Molecular characterization of an Indian isolate of Japanese encephalitis virus that shows an extended lag phase during growth

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The biological properties of an Indian isolate (GP78) of Japanese encephalitis virus (JEV) were characterized in tissue-cultured cells and mice and these were compared with the JaOArS982 strain from Japan. The GP78 strain had a markedly extended lag phase during its growth in porcine stable kidney (PS) cells. There were no obvious defects in the penetration of GP78 into PS cells. However, viral RNA and protein synthesis were significantly delayed in GP78-infected PS cells. Fusion-from-within assays carried out in C6/36 cells indicated that GP78 was less fusogenic than the JaOArS982 strain of JEV. Moreover, maximum fusion in GP78-infected cells occurred at pH 5.5, whereas JaOArS982-infected cells showed maximum fusion at pH 6.0. These results suggested that there may be a lesion in the virus–cell fusion process. The GP78 strain also showed delayed growth in brains of 1-week-old BALB/c mice. Although JEV GP78 was as virulent as the JaOArS982 strain in these mice, the appearance of clinical symptoms of JEV infection was delayed by a day in mice infected with the GP78 strain and these animals showed an increased average survival time. Comparison of the nucleotide sequences of the GP78 and the JaOArS982 strains of JEV identified a number of amino acid substitutions in structural proteins. Of these, a Thr → Met substitution at residue 76 of the envelope protein is predicted to be causally associated with the altered biology of the GP78 strain during growth.

Introduction

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus responsible for acute encephalitis in humans, with high fatality rates ranging from 20% to as high as 50% (Burke & Leake, 1988; Vaughn & Hoke, 1992). The genome of the virus is a plus-sense single-stranded RNA of about 11 kb that contains a single open reading frame (ORF) encoding a polyprotein that is subsequently cleaved into a number of structural and non-structural proteins. The 5’ one-third of the ORF encodes three viral structural proteins, namely, capsid (C), membrane (M) and envelope (E), while the 3’ two-thirds encodes non-structural proteins designated NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (reviewed in Chambers et al., 1990; Rice, 1996). Amino acid sequences of these proteins have been predicted on the basis of the nucleotide sequence of the viral RNA and functions have been attributed on the basis of their homology to proteins of known function or on the basis of experimental evidence. For example, the E protein induces neutralizing antibodies that provide protection against virus challenge in animals (Mason et al., 1991; Huong et al., 1993; Seif et al., 1995; Konishi et al., 1998). The protein is believed to be involved in virus attachment, fusion, penetration and haemagglutination. These properties of the E protein may determine virus host-range and cell tropism and thus could have a bearing on virus virulence and attenuation (Monath & Heinz, 1996; reviewed in McMinn, 1997).

In order to understand the genetic determinants of JEV virulence, we have been studying JEV isolates that show marked differences in their biological properties that have the potential to affect virus virulence. In this process, we have characterized an Indian isolate, GP78, of JEV that had an extended lag phase during its growth in cultured cells, resulting in smaller plaques. A similar phenomenon of delayed growth in mouse brain coupled with slower development of clinical symptoms was also noted. In this paper, we compare the biology of the GP78 strain with the JaOArS982 strain of JEV in tissue-cultured cells and in mice. We present data that indicate that the extended lag phase during the growth of JEV GP78 may be due to a lesion in the virus–cell fusion event. We have compared the amino acid sequences of the E proteins of the GP78 and JaOArS982 isolates to identify a Thr → Met substitution...
substitution at residue 76, which is spatially close to the predicted fusion sequence of the virus.

Methods

**Viruses and cells.** JEV isolate 78668A, obtained from the post-mortem brain tissue of a 38 year old female patient from an epidemic of encephalitis that broke out in Gorakhpur, India, in October 1978 (Mathur et al., 1982), was kindly provided by Asha Mathur (King George’s Medical College, Lucknow, India). The virus was plaque-purified twice on monolayers of porcine stable kidney (PS) cells and the isolate was subsequently referred to as the GP78 strain. The complete genome sequence of the GP78 strain is available in GenBank (accession no. AF075723). The JaOArS982 strain of JEV was obtained from Akira Igarashi (University of Nagasaki, Japan). Baby hamster kidney (BHK-21) and PS cells were obtained from NCCS, Pune, India. Cells were grown at 37 °C in Eagle’s minimal essential medium (MEM) supplemented with 10% foetal calf serum (FCS).

**Penetration assays.** The rate of virus penetration into cells was measured in terms of the rate at which resistance to neutralization by added polyclonal antibody was acquired by the infecting virus (Olmsted et al., 1986; Vrati et al., 1996). Briefly, PS monolayers in 60 mm dishes were inoculated with approximately 100 p.f.u. virus and incubated with intermittent shaking at 37 °C. At various times after infection, the inoculum was removed and the monolayers were overlaid with MEM containing 4% FCS, 1% low-melting-point agarose and a cocktail of antibody–antimycotic solution (Gibco) containing penicillin, streptomycin and amphotericin B. Plates were incubated at 37 °C for 3–4 days until plaques became visible. To allow counting of the plaques, the cell monolayer was stained with crystal violet after fixing the cells with 10% formaldehyde.

**Fusion-from-within (FFWI) assay.** C6/36 cells were used for the FFWI assays to study the fusogenic property of the virus and to determine the pH threshold of fusion activity (Randolph & Stollar, 1990). Briefly, cell monolayers were infected with JEV at an m.o.i. of ~ 100. At 72 h post-infection (p.i.), the monolayers were incubated in culture medium adjusted to the indicated pH for 15 min and returned to neutral pH for a further 60 min following by fixing and staining. The numbers of nuclei and cell bodies in three high-power fields were counted and the extent of fusion was calculated as the fusion index, 1 – (number of cells/number of nuclei).

**Virus growth in mice.** One-week-old BALB/c mice of either sex were injected intra-cerebrally with approximately 100 p.f.u. virus. Groups of three mice were sacrificed at each time-point by cervical dislocation and brain tissue was removed and stored at −70 °C. For virus titration, the brain tissue was homogenized in ice-cold MEM with a teflon-coated glass homogenizer to give a 20% suspension.

**Virulence assay.** The LD<sub>50</sub> of the virus and average survival time (AST) of infected mice were used to define the virulence of the virus. One-week-old BALB/c mice of either sex were used. For LD<sub>50</sub> determination, groups of 8–10 mice were injected intra-cerebrally with 30 µl samples of 10-fold virus dilutions in MEM. The lethal dose was calculated according the method of Reed & Muench (1938). The incidence of clinical signs and deaths were recorded every day. The AST is the arithmetic mean of the life span of all mice that died subsequent to the intra-cerebral inoculation of 100 p.f.u. virus.

Results

**Virus growth in tissue-cultured cells.** JEV GP78 was cloned by plaque purification of isolate 78668A on PS cells. At 96 h p.i., both GP78 and its parent stock 78668A made smaller plaques of < 1 mm diameter compared with the JaOArS986 strain, the plaque size of which was ~ 1.5 mm. A similar difference in plaque size was observed on BHK monolayers; GP78 made plaques of ~ 1 mm diameter whereas JaOArS986 plaques were ~ 2 mm in diameter. The plaque size of both strains increased further if the plates were incubated for longer. This suggested that the GP78 strain may be growing slower in the PS or BHK cells compared with the JaOArS982 strain. We have, therefore, studied the growth kinetics of the two viruses in tissue-cultured cells (Fig. 1). In PS cells, at an m.o.i. of ~ 1, the JaOArS982 strain had a lag period of about 12 h, after which there was a gradual increase in virus titre until 30 h p.i., when a peak titre of ~ 6 × 10<sup>7</sup> p.f.u./ml was reached. In contrast, the lag phase for GP78 lasted for 36 h, after which the virus grew
Kinetics of virus penetration

The 24 h-extended lag period of the GP78 virus could result from a lesion in virus attachment and/or penetration of cells. The virus penetration rate was studied by measuring the amount of virus that had gone inside the cell and had established a productive infection resulting in plaque formation. At a given time-point, the infectivity of the virus remaining outside the cells was destroyed either by neutralizing the virus with antibody or by exposure to low pH. Fig. 2 shows that the GP78 virus penetrated the PS cells faster than the JaOArS982 strain, although by 60 min p.i. the difference in the amounts of the two viruses that had penetrated the PS cells was statistically insignificant. A similar higher rate of penetration for the GP78 virus was observed when using the low-pH citrate buffer protocol (data not shown).

Kinetics of viral RNA and protein synthesis

The extended lag phase of the GP78 virus could be due to altered kinetics of viral RNA replication and/or protein synthesis. The kinetics of viral RNA synthesis were studied by following actinomycin D-resistant RNA synthesis in virus-infected cells. Fig. 3(a) shows that in JaOArS982-infected PS cells there was a clear increase in the incorporation of [5-3H]uridine over the mock-infected cells between 18 and 24 h p.i., whereas at this stage no viral RNA synthesis was detected in GP78-infected cells. Virus-specific RNA synthesis in GP78-infected cells was clearly detectable between 36 and 48 h p.i.
The peak of viral RNA synthesis in JaOArS982-infected cells occurred at 24 h p.i., whereas it was seen at 48 h p.i. in cells infected with GP78.

The kinetics of viral protein synthesis were studied by following the synthesis of the NS1 protein in virus-infected cells (Fig. 3b). The NS1 protein could be detected in JaOArS982-infected PS cells at 24 h p.i. At this stage, NS1 synthesis in GP78-infected cells was only barely detectable. At 36 h p.i., and subsequently at 48 h p.i., JaOArS982- and GP78-infected cells showed similar levels of NS1 synthesis.

Cell-to-cell fusion in JEV-infected cells

It is possible that the delayed RNA and protein synthesis in JEV GP78-infected cells, as seen above, may be due to less-efficient fusion of the viral envelope with endosomal membranes, resulting in slower/delayed availability of viral RNA in the cytoplasm for subsequent steps of replication. To determine whether this was the case, FFWI assays were carried out at different pH values with C6/36 cells infected with GP78. The extent of fusion, as measured by the fusion index, was significantly lower in GP78-infected cells compared with JaOArS982-infected cells (Fig. 4). In addition, maximum FFWI in JaOArS982-infected cells was seen at pH 6.0, whereas GP78-infected cells showed maximum fusion at pH 5.5. These differences in the fusogenicity and the pH threshold of fusion of the two viruses were reproducible.

Virulence in 1-week-old mice

The virulence of JEV GP78 was studied in 1-week-old mice to see if the extended lag phase of this strain seen in tissue-cultured cells had any effect on its in vivo biological properties. Mice inoculated intra-cerebrally with the GP78 strain developed classical symptoms of JEV infection. However, the onset of clinical symptoms was delayed by at least a day, although there were no obvious differences in the severity of clinical symptoms. Thus, the AST for GP78-infected mice was 8.1 ± 0.46 days, against 6.4 ± 0.40 days for JaOArS982-infected mice. The difference between the ASTs of the two viruses was statistically significant (P < 0.001). Even though the AST of mice inoculated with the GP78 strain was higher than that of those inoculated with the JaOArS982 strain, the two strains were equally neurovirulent; their LD50 values were around 0.5 p.f.u. Both viruses were non-lethal to mice when inoculated by the peripheral route.

Virus growth in mouse brain

Virus growth was studied in the brains of 1-week-old mice infected intra-cerebrally with JEV GP78, to examine whether the extended lag phase seen in vitro was reflected in virus growth in vivo. Fig. 5 shows the kinetics of growth of the GP78 and JaOArS982 strains of JEV in mouse brain. The JaOArS982 strain had begun to grow by day 1 p.i., when titres of ~ 200 p.f.u./g were recorded. Virus titres increased gradually until day 4 p.i., when peak titres of ~ 107 p.f.u./g were reached. No virus was detectable on day 1 p.i. in brains of mice inoculated with the GP78 strain of JEV. It was first detected on day 2 p.i., after which its titre increased gradually to peak on day 5 p.i. The mean titres of the GP78 virus were consistently lower than those of the JaOArS982 strain until day 4 p.i., although the differences were not statistically significant (P > 0.1).
Discussion

The question of which properties of a virus determine its degree of virulence is important for the development of effective control measures against the virus. Two important properties that can affect the virulence of a virus are its growth rate and tissue tropism. These properties can be altered, at least in part, by changes in structural proteins, which may be involved in receptor recognition, virus attachment and penetration or membrane fusion to initiate virus infection. We have characterized an Indian strain of JEV that shows an extended lag phase during its growth in cultured cells. This increase in the length of the lag phase was not due to slow attachment/penetration of the virus with the host cells, as the GP78 strain penetrated PS cells faster than the JaOArS982 strain. Subsequent events such as viral RNA and protein synthesis were also delayed in GP78-infected cells. However, once the synthesis of these macromolecules had started, there were no obvious defects in the two processes.

Flaviviruses enter cells by receptor-mediated endocytosis followed by fusion of the viral envelope with the cell membrane, which occurs in the low-pH environment of the endosome. This results in the release of the virion capsid into the cytoplasm. Subsequent events involve unfolding of the capsid, making the viral RNA available in the cytoplasm for translation and replication. There was no lesion in the very early stages of GP78 infection, as the endocytosis rate, measured in terms of virus penetration rate, was in fact faster compared with the JaOArS982 strain. However, the synthesis of viral proteins was delayed in GP78-infected cells, as seen by the kinetics of JEV NS1 synthesis. Similarly, the kinetics of viral RNA synthesis showed that the start of viral genome replication was delayed for the GP78 strain compared with the JaOArS982 strain, although the rate of RNA synthesis in these two cases, as indicated by the slope of the graph, was not much different. These observations suggest that the GP78 strain may have a lesion either in the fusion event, resulting in delayed availability of the viral genome for translation and replication, or in the translation process itself, resulting in delayed synthesis of viral non-structural proteins that are needed for the replication of the viral genome. In order to clarify this, we carried out FFWI assays that may reflect the ability of the two viruses to carry out fusion with the endosomal membranes to release their genome into the cytoplasm. These assays indicated that GP78 had poor fusion ability compared with the JaOArS982 strain of JEV. Furthermore, maximum fusion in GP78-infected cells was achieved at a lower pH than that in JaOArS982-infected cells.

In the case of the flavivirus replication, translation initiates near the 5′ end of the genome (Rice, 1996). Thus, the nucleotide sequence of the 5′ non-coding region of the genome has the potential to affect viral protein translation. We have established the complete genome sequence of the GP78 strain of JEV (GenBank accession no. AF075723). When we compared this sequence with the JEV JaOArS982 sequence (Sumiyoshi et al., 1987), we found no mutations in the 5′ non-coding region of the GP78 genome. However, there were a number of amino acid substitutions in the C, M and E proteins of the GP78 strain (Table 1), and many of these changes were non-conservative with the potential to affect the conformation and thus the function of the proteins.

The flavivirus E protein mediates membrane fusion and virus entry into the cell (Monath & Heinz, 1996; reviewed by McMinn, 1997). On the basis of the deduced amino acid

<table>
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<th>Protein</th>
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<td>657</td>
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<tr>
<td></td>
<td>692</td>
<td>Lys → Arg</td>
<td>Conservative</td>
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* Amino acid positions are according to the sequence of the JEV JaOArS982 polyprotein (Sumiyoshi et al., 1987).
† All changes except the following were designated as non-conservative: Arg ↔ Lys, Ser ↔ Thr, Asp ↔ Glu, Gln ↔ Asn, Val ↔ Leu ↔ Ile ↔ Met, Ala ↔ Gly, Ala ↔ Val and Tyr ↔ Phe.
sequence of the E protein of various flaviviruses, together with monoclonal antibody-based topological analysis, a two-dimensional model of the flavivirus E protein has been developed (Mandl et al., 1989). It contains a highly conserved, hydrophobic peptide between residues 98–111 that has been predicted to be involved in fusion activity (Roehrig et al., 1989). Compared with the JaOArS982 strain of JEV, the E protein of the GP78 strain contained four amino acid substitutions (Table 1). We have compared the predicted two-dimensional structures of the E proteins from the two strains (Fig. 6a). No changes in the secondary structure of the E protein were seen around Ile$^{169}$ → Val or Thr$^{363}$ → Ala substitutions. The other two substitutions, Thr$^{276}$ → Met and Lys$^{598}$ → Arg, seemed to affect the protein conformation (Fig. 6a). Interestingly, the predicted two-dimensional structure of the GP78 E protein shows that the Thr$^{276}$ → Met substitution in the GP78 strain could affect the conformation of the fusion peptide and its neighbouring amino acids (Fig. 6a). Thus, a stretch of amino acids (positions 79–93) just upstream of the fusion peptide, which had a random-coil conformation in the JaOArS982 strain, is likely to adopt a predominantly helical conformation in the GP78 strain. In addition, the fusion peptide of GP78 is predicted to have amino acids in a random-coil conformation, whereas this sequence in the JaOArS982 strain had amino acids in both random-coil and β-strand conformations.

X-ray crystallographic studies have established the three-dimensional structure of the external domain of the E protein of tick-borne encephalitis virus (TBEV) (Rey et al., 1995). There is about 40% amino acid sequence identity between the E protein sequences of different flaviviruses such as TBEV, dengue virus, yellow fever virus and JEV, and all 12 cysteine residues are conserved (Heinz & Roehrig, 1990). The three-dimensional structure of the E protein of TBEV could, therefore, be taken to show the folded structure of all flavivirus E proteins for the analysis of biological properties (Rey et al., 1995). The three-dimensional structure of the TBEV E protein shows that the cysteine residues at positions 74 and 116 form a disulphide bond, giving rise to a loop containing the putative fusion sequence (amino acids 98–111) predicted to be involved in flavivirus fusion with the cell membrane to initiate virus infection (Rey et al., 1995). It may be interesting to note that amino acid 76 and the stretch of amino acids showing altered conformation in the predicted secondary structure of the GP78 E protein are in close proximity to the putative fusion sequence (aa 76–116). No changes in the secondary structure of the E protein were seen around Ile$^{169}$ → Val or Thr$^{363}$ → Ala substitutions. The other two substitutions, Thr$^{276}$ → Met and Lys$^{598}$ → Arg, seemed to affect the protein conformation (Fig. 6a). Interestingly, the predicted two-dimensional structure of the GP78 E protein shows that the Thr$^{276}$ → Met substitution in the GP78 strain could affect the conformation of the fusion peptide and its neighbouring amino acids (Fig. 6a). Thus, a stretch of amino acids (positions 79–93) just upstream of the fusion peptide, which had a random-coil conformation in the JaOArS982 strain, is likely to adopt a predominantly helical conformation in the GP78 strain. In addition, the fusion peptide of GP78 is predicted to have amino acids in a random-coil conformation, whereas this sequence in the JaOArS982 strain had amino acids in both random-coil and β-strand conformations.

Fig. 6. Mutations in the E protein of JEV GP78 and their effect on its conformation. (a) The nucleotide sequence of the genome segment encoding the E protein was translated to give the predicted amino acid sequence of the E protein. The secondary structure of the E protein was predicted by using the 3D–1D compatibility algorithm (to et al., 1997). The upper panel shows the predicted secondary structure of the E protein of the JaOArS982 strain while the lower one shows that of the GP78 strain. (AA), Amino acid sequence; (SST), predicted secondary structure; H, α-helix; E, β-strand; C, random coil. The putative fusion peptide (residues 98–111) is boxed. Mutations in the GP78 E are encircled. Changes in the predicted secondary structure of the GP78 E are underlined. (b) A ribbon diagram of the external domain (amino acids 1–395) of the E polypeptide of TBEV (Rey et al., 1995) was drawn by using coordinates derived from the Brookhaven protein structure database (accession number 1SVB). Locations are indicated of amino acid residues 98–111, representing the putative fusion sequence, and other amino acids described in the text.
protein (Rey et al., 1995; McMinn et al., 1996; McMinn, 1997; see Fig. 6 b). In view of these observations and the likely effect of the Thr\(^{26}\) → Met substitution on the conformation of the putative fusion sequence of GP78, a causal link between this mutation and an altered fusion process in GP78 infection can be suggested.

An interesting parallel to the delayed growth of GP78 in tissue-cultured cells was seen in mice. The GP78 strain grew more slowly than the JaOArS982 strain; its titre in mouse brain on day 1 p.i. was not detectable, whereas the JaOArS982 strain had started to grow by then. There were no differences in the neurovirulence of the two viruses in 1-week-old BALB/c mice, although mice inoculated intra-cerebrally with the GP78 strain of JEV showed a delayed appearance of clinical symptoms and an extended AST. These may perhaps be related to the slower growth of GP78 in mouse brain.

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References


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