A model to study neurotropism and persistency of Japanese encephalitis virus infection in human neuroblastoma cells and leukocytes

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Japanese encephalitis (JE) is the most common mosquito-borne encephalitis in the Asia–Pacific region. Patients with JE usually present neuronal involvement, but other organ involvement is relatively rare. Employing human neuroblast-derived (NB) cell lines and different blood cells (erythrocytes, lymphocytes, granulocytes and monocytes), the neurotropism and persistency of Japanese encephalitis virus (JEV) in human cells was investigated. It was found that JEV could not replicate in erythrocytes, granulocytes or lymphocytes. Monocytes and NB cell lines could support replication of JEV as demonstrated by expression of viral NS3 antigen and virus plaque-forming units (p.f.u.). JEV could replicate more efficiently in neuroblastoma (HTB-11) cells than in monocytes after infection for 48 h ($2\times10^7$ vs $2\times10^2$ p.f.u. ml$^{-1}$). Two different strains of JEV revealed a similar infectivity to different leukocytes and four NB cell lines. In a kinetic study, it was found that JEV-infected monocytes possessed a high viability (90%) after infection for 5 days, while JEV-infected neuroblastoma cells suffered cell apoptosis in 2 days and decreased viability to less than 1% in 5 days. Further studies showed that monocytes could take up JEV rapidly, displaying a log scale increase of intracellular JEV titres in 9 h after infection. Significantly, extracellular production of JEV by monocytes started in 12 h, peaked in 3 days and persisted for more than 3 weeks. These results suggest that JEV-infected monocytes may play an important role in harbouring JEV for eventual transmission to NB cells and that modulation of JEV-induced NB cell apoptosis may be useful in treating patients with JE.

INTRODUCTION

Japanese encephalitis (JE) is a mosquito-borne disease that is widely prevalent in the Asia–Pacific area from the maritime provinces of the former Soviet Union to southern India and Sri Lanka (Umenai et al., 1985). This disease is caused by Japanese encephalitis virus (JEV), a positive-sense single-stranded RNA virus, and is transmitted mainly by Culex mosquitoes, such as Culex tritaeniorhynchus and Culex annulus. JEV infects a variety of mammals and birds. It is believed that birds are the reservoir hosts (Hammon et al., 1985a) and that pigs are the amplifying hosts (Cross et al., 1971). Mosquitoes act as vector hosts, transporting JEV infections from the reservoir or amplifying hosts via blood-sucking. JEV takes 7–14 days to replicate inside the salivary gland of mosquitoes and be transmitted to a susceptible host through a mosquito bite (Halstead, 1992). Although humans may develop a life-threatening disease within 5–14 days after infection, evidence suggests that humans are not natural hosts but dead-end hosts for JEV infections (Rosen, 1986).

It is not clear why JEV causes overwhelming infections in humans yet is not transmissible from human beings. Studies of autopsies showed that JEV can only be isolated from brain tissues, but not tissue fluids or other organs (Iwasaki et al., 1986; Mukherji & Biswas, 1976). This suggests that JEV tends to cause a neurotropic infection, attacking neural rather than non-neural tissues in humans. Experimental studies in rats have demonstrated that neuron cells, especially developing neurons, were the major infective source (Kimura-Kuroda et al., 1993). Furthermore, animal studies have also shown that direct intracerebral inoculation of JEV induces extensive encephalitis in mice, while intraperitoneal inoculation does not (Hase et al., 1990a). These studies indicate that JEV may not actively infect blood cells or non-neural cells after delivery into the subcutaneous tissues and blood through a mosquito bite.
Although JEV does not replicate efficiently in blood cells, it may reside in certain leukocytes, enabling it to enter the central nervous system, resulting in encephalitis. Sharma et al. (1991) showed that no JEV antigen or virus isolation could be found in blood or leukocytes from JE patients, although its antigen could be detected by indirect immunofluorescence in mouse embryonic fibroblasts after co-culturing with human mononuclear cells obtained from patients with JEV infections. We have attempted to establish a model suitable for identifying neurotropism and persistence of JEV infections in human cells. Employing human neuroblast-derived (NB) cell lines and human blood cells, including erythrocytes, lymphocytes, granulocytes, and monocytes, we investigated whether JEV causes persistent infection in certain human leukocytes while causing neurocytotropic infections with cell apoptosis in human NB cells.

METHODS

Viruses and virus culture. Two strains of JEV were used in this study. The Nakayama-NIH strain was obtained from the American Type Culture Collection (ATCC; Manassas, VA) and a Taiwan local strain, NTI113, was obtained from the Institute of Preventive Medicine, National Defense Medical Center, Taipei, Taiwan. The strains of JEV were propagated at an m.o.i. of 0·1 in C6/36 cells grown to 80% confluence in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS; Gibco Biochemicals) at 28 °C. The JEV strains were harvested from the JEV-infected C6/36 cell-culture supernatants after incubation for 6 days. The JEV titres in the supernatants were measured by a standard plaque-forming unit (p.f.u.) assay in BHK-21 cells, as described below, and concentrated to the titre of 2 × 10^6 p.f.u. ml⁻¹ as the seeding source for studies.

Determination of viable JEV titres by the p.f.u. assay. The JEV titres were measured by the p.f.u. assay as modified from a previous description (Mores et al., 1985). In brief, baby hamster kidney (BHK-21) cells (1 × 10^5 cells per well) were cultured into monolayers on 24-well plates (Corning Laboratory Science). The BHK-21 cells were adsorbed with a series of 10-fold dilutions of virus-infected culture supernatants or virus-infected cell extracts for 60 min at 37 °C before they were overlaid with Eagle’s minimal essential medium (MEM) containing 2% FCS and 1·5% carboxymethylcellulose (Sigma Biochemicals) for 4 more days of incubation. The plaque-forming wells were fixed with 10% formalin in phosphate-buffered saline (PBS) for 1 h, and stained with 0·5% crystal violet for 30 min before counting (Shiao et al., 1994; Yang, K. D. et al., 1995). Results are presented with the standard error of the mean calculated from three triplicate experiments.

Determination of JEV titres by a real-time quantitative RT-PCR. Viral RNA extracted from JEV-infected monocytes and their culture supernatants were subject to a fluorogenic quantitative RT-PCR detection of total JEV titres as modified from our previous description (Chen et al., 2001; Li et al., 2002). In brief, 0·25 ml of culture supernatants or cultured cell pellets were added individually with 0·75 ml of Tri-reagent (Sigma) to separate RNA from DNA and protein fractions (Chen et al., 2001). Fluorescent RT-PCR was carried out in an ABI 7700 quantitative PCR machine (Applied Biosystems) for 40 cycles using TaqMan technology, as described previously (Chen et al., 2001). The forward primer used to amplify the JEV RNA was 5′-GGG TTA GGC CTC ACA TCC A-3′; the reverse primer was 5′-GCT GGC CAC CCT TTC TT-3′; the nested fluorescent probe sequence was FAM-GCG CCA CCA CTA TAG CTG CCG GA-TAMRA. Results were calculated from a standard curve made from a series of well-known virus titres (Chen et al., 2001; Li et al., 2002).

Preparation of human NB cells and myeloid leukemic (HL60) cells. Two human neuroblastoma cell lines, HTB-11 and IMR-32, and one human glioblastoma cell line, DBTGR-05MG, were obtained from the ATCC. Another human glioblastoma cell line, GST/VGH, was obtained from the National Health Research Institute Culture Collection, Shin-Chu, Taiwan. These cells were cultured in EMEM supplemented with 10% FCS, 1 mM glutamine, 0·1 mM non-essential amino acids, 1 mM sodium pyruvate, 50 μg gentamicin ml⁻¹ and 100 μg penicillin ml⁻¹ in a 5% CO₂ humidified incubator at 37 °C. Cells were regularly propagated at a density of 2 × 10⁶ cells ml⁻¹ every 7 days and harvested using 0·02% EDTA in PBS (0·15 M, pH 7·3–7·4), as described previously (Yang, K. D. et al., 1993, 1995). HL60 cells were cultured in RPMI 1640 medium with 10% FCS as described previously (Yang, K. D. et al., 1994a).

Preparations of human erythrocytes and different leukocytes. Human erythrocytes and various leukocytes were isolated from peripheral whole blood donated by healthy volunteers after informed consent had been obtained. Heparinized peripheral blood was separated into plasma, leukocyte buffy-coat and erythrocyte layers by centrifugation at 1500 g for 15 min. After removing plasma, leukocytes were harvested from the buffy-coat layers; erythrocytes were harvested from the bottom layer and resuspended to the original volume in PBS for studies. Leukocytes in the buffy-coat layers were separated into polymorphonuclear (PMN) and mononuclear leukocytes (MNls) by a gradient centrifugation in Ficoll-paque (Pharmacia Fine Chemicals) at 1500 g for 20 min (Yang, K. D. et al., 1988). PMNs were harvested from the cell pellet in the bottom. Residual erythrocytes were lysed twice with a hypotonic solution (0·2% NaCl) for 30 s, followed by removal of the osmolarity with 1·6% NaCl. The PMNs were washed in PBS and suspended to 5 × 10⁶ cells ml⁻¹ in Hanks balanced salt solution (HBSS) containing 0·1% human serum albumin (Hyland Baxter Healthcare). The purity and viability of the PMNs were greater than 95 and 98%, respectively (Yang, K. D. et al., 1988; Yang, M. Y. et al., 2002). The MNl fraction was separated further by allowing monocytes to adhere onto a 6-well plate (Corning) for 90 min (Wang et al., 1996; Yang, K. D. et al., 1994b). PMNs were harvested from the non-adherent cells, whereas monocytes were harvested from the adherent layer and stained with the anti-NS3 antibody at a dilution of 1:50 in PBS (0·1 M, pH 7·3–7·4), as described previously (Yang, K. D. et al., 1995b). All cells were resuspended at 5 × 10⁶ cells ml⁻¹ in RPMI 1640 medium (Gibco) containing 10% FCS with penicillin G (100 μg ml⁻¹) and streptomycin (100 μg ml⁻¹) for studies.

JEV infectivity as determined by indirect immunofluorescence assay. The JEV infectivity in leucocytes and NB cells was monitored by an indirect immunofluorescent analysis of NS3 antigen expression as modified from a previous description (Read et al., 1986). Cells infected with JEV at an m.o.i. of 1:1 for 60 min were washed twice in PBS to remove extracellular virus before the cells were set for culture. The mAb (RP9-2) directed against the NS3 antigen of JEV (kindly provided by the Institute of Preventive Medicine, National Defense Medical Center, Taipei, Taiwan) (Ma et al., 1995) was used as the specific antibody. Cells with and without JEV infection for the specified periods of time were spotted in triplicate onto a Teflon-coated chamber slide. These slides were fixed in acetone and stained with the anti-NS3 antibody at a dilution of 1:50 in PBS for 30 min, followed by an FITC-conjugated goat anti-mouse IgG at a dilution of 1:100 as the secondary antibody for another 30 min. After washing, these slides were observed under a Zeiss fluorescent microscope. Results are presented as the percentage of positive cells per 500 cells.
JEV replication in different human leukocytes and NB cell lines. JEV replication in human monocytes and NB cells was assessed by standard p.f.u. assay and RT-PCR analysis as described above. In brief, monocytes or NB cells adsorbed with JEV at an m.o.i. of 1:1 for 60 min were washed twice in PBS to remove extracellular virus before the cells were cultured in medium for the indicated periods of time. In studying persistency of JEV infection in human monocytes, culture medium was changed every 7 days for 60 days. The culture supernatants and cell pellets from monocytes or neuroblastoma cells infected with JEV for the indicated periods of time were subjected to viral p.f.u. determination in BHK-21 cells as well as RT-PCR analysis of JEV titres. For those with positive RT-PCR but negative p.f.u. assays from JEV-infected monocytes, co-culture (0.1 ml) of the culture supernatants of JEV-infected monocytes with neuroblastoma (HTB-11) cells at 2 ml (5 × 10⁵ cells ml⁻¹) for 5 days was performed before a second round of assessment of p.f.u. ml⁻¹ in the culture supernatants of neuroblastoma (HTB-11) cells. The virus titres determined by the p.f.u. assay were reciprocally compared with the host-cell viability as described below.

Cell viability as determined by trypan-blue exclusion. Cell viability was monitored by the trypan-blue-exclusion assay (Yang, K. D. et al., 1988; Yang, M. Y. et al., 2002). Monocytes and NB cells with and without JEV infections for the indicated periods of time were incubated with 0·4 % trypan blue for 5 min before observation under a microscope (400 ×). Viable cells capable of excluding trypan blue showed no blue stain, whereas non-viable cells were incapable of excluding the dye and were stained blue by it. Results are presented as the percentage of viable cells per 500 cells.

Cell apoptosis as assessed by DNA fragmentation and flow cytometric analysis of apoptotic cells. Cells undergoing apoptosis tend to express endonuclease resulting in DNA fragmentation in association with exposure of phosphatidylserine (PS) to the outer leaflet of the cell membrane. Employing flow cytometric analysis of PS expression and gel electrophoretic analysis of DNA fragmentation, we studied cell apoptosis in the NB cells and monocytes with and without JEV infection. Cells (2 × 10⁵ per 0·1 ml) from monocytes and NB cells with and without JEV infection for 2 days were stained with FITC-labelled Annexin-V (50 μg ml⁻¹; PharMingen) for 20 min at room temperature and analysed by flow cytometry (FACScan apparatus; Becton Dickinson), as described previously (Yang, M. Y. et al., 2002). DNA extracted from the culture supernatants of JEV-infected NB cells and monocytes was subjected to gel electrophoretic analysis of DNA fragmentation as modified from previous descriptions (Mabbott et al., 1995; Yang, M. Y. et al., 2002). In brief, culture supernatants (5 ml) were collected and subjected to DNA extraction by using an equal volume of chloroform/phenol solution. After this extraction, the DNA in the upper aqueous phase was further precipitated with 0·1 vol. of 5 M NaCl and 1 vol. of 2-propanol at −20 °C for 16 h. The DNA precipitates were suspended in 10 μl TE (10 mM Tris salt, 1 mM EDTA) buffer and incubated with 1 μg RNase A ml⁻¹ at 65 °C for 10 min followed by another 60 min at 37 °C before electrophoresis in a 2 % agarose gel containing 1 μg ethidium bromide ml⁻¹. The DNA fragmentation showing approximately 200 bp ladder was confirmed by a series of 100 bp reference markers (Sigma).

RESULTS

Infectivity of JEV in human blood cells and neuroblastoma cells

Employing human neuroblastoma (HTB-11) cells and human peripheral blood cells, experiments were performed to examine the infectivity of JEV (Nakayama-NIH strain) in these human cells at an m.o.i. of 1:1 (5 × 10⁵ viruses: 5 × 10⁵ cells ml⁻¹). Viral NS3 antigen expression was not detected in erythrocytes, lymphocytes or granulocytes after JEV infection for 48 h, as determined by an indirect immunofluorescence assay with a specific mAb (RP92-2) directed against the NS3 antigen. In contrast, viral NS3 antigen was prominently expressed in the human neuroblastoma (HTB-11) cells and to a lesser extent in human monocytes (Fig. 1). In a kinetic study, we found that expression of the NS3 antigen in neuroblastoma cells was detected 6 h after JEV infection. Almost all the neuroblastoma cells were infected and expressed the viral NS3 protein in 2–3 days. By contrast, the expression of viral NS3 antigen in monocytes was not significant until 12 h after

Fig. 1. Viral NS3 antigen expression in human neuroblastoma cells and blood cells after JEV infection as demonstrated by indirect immunofluorescence assay. Cells (5 × 10⁵ cells ml⁻¹) were infected with JEV (Nakayama-NIH strain) at an m.o.i. of 1 for 48 h before being harvested for determination of viral NS3 expression using the specific mouse mAb (RP92-2) directed against the NS3 antigen as the primary antibody and a FITC-labelled goat anti-mouse IgG as the secondary antibody. Results were read under a light microscope (left lane) and fluorescent microscope (right lane) using a 10 × 16 magnifier, simultaneously. (A) and (B), results from erythrocytes; (C) and (D), results from lymphocytes; (E) and (F), results from PMNs; (G) and (H), results from monocytes; (I) and (J), results from neuroblastoma (HTB-11) cells.
infection, and the infection rate peaked on day 2, but showed a lower infection rate as demonstrated by NS3 expression (78 ± 5% vs 99 ± 1%) as well as a weaker fluorescence of NS3 expression than that of neuroblastoma cells (Fig. 1). The NS3 expression was compatible with the virus-replication titres described below.

Replication of different JEV strains in human monocytes and NB cells

To discern the replication rates of different JEV strains in monocytes and different NB cells, we used two strains of JEV to infect human neuroblastoma (HTB-11 and IMR-32) or glioblastoma (DBTRG-05MG and G5T/VGH) cells, and different blood cells to differentiate the possible discrepancies of virus replication between NB cells and non-NB human cells. As shown in Table 1, the Nakayama-NIH strain of JEV could replicate in all four kinds of NB cells and revealed 3- to 4-log scale higher titres than those in monocytes in the first 24 h. Similarly, the local strain (NT113) of JEV could replicate in both neuroblastoma cell lines but not granulocytes or erythrocytes (Table 1). Low JEV titres were recovered from the culture of lymphocytes with local strain NT113 but not the Nakayama-NIH strain of JEV, suggesting that certain JEV strains may also infect lymphocytes or that the lymphocyte suspensions may contain a small percentage of monocytes. Taken together, these results suggest that different strains of JEV reveal a similar pattern of infectivity in human monocytes and NB cells, with a 4-log scale difference in the virus-replication rate between monocytes and neuroblastoma cells.

Table 1. Replication of different strains of JEV in different human cells

Cells (5 × 10⁵ cells ml⁻¹) were infected with JEV (Nakayama-NIH or local Taiwan strain NT113) at an m.o.i. of 1 for 60 min at room temperature. After washing out extracellular virus, leukocytes (PMNs, lymphocytes and monocytes) were resuspended in RPMI 1640 medium and human NB cells (HTB-11, IMR-32, DBTRG-05MG or G5T/VGH) were suspended in EMEM for 24 h of culture. Virus titres in the culture supernatants were determined by standard p.f.u. assay in BHK-21 cells. Data presented are calculated from three replicate experiments. NT, Not tested.

<table>
<thead>
<tr>
<th>Human cells</th>
<th>JEV strain</th>
<th>NT113 (p.f.u. ml⁻¹)</th>
<th>Nakayama-NIH (p.f.u. ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PMNs</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td></td>
<td>3 ± 5</td>
<td>0</td>
</tr>
<tr>
<td>Monocytes</td>
<td></td>
<td>1.7 ± 0.4 × 10⁵</td>
<td>1.2 ± 0.3 × 10²</td>
</tr>
<tr>
<td>HTB-11</td>
<td></td>
<td>8.4 ± 1.3 × 10⁵</td>
<td>4.9 ± 0.8 × 10⁵</td>
</tr>
<tr>
<td>IMR-32</td>
<td></td>
<td>4.4 ± 0.5 × 10⁵</td>
<td>5.9 ± 0.3 × 10⁵</td>
</tr>
<tr>
<td>DBTRG-05MG</td>
<td></td>
<td>NT</td>
<td>7.0 ± 1.2 × 10⁵</td>
</tr>
<tr>
<td>G5T/VGH</td>
<td></td>
<td>NT</td>
<td>8.9 ± 1.7 × 10⁵</td>
</tr>
</tbody>
</table>

Virus replication and cell cytotoxicity in monocytes and neuroblastoma cells

To assess the relationship between virus replication and host-cell viability, we monitored JEV replication titres in monocytes and neuroblastoma cells for 5 days with a standard p.f.u. assay in BHK-21 cells. We found that JEV titres in monocytes and HTB-11 neuroblastoma cells both peaked 2 days after infection with m.o.i. 1:1. The pattern of JEV (Nakayama-NIH strain) replication in monocytes was similar to that of neuroblastoma cells (Fig. 2), whereas JEV titres in monocytes were 5-log scale lower than those of neuroblastoma cells (2 ± 1 ± 1.2 × 10⁷ vs 2 ± 8 ± 0.7 × 10⁵ p.f.u. ml⁻¹). Looking at cell cytotoxicity, an interesting finding of our study was that JEV-infected monocytes remained viable (> 90%) after JEV infection for 5 days, whereas the

Fig. 2. Virus replication and cell cytotoxicity in monocytes and neuroblastoma cells after JEV infection. Cells (5 × 10⁵ cells ml⁻¹) were infected with JEV for 1, 2, 3, 4 and 5 days before cells and culture supernatants were harvested for determination of cell viability and virus titres, respectively. Results are presented as the percentage of viable cells per 500 cells (○) and JEV replication titres (▲). (A) shows the kinetic changes of virus replication and cell viability in JEV-infected monocytes; (B) shows the kinetic changes of virus replication and cell viability in JEV-infected neuroblastoma (HTB-11) cells.
viability of JEV-infected human neuroblastoma (HTB-11) cells declined dramatically on day 3 and was down to less than 1% on day 5 (Fig. 2). To confirm JEV-induced neuroblastoma cell death is related to neurotropic effect but not neoplastic destination, additional studies with both human neuroblastoma (HTB-11) cells and myeloid leukaemic (HL60) cells were performed to differentiate virus cytotoxicity from neoplastic cytotoxicity. It was found that although JEV infection caused extensive neuroblastoma cell death (only 1·0% viable) in 5 days it did not affect the cell viability of myeloid leukaemic HL60 cells (93% viable) in 5 days.

**Kinetic assessment of JEV persistency in human monocytes**

JEV caused a heavy infection with lethal insult in human neuroblastoma (HTB-11) cells but raised a light infection without lethal effect in human monocytes. With this in mind, experiments were performed to explore whether JEV caused a persistent infection in human monocytes using a sensitive, quantitative RT-PCR. Results showed that JEV caused a heavy infection with lethal insult in human monocytes while JEV caused a light infection in neuroblastoma (HTB-11) cells but raised a light infection without lethal effect in human monocytes. With this in mind, experiments were performed to explore whether JEV caused a persistent infection in human monocytes using a sensitive, quantitative RT-PCR. Results showed that JEV appeared to be taken into monocytes in 3 h and rapidly increased by 1-log scale of total intracellular virus titres in 9 h although the intracellular viruses detected by p.f.u. assay were 2-log scale lower than those detected by quantitative RT-PCR (4·3×10^6 ml^-1 vs 5·0×10^6 ml^-1).

The early (3 h) higher extracellular JEV titres detectable by RT-PCR but not by p.f.u. assay might be due to input virus levels instead of new virus replication. A detectable increase of extracellular JEV titres between 6 and 12 h after infection reflected actual new virus replication and release by monocytes. During the first 24 h, extracellular JEV titres were much lower than those in intracellular titres, indicating a lag time for virus replication and release by monocytes. As time passed, extracellular JEV titres increased and the intracellular titres decreased in 2–3 days (Table 2). Afterwards, both intracellular and extracellular titres declined. Although the intracellular and extracellular viable JEV were only detectable by p.f.u. assay for 7 days, JEV was detectable inside the JEV-infected monocytes for 35 days (Table 2). In the co-culture of neuroblastoma (HTB-11) cells with the supernatants from JEV-infected monocytes, JEV was detectable for 3 weeks (Table 2). Taken together, these results suggest that JEV can cause a persistent infection in human monocytes for more than 3 weeks in an *in vitro* culture under change of fresh medium every 7 days.

**Induction of apoptosis by JEV in neuroblastoma cells but not monocytes**

Cell death is generally classified into two types, cell cytolysis and apoptosis (programmed cell death). Experiments were

<table>
<thead>
<tr>
<th>Time</th>
<th>JEV titre (p.f.u. ml^-1)</th>
<th>RT-PCR assay (virions ml^-1)</th>
<th>IFA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extracellular</td>
<td>Cell-associated</td>
<td>Extracellular</td>
</tr>
<tr>
<td>3 h</td>
<td>6·0×10^6</td>
<td>1·2×10^4</td>
<td>1·4×10^4</td>
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<tr>
<td>6 h</td>
<td>2·1×10^1</td>
<td>4·3×10^1</td>
<td>8·5×10^2</td>
</tr>
<tr>
<td>12 h</td>
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<td>2·0×10^4</td>
<td>7·0×10^3</td>
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<td>8·3×10^3</td>
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<tr>
<td>3 days</td>
<td>7·0×10^2</td>
<td>2·5×10^5</td>
<td>2·6×10^4</td>
</tr>
<tr>
<td>5 days</td>
<td>1·0×10^2</td>
<td>1·4×10^2</td>
<td>1·6×10^5</td>
</tr>
<tr>
<td>7 days</td>
<td>3·3</td>
<td>5·8</td>
<td>8·2×10^4</td>
</tr>
<tr>
<td>14 days</td>
<td>0·0</td>
<td>0·0</td>
<td>5·0×10^3</td>
</tr>
<tr>
<td>21 days</td>
<td>0·0</td>
<td>0·0</td>
<td>4·0×10^3</td>
</tr>
<tr>
<td>35 days</td>
<td>0·0</td>
<td>0·0</td>
<td>1·5</td>
</tr>
<tr>
<td>45 days</td>
<td>0·0</td>
<td>0·0</td>
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<td>60 days</td>
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</table>
performed to differentiate whether JEV-induced cell cytotoxicity was due to cell lysis or programmed cell death. As shown in Fig. 3, DNA fragmentation was found in the DNA collected from the culture supernatants of neuroblastoma (HTB-11) cells after JEV infection for 3 days. Monocytes did not show DNA fragmentation after infection for 3 days. In a kinetic study to demonstrate when the JEV-infected HTB-11 cells began to go on to DNA fragmentation after infection, it was found that JEV-induced neuroblastoma cell DNA fragmentation appeared 3 days after infection. However, early cell apoptosis as assessed by flow cytometric analysis of PS expression occurred in the JEV-infected neuroblastoma (HTB-11) and glioblastoma (DBTRG-05MG) cells but not monocytes 2 days after infection (Fig. 4). The fact that JEV-induced neuroblastoma and glioblastoma cell death was mediated by programmed cell death suggests that blockage of programmed cell death may enable patients with JE to avoid long-term neurologic sequelae.

**DISCUSSION**

The transmission route of JE in human beings is not well understood. A possible retrograde transmission pathway from olfactory nerve fibres to the central nervous system (CNS) has been deciphered (Halstead, 1992). Studies with...
mice by Mathur et al. (1992) suggest that inflammatory mediators from a JEV-induced immune reaction may be responsible for breaching of the blood–brain barrier, resulting in infections of the CNS. In this study, we employed human NB cell lines as well as non-NB leukocytes to explore neurotropic infections of JEV in human cells. We showed that JEV has a neurotropic tendency in NB cells whose replication rate is 4- to 5-log scale higher than that in monocytes. By contrast, certain blood cells, such as erythrocytes and granulocytes, did not support JEV replication. Based on these studies, three clinical implications may be addressed. (i) The fact that JEV replicates more efficiently in human NB cells than in human blood leukocytes may help explain why JE tends to involve infections of the CNS rather than of other organs. (ii) The zero or lower JEV titres in various human blood cells may help explain in part why human beings are dead-end hosts for JEV infection and why JEV does not transmit from human-to-human via mosquito bite. (iii) The fact that JEV-infected human neuroblastoma cells tend to die out in 5 days, but monocytes do not, may explain in part why patients with JE generally have a high rate of long-term neurologic sequelae. (iv) JEV, which is able to replicate, though at a low titre in human monocytes, did not induce monocyte apoptosis or cytotoxicity. This suggests that monocytes circulating in the blood for weeks to months may transmit the virus into the CNS. In the literature, there is only one report describing the possibility of JEV latency in human peripheral blood from three children with asymptomatic JEV infection (Sharma et al., 1991). The latency was demonstrated, although not virus isolation or direct antigen detection, by JEV antigen expression in mouse embryonic fibroblasts after co-culturing with blood leukocytes from the children (Sharma et al., 1991). We have directly identified JEV antigen (NS3) expression in, and isolated JEV from, monocytes during the first 7 day culture period. Moreover, we showed that JEV could cause a persistent infection for more than 3 weeks in monocytes as detected by a quantitative RT-PCR.

We also found that the JEV titres in monocytes detected by RT-PCR were 1- to 3-log scale higher than those detected by p.f.u. assay, especially in the early initial infection period (Table 2). This indicates that non-viable JEV is present at much higher levels than viable JEV in human monocytes. In other words, this also suggests that certain mediators from JEV-infected monocytes may inactivate or, in part, kill the virus, resulting in poor transmission of JEV from the blood of human beings.

Pathogenesis of JE is still controversial. Two hypotheses are currently proposed. Studies with murine brain cell culture have shown that neuron cells, especially developing neurons, are the major infection sites (Kimura-Kuroda et al., 1993). JEV-infected mouse brain cells revealed impaired organelle functions before cell death (Hase et al., 1990b), suggesting a direct virus-induced cell cytotoxicity. In contrast, some evidence demonstrates that damage of the CNS by JEV infection was indirectly mediated by immune reactions including antibody-mediated cytotoxicity or suppressor T cell activity (Burke et al., 1985; Webb & Smith, 1966). An understanding of the disease pathogenesis is crucial for prevention of neurologic sequelae mediated by JE in human beings. In support of direct cell cytotoxicity of JEV infections in humans, our study showed that JEV caused a massive infection in four human NB cell lines, followed by cell apoptosis in 2 days and almost 100% cell death in 5 days. In addition, the fact that JEV can be only isolated in brain tissues during autopsies, but not cerebrospinal or tissue fluids (Iwasaki et al., 1986; Mukherji & Biswas, 1976), also suggests that active JEV replication is correlated to cerebral morbidity and mortality in JEV infections. In fact, evidence accumulated indicates that the titres of JEV neutralizing antibody were inversely correlated to JEV susceptibility in human beings (Burke et al., 1985). Moreover, the case-fatality rate of JEV infections in dengue immunes is only one-third that of non-immunes (Grossman et al., 1974; Hammon et al., 1958b). These results suggest that JEV may specifically target NB cells and that specific or cross-reactive antibodies are protective but not offensive in human JEV infections. Further studies will investigate whether JEV-mediated programmed cell death of NB cells is preventable or ameliorable. An effective control of JEV-mediated programmed cell death may be useful in treating patients with JE.

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REFERENCES


