brain (Kimura-Kuroda *et al.*, 1992). Hyperactivity of glial cells has been observed histologically in human cases (Miyake, 1964; Johnson *et al.*, 1985; Mukherji and Biswas, 1976) as well as in experimentally infected rodent brains (Hase *et al.*, 1990). The virus has been shown to infect neurons in the embryonic rat brain cell cultures (Kimura-Kuroda *et al.*, 1992). The multiplication of JEV in astrocytes in brain cell cultures has not been well documented. In our previous communication, JEV-specific immunofluorescence (IF) was demonstrated in neurons as well as in astrocytes in the embryonic mouse brain cell cultures (Suri and Banerjee, 1987b).

In the present study, we have shown infection and growth of JEV in neuron-free astrocyte-enriched cultures prepared from brains of 3 day-old mice.

**Abbreviations:** CPE = cytopathic effect; DMEM = Dulbecco's Minimum Essential Medium; EM = electron microscopy; FBS = foetal bovine serum; FITC = fluorescein isothiocyanate; GC = galactocerebroside; IF = immunofluorescence; JE(V) = Japanese encephalitis (virus); MBP = myelin basic protein; MoAb = monoclonal antibody; NFP = neurofilament protein; NIV = National Institute of Virology, Pune; PBS = phosphate buffered saline; PDL = poly-D-lysine; p.i. = post infection; TCF = tissue culture fluid; TRITC = tetramethyl rhodamine isothiocyanate; WN = West Nile

## Materials and Methods

*Virus.* The Nakayama strain of JEV, kept at the National Institute of Virology, Pune (NIV) was employed at its 62nd and 95th mouse brain passages.

*Glassware, media and IF reagents.* Twenty-five $cm^2$ tissue culture flasks (Nunc) and plastic Petri dishes (diameter 55 mm, Laxbro) were used for cell growth after coating with poly-D-lysine (PDL, Boehringer). Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) was used as growth medium. Cell types were identified by indirect IF by use of following cell-specific markers: rabbit anti-glial fibrillary acidic protein (GFAP, Dakopats) for astrocytes; rabbit anti-myelin

basic protein (MBP, Dakopats) and rabbit anti-galactocerebroside (GC, prepared at NIV) for oligodendrocytes. Neurons were identified by using monoclonal antibody (MoAb) to neurofilament protein (NFP) of 160 K (Pelfreez) and 68 K (Dakopats). To detect JEV or its proteins in cells by IF, mouse or rabbit anti-JEV antibodies (prepared at NIV) were used. Simultaneous detection of viral and cell proteins was carried out by double staining with fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) conjugated anti-rabbit or anti-mouse antibodies (Sigma, Walker *et al.*, 1984).

*Cell cultures.* Primary mixed brain cell cultures were prepared form mice as described earlier (Suri and Banerjee, 1987) except that brains of 3 day-old mice were used. Oligodendrocytes and microglia were removed by methods described by McCarthy and De Vellis (1980) and Frei *et al.* (1986), respectively. Briefly, confluent primary brain cell cultures were agitated on a rotary shaker at 250 rpm for 2 hrs on the 8th and 9th days after seeding. The detached cells were removed. The cells that remained attached were enriched astrocytes which displayed GFAP in IF test. These cultures were maintained further for 20 days before virus infection. To make secondary astrocyte cultures, some of the astrocyte-enriched monolayer cultures were dispersed and seeded on PDL-coated glass coverslips as described by Frei *et al.* (1986). The cells were allowed to grow for 10 days before infection.

*Virus growth studies.* The primary and secondary astrocyte-enriched cultures were infected with JEV. The tissue culture fluid (TCF) from virus-infected cultures was harvested on day 1, 2, 5, 7, 9, 13 and 16 p.i. and assayed for virus infectivity by plaque formation in Vero cell cultures. To detect virus in cells by IF, the detached cells from the infected cultures were removed by centrifugation, resuspended in a drop of PBS, smeared on a glass slide, dried on air and fixed with formalin-methanol. The infected and non-infected primary astrocyte cell monolayers in culture flasks and secondary astrocytes on coverslips were fixed in the same manner before staining for IF.

For the observation of IF in the cells remaining attached on the plastic surface, discs were cut out from the flask bottom using a heated cork borer (Walker *et al.*, 1984) and immediatelly plunged into chilled PBS and immunostained.

## Results

By the 8th day of cultivation *in vitro* the primary brain cell cultures became confluent, the cell clumps disappeared and small polygonal cells with processes (oligodendrocytes) appeared on the top of flat cells (astrocytes).

After agitating the flask culture on a rotary shaker, most of the polygonal cells of the surface layer got detached from the layer of flat epitheloid cells. By the 9th day, cells resembling microglia (round to bizzare-shaped) appeared on the top of the monolayer which also got detached after a repeated agitation together with a few cells of the astrocyte layer. The astrocyte layer grew to confluency and did not show any deterioration or change till the 20th day. The cells in the primary astrocyte-enriched monolayers showed more than 95% enrichment when observed morphologically (Fig. 1) and through IF staining for GFAP. The secondary astrocytes showed growth but did not form confluent monolayers. In these cultures, both protoplasmic and fibrous type GFAP-positive astrocytes were seen along with a few oligodendrocytes (IF stained with anti-GC antibody) and occassionally microglia recognized morphologically (Suri and Banerjee, 1987a). None of the astrocytes either from primary or secondary cultures showed IF staining with anti-NFP antibodies thus confirming the absence of neurons.

When infected, the primary cell cultures containing predominantly astrocytes exhibited distortion and withdrawal of fine proccesses on day 2 p.i. but no other change up to day 11 p.i. On day 12 p.i., rounding of some cells of the monolayer was seen along with shrinkage of the astrocyte

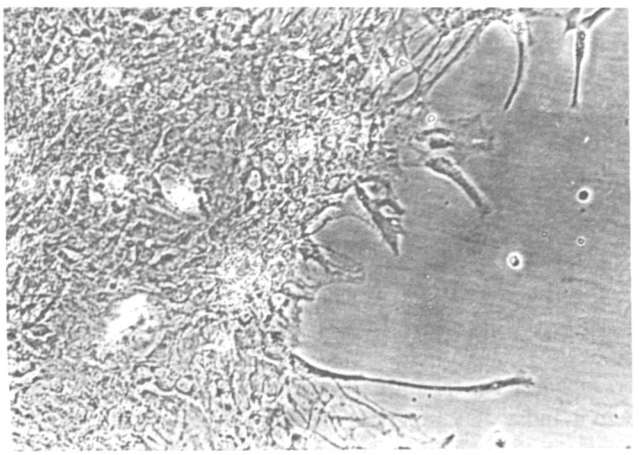

**Fig. 1**
**Thirty-three day-old uninfected primary neuron-free astrocyte-enriched monolayer culture**
Phase contrast, magnification x 350.

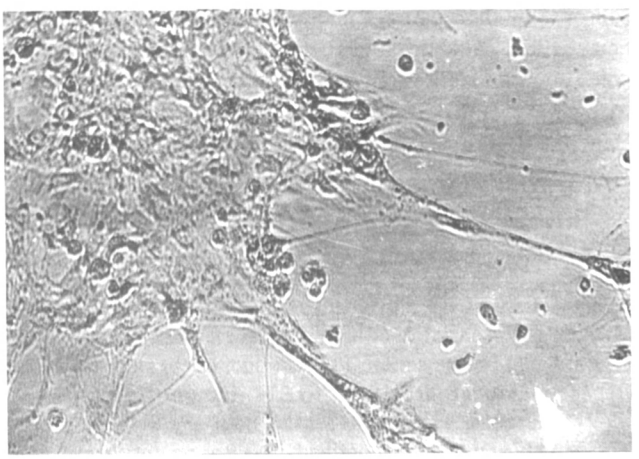

**Fig. 2**
**The same culture as in Fig. 1 showing CPE 13 days p.i. with JEV**
Note detachment of round cells and trunk-like structures (arrowhead).
Phase contrast, magnification x 350.

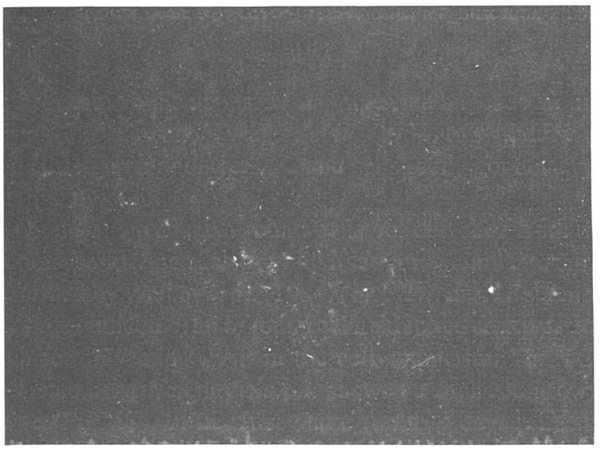

**Fig. 3**
**Thirty-eight day-old uninfected astrocyte culture after IF staining for JEV and GFAP**
Culture on cut-out plastic disc stained for JEV and GFAP with FITC- and TRITC-conjugates, respectively. Note negative staining for JEV. FITC filter used, magnification x 280.

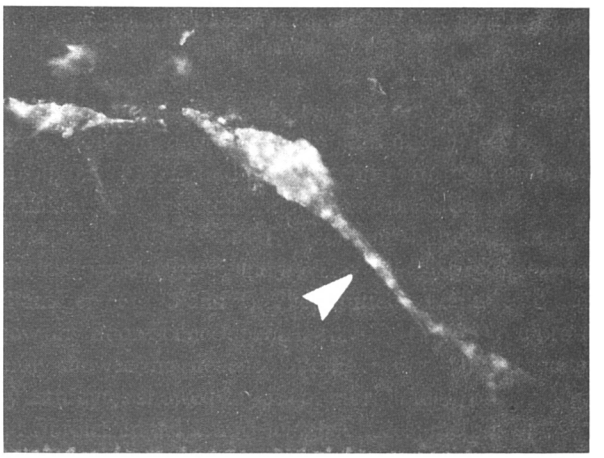

**Fig. 5**
**Astrocyte-enriched culture 18 days p.i. with JEV**
Double IF staining as in Fig. 3. Note JEV-positive trunk-like structures (arrowhead). FITC filter used, magnification x 280.

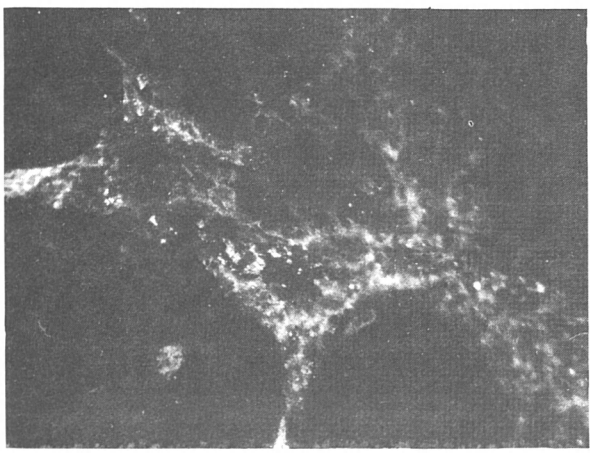

**Fig. 4**
**The same field as in Fig. 3 but viewed through TRITC filter**
Note bright fluorescence of GFAP-positive cells, magnification x 280.

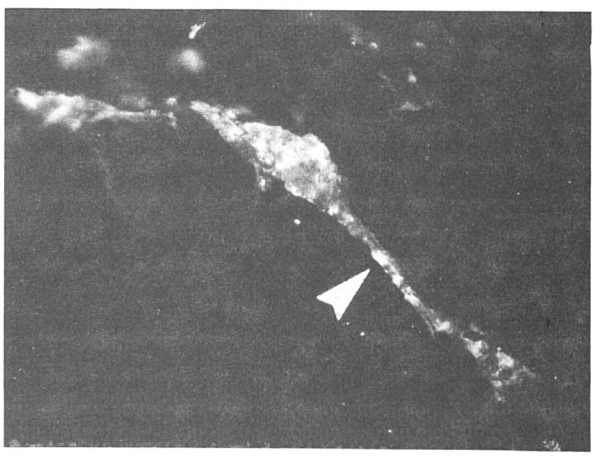

**Fig. 6**
**The same field as in Fig. 5 but viewed through TRITC filter**
Positivity for both the JEV and GFAP (arrowhead). Magnification x 280.

sheet. Round cells appeared as grape-like bunches around "trunk-like network" (Walker *et al.,* 1984) of withdrawing and curled up monolayers. Round cells easily got detached on mild agitation.

On day 13 p.i., most round cells got detached from the monolayer, leaving back trunk-like structures similar to those described by Walker *et al.* (1984) (Fig. 2). After IF staining the slide with thin film of round cells exhibited coexpression both for JEV and GFAP in the cell cytoplasm. The TCF collected on different days p.i. showed a biphasic growth curve (Fig. 13). In the first phase (up to 9 days p.i.), the titer was maximum on day 2 p.i., and decreased on days 3 – 9 p.i., when it reached a minimum. The titer rose again in the 2nd phase (from the 10th day onward) and the increase coincided with the appearance of CPE in the astrocytes (Fig. 2 and 13). The remaining patches of shrunken JEV-infected monolayers on the plastic discs showed cells positive for GFAP staining, implying that these were astrocytes (Fig. 6 and 8). The cells also expressed viral antigen (Fig. 5 and 7). The uninfected cultures did not show any fluorescence specific for JEV (Fig. 3) but prominently showed the presence of GFAP (Fig. 4). The secondary astrocytes showed 2 types of morphology, protoplasmic and fibrous. In the astrocyte subcultures, the protoplasmic astrocytes showed viral antigen in the perinuclear area (Fig. 9), and in the fibrous astrocytes IF was seen in the

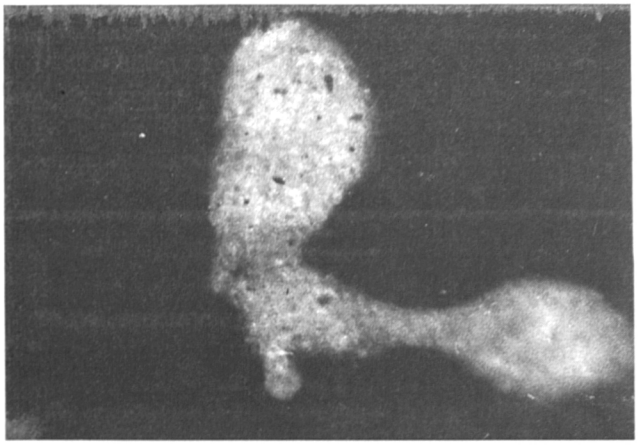

**Fig. 7**
**Portion of shrunken, back-rolled primary astrocyte monolayer 18 days p.i. with JEV**
Double IF staining as in Fig. 3. Note JEV-positive cells. FITC filter used, magnification x 320.

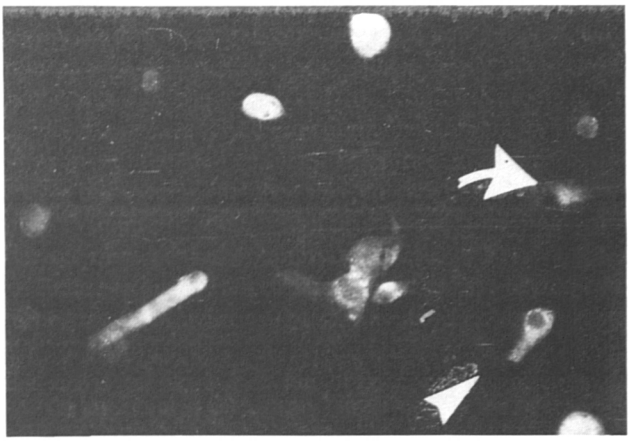

**Fig. 9**
**Secondary astrocyte culture 36 hrs p.i. with JEV**
Double IF staining as in Fig. 3. Note protoplasmic astrocytes showing profuse cytoplasmic (arrowhead) and perinuclear (curved arrow) localization of JEV. FITC filter used, magnification x 560.

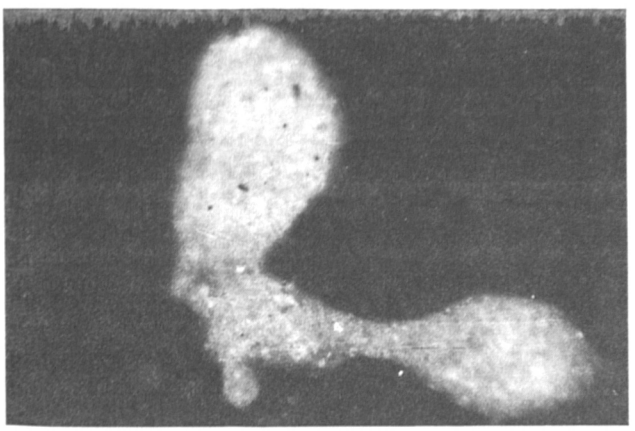

**Fig. 8**
**The same field as in Fig. 7 but viewed through TRITC filter**
Most cells are positive for both the GFAP and JEV.
Magnification x 320.

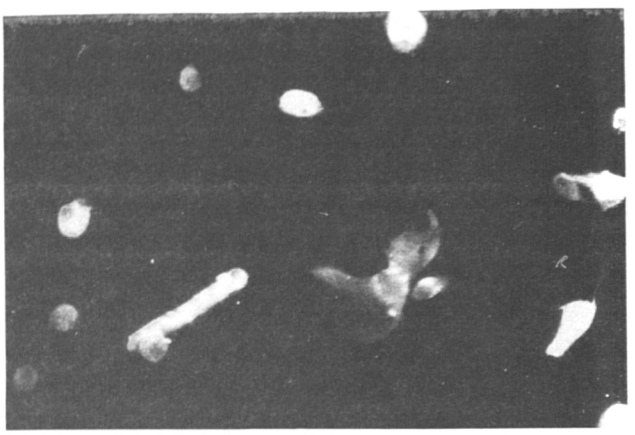

**Fig. 10**
**The same field as in Fig. 9 but viewed through TRITC filter**
Most cells are positive for both the GFAP and JEV.
Magnification x 560.

fine processes and cytoplasm of cells (Fig. 11). Both the types stained heavily with GFAP (Fig. 10 and 12). Thus the infected astrocytes coexpressed both the antigens.

In subcultured astrocyte monolayers, a few oligodendrocytes were also present. They could be distinguished by their reactivity with anti-GC antibody. They also expressed JEV thus showing that oligodendroglia were also infected by the virus.

## Discussion

The neuronotropic nature of JEV has been well described in histopathological studies of JE cases, (Dropulic and Masters, 1990; Johnson *et al.,* 1985; Mukherjee and Biswas, 1976; Miyake, 1964) and in the experimental infection of rodent brains (Hase *et al.,* 1990; Ogata *et al.,* 1991). In our earlier studies of primary mouse brain cell cultures, neurons as well as astrocytes were shown to be infected with JEV (Suri and Banerjee, 1987b). Kimura-Kuroda *et al.* (1992), however, showed infection of neurons only with this virus in rat brain cell cultures. Therefore, to reconfirm our earlier observation that the astrocytes are capable of allowing JEV multiplication, we set up cultures from brains of 3 day-old mice instead of embryonic mice.

Brain cell cultures from 3 day-old mice have been shown to be free from neurons (McCarthy and De Vellis, 1980; Walker *et al.,* 1984). In the cell cultures derived from brain, the astrocytes grow first and form confluent monolayer,

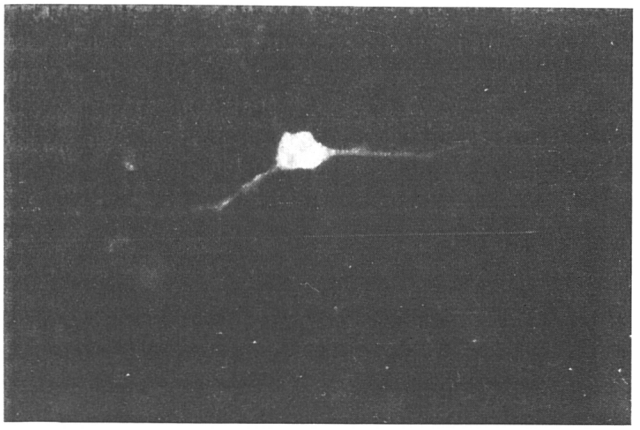

**Fig. 11**
**Secondary astrocyte culture 36 hrs p.i. with JEV**
Double IF staining as in Fig. 3. Note JEV-specific IF in the cell processes and the perikaryon of a fibrous astrocyte. FITC filter used, magnification x 560.

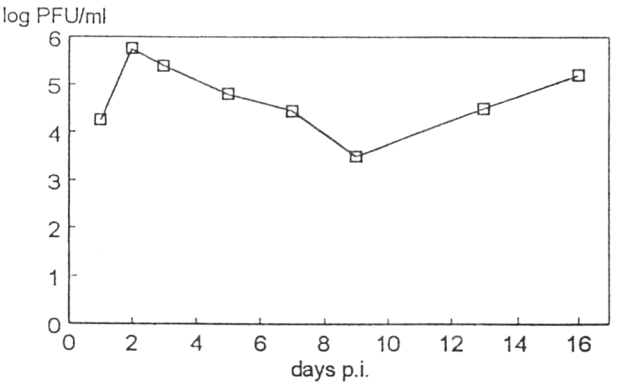


**Fig. 13**
**Growth of JEV in neuron-free astrocyte-enriched primary mouse brain cell culture**

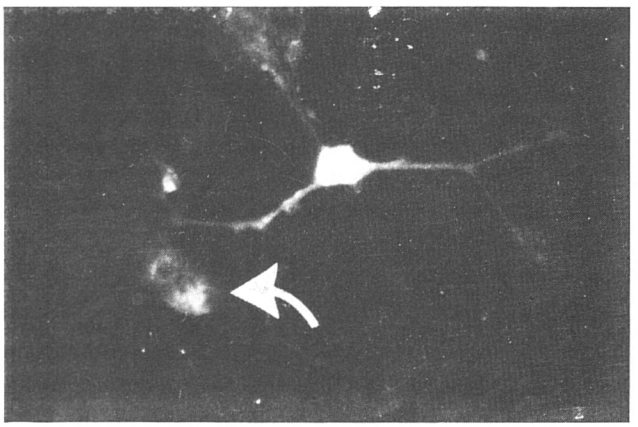

**Fig. 12**
**The same field as in Fig. 11 but viewed through TRITC filter**
The astrocyte positive for JEV in Fig. 11 is positive also for GFAP. However, some GFAP-positive cells are negative for JEV (curved arrow). Magnification x 560.

while cell like neurons, oligodendrocytes and microglia grow on top of the flat cell layer without firm contact (McCarthy and De Vellis, 1980; Walker *et al.,* 1984; Suri and Banerjee, 1987). Neurons differentiate fully before birth and fail to regrow in cultures from brains after birth (McCarthy and De Vellis, 1980). Since the cultures in this study were made from 3 day-old mouse brains, they could not have contained neurons.

When shaken on a rotary shaker on days 8 – 12 p.i., the cultures grown from 3 day-old mice (McCarthy and De Vellis, 1980; Walker *et al.,* 1984) release cells of the superficial layer, and only a purer population of astrocytes remains in the monolayer (Walker *et al.,* 1984). When such neuron-free monolayers (negative for NFP) containing about 95% astrocytes were cultivated for 20 days *in vitro* and then were infected with JEV, sufficient progeny virus was produced and released in the TCF (by 24 hrs p.i. onwards). In the biphasic growth curve, the second phase of titer rise beginning around days 10 – 16 p.i. and coinciding with the appearance of CPE indicates increased virus activity. Both the detached and adherent cells exhibited label for GFAP (astrocytes) as well as JEV antigen. The infection of secondary astrocyte cultures not only confirmed susceptibility of astrocytes but also of oligodendrocytes to JEV. The possibility of the infected cells being A2B5-plus glial progenitor cells, as suggested by Kimura-Kuroda *et al.* (1992), is ruled out as (1) the differentiation of astrocytes is reportedly completed prenatally in mouse brain (Norton, 1983), and (2) the cultures used in this study originated from 3 day-old post-natal brains and were grown for up to 20 days *in vitro* and then infected with JEV.

We have already shown that besides JEV dengue and West Nile (WN) viruses also infect astrocytes in cultures from embryonic mouse brains (Suri and Banerjee, 1987b). Liu *et al.* (1989) also confirmed our finding by showing the growth of WN virus in secondary astrocyte cultures. Infection and growth of other arboviruses causing encephalitis, like Murrary Valley encephalitis virus (Mims, 1960) and Eastern equine encephalitis virus (Murphy and Whitefield, 1970), have been observed in astrocytes in brains of experimentally infected mice by EM. These observations provide substantial evidence for infection of glial cells with arboviruses.

The infection of primary astrocyte-enriched cultures with JEV, resulting in virus growth and CPE, presented in this study confirms beyond doubt the view that the lysis of brain

cells in JE is due to direct damage of the infected cells by the virus. The rounding of virus-infected astrocytes due to CPE also confirms the degeneration of cell cytoskeleton after JEV infection (Suri and Banerjee, 1987b). The late appearance of CPE after 11 days of infection confirms the view of Pfefferkorn and Shapiro (1974) that in comparison to alphaviruses, the growth of flaviviruses in cultured cells is slower, since the host cell macromolecular synthesis is inhibited gradually till late after infection. The appearance of CPE in JEV-infected cultured brain astrocytes is likely to be delayed because flaviviruses induce the expression of class II major histocompatibility complex in these cells, rendering them immunocompetent; these cells are also triggered to secrete cytokines including interferons alpha and beta (Liu *et al.*, 1989). The probable reason as to why JEV antigen could not be detected in brain astrocytes in terminal JE cases or experimentally infected rodent brains, might be the death of the subject through fast neuronal depletion, before the virus could infect the astrocytes to the level detectable by the conventional tests. Thakare *et al.* (1991) have shown mild demyelination in JE cases with shedding of increased amounts of MBP in the serum of JE cases. This could be due to the infection of oligodendrocytes with the virus as seen in the secondary culture.

The phenomenon of reactive gliosis (astrogliosis) which has been described histopathologically in *post mortem* examination of JE cases, however, could not be observed as no p.i. cell hypertrophy or proliferation was seen in the infected cultures.

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