A mouse-attenuated envelope protein variant of Murray Valley encephalitis virus with altered fusion activity

Peter C. McMinn, Ronald C. Weir and Lynn Dalgarno

Division of Biochemistry and Molecular Biology, School of Life Sciences, Australian National University, Canberra, ACT 0200, Australia

Murray Valley encephalitis virus (MVE) is a member of the family Flaviviridae, a group of small, lipid-enveloped, plus-stranded RNA viruses, many of which are important human pathogens. Flaviviruses enter cells by receptor-mediated endocytosis (Marsh & Helenius, 1989), with fusion of the viral envelope and cell membrane occurring in the low pH environment of the endosome (Gollins & Porterfield, 1985, 1986a). The major envelope glycoprotein (E) of the virion is thought to initiate fusion between the envelope and endosomal membrane following low pH-induced conformational change in E which renders the protein ‘fusion active’ (Guirakhoo et al., 1989; Allison et al., 1995). However, a detailed understanding of flavivirus entry into cells during productive infection has not yet been developed.

In a previous study, McMinn et al. (1995) showed that the MVE neutralization (N)-escape variant BHv1, altered in E at residue 277 (Ser to Ile), had lost haemagglutination activity and was of reduced neuroinvasiveness in 21-day old Swiss mice compared to both the wild-type BH3479 (Marshall et al., 1982) and another N-escape variant BHv2 (E-277, Ser to Asn). A Japanese encephalitis virus (JE) N-escape variant, mutated in E at residue 270 (Ile to Ser), a site close to E-277 in MVE, had also lost haemagglutination activity and was of low neuroinvasiveness in mice (Cecilia & Gould, 1991). Thus a region encompassing residues 270–277 in the E proteins of MVE and JE appears to be a determinant of haemagglutination and neuroinvasiveness in mice. Although the nature of the link between changes in haemagglutination and neuroinvasiveness is unknown, the observation that growth of BHv1 in Vero cells was less than that of BH3479 during the early phase of infection (McMinn et al., 1995) suggests that a defect in an early event in BHv1 replication may be causally linked to the loss of neuroinvasiveness in mice.

To further examine this possibility, the abilities of MVE-BH3479 and -BHv1 to initiate replication were compared by measuring rates of virus-specific RNA synthesis in infected Vero cells (Fig. 1a). MVE stocks were derived from Aedes albopictus (C6/36) cell supernatants. Cells were mock-infected or infected with MVE (m.o.i. 1) and labelled (2 h) in medium containing [3H]uridine (25 μCi/ml) and actinomycin D (10 μg/ml) at the indicated times. Cytoplasmic lysates of the labelled cells were spotted onto glass fibre discs, washed with 5% trichloroacetic acid (TCA) and TCA-precipitable [3H]-labelled RNA was determined. Between 10 and 12 h post-infection (p.i.), the rate of RNA synthesis was similar in BH3479-, BHv1- and mock-infected cells. Between 12 and 18 h p.i., the rate of virus-specific RNA synthesis in BH3479-infected cells increased to a level approximately four times higher than in mock-infected cells. A more gradual rise in virus-specific RNA synthesis occurred in BHv1-infected cells from 12 h p.i., the maximal rate (at 20–22 h p.i.) being approximately threefold higher than in mock-infected cells. At 14 h p.i., cumulative titres of BH3479 released from the Vero cells were 20-fold greater than for BHv1 but the difference in extracellular virus (EV) titre became progressively smaller from 16 to 22 h p.i. (Fig. 1b). Delayed synthesis of virus-specific...
RNA by BHv1 suggested that this virus enters the cytoplasm of Vero cells more slowly than does BH3479.

As we had previously shown that rates of BHv1 and BH3479 penetration of Vero cell surfaces are similar (authors, unpublished data), it is possible that the delayed BHv1 RNA synthesis is due to less efficient fusion of the viral envelope with endosomal membranes during receptor-mediated endocytosis. To determine whether the fusion activities of BH3479, BHv1 and BHv2 correlated with rates of initiation of RNA synthesis, fusion-from-within (FFWI) assays (Randolph & Stollar, 1990) were used. Monolayers of C6/36 cells were mock-infected or infected with MVE (m.o.i. ~ 10); at 48 h p.i. the cells were incubated in medium adjusted to the indicated pH values for 15 min, returned to neutral pH for a further 30 min, then fixed and stained. The numbers of nuclei and cell bodies in three high-power fields were counted, and the extent of fusion expressed as a fusion index (FI). Maximum FFWI in BHv1-infected cells was at pH 5.5 (FI = 0.43), and was significantly lower than in BH3479- and BHv2-infected cells at pH 5.5 (FI > 0.9) (Fig. 2a). The threshold for fusion by BHv1 was at pH 6.2, 0.2 pH units lower than for BH3479 and BHv2 (Fig. 2a); this difference in pH threshold was consistent in repeat assays. Thus BHv1-infected cells showed a lower FI after exposure to mildly acidic pH than either BH3479 or BHv2. The poor fusion activity was not due to reduced E protein expression, as rates of E protein synthesis were similar in BH3479-, BHv1- and BHv2-infected cells (data not shown).

Randolph & Stollar (1990) showed that FFWI in flavivirus-infected C6/36 cells was inhibited by an anti-E protein monoclonal antibody (MAb). To determine which MVE E protein epitopes were responsible for fusion, MAbs 4B6C-2, 4B5A-2, 4B3B-6 (Hawkes et al., 1988), 6B4A-10 and 6A4D-1 (Guirakhoo et al., 1992), which define epitopes E-lc, E-ld, E-5, E-7 and E-8 respectively, were tested for the ability to inhibit FFWI in MVE-infected C6/36 cells. Epitopes E-1c, E-1d, E-5 and E-8 form a single neutralization domain on E; epitope E-7
is part of a separate neutralization domain (McMinn et al., 1995). At 48 h p.i., MVE-infected cells were incubated with serial twofold dilutions of anti-MVE immune mouse ascitic fluid (IMAF) or MAbs for 15 min; subsequently, the cells were exposed to low pH medium (pH 6.0) containing identical dilutions of IMAF or MAbs for 15 min and then incubated for a further 30 min at pH 7.4; the cells were then fixed, stained and examined by light microscopy. The anti-E-5 and anti-E-8 MAbs inhibited FFWI in BH3479-infected cells at dilutions of 1:1280 and 1:640 respectively (Table 1). In contrast, the anti-E-1c, anti-E-1d and anti-E-7 MAbs did not inhibit FFWI at a 1:20 dilution. Anti-MVE IMAF inhibited FFWI in BH3479-, BHv1- and BHv2-infected cells. The titres for complete fusion inhibition (approx. 1:160) were similar, and thus anti-MVE IMAF did not distinguish between the variants.

Differences in haemagglutination (McMinn et al., 1995) and in pH threshold and extent of FFWI (see above) suggested that BH3479 and BHv1 virion infectivity may differ in pH sensitivity. To test this, the viruses were incubated at the indicated pH values for 15 min and then diluted 100-fold (at pH 8.0) and residual infectivity determined (Fig. 2b). BH3479 and BHv2 Vero-p.f.u. titres were unaffected at pH 6.6 and above. In contrast, BHv1 was reproducibly more acid sensitive than BH3479 and BHv2, and underwent a ~10-fold reduction in infectivity at pH 6.6 compared to pH 7.0. Between pH 6.4 and pH 6.0, the infectivities of BH3479, BHv1 and BHv2 decreased ~1000-fold, in a similar pH range to the induction of FFWI in MVE-infected cells (Fig. 2a).

The poor growth of the mouse-attenuated N-escape variant BHv1 during the early phase of its replication cycle in Vero cells (McMinn et al., 1995) suggested that the mutation (E-277, Ser to Ile) responsible for loss of virulence in mice may also cause delayed entry of virus into cells or delayed initiation of viral replication. Rates of virus-specific RNA synthesis and release of extracellular virus were lower in BHv1-infected Vero cells than for BH3479 in the period from 14 to 20 h p.i., suggesting that the kinetics of viral RNA entry into the cytoplasm and initiation of viral replication by BHv1 is slower than for BH3479. In the early steps of virus entry, BHv1 acquired resistance to antibody neutralization at the same rate as BH3479. This implies that endocytosis occurred at similar rates for both viruses. A subsequent step in the flavivirus entry pathway which may be growth-rate limiting involves fusion of the viral envelope with the endosomal membrane. An indication that BHv1 had a defect in its ability to interact with cellular membranes arose from the observation of its failure to haemagglutinate (McMinn et al., 1995). This has been supported by FFWI assays in which the rate and extent of cell-to-cell fusion by BHv1 was greatly reduced compared to BH3479 (Fig. 2a). Although this result has only been observed in mosquito (C6/36) cells, a defect in low pH-dependent fusion of the viral envelope with endosomal membranes during receptor-mediated endocytosis in vertebrate cells may explain the lower growth rate observed early in the replication cycle of BHv1.

Polyclonal neutralizing antibodies prevent fusion of the viral envelope and endosomal membrane during entry of West Nile virus into murine macrophage cells (Gollins & Porterfield, 1986b). We have extended this observation to show that MAbs which define epitopes E-5 and E-8 inhibit fusion in MVE-infected C6/36 cells. Interestingly, the anti-E-1c MAb strongly inhibits haemagglutination by MVE whereas the anti-E-5 and anti-E-8 MAbs have no haemagglutination inhibition activity (Hawkes et al., 1988). This apparent inverse relationship between haemagglutination inhibition and fusion inhibition by MAbs whose epitopes form part of a complex neutralization domain on E (McMinn et al., 1995), suggests that epitopes E-1c and E-5/E-8 may represent membrane attachment and fusion determinants on E respectively. Epitope E-8 has been mapped to residues within the sequence 201–224

### Table 1. Inhibition of MVE-induced cell-to-cell fusion by polyclonal antibody and by E protein monoclonal antibodies

<table>
<thead>
<tr>
<th>Virus</th>
<th>IMAF</th>
<th>4B3B-6 (anti-E-5)</th>
<th>6B4A-10 (anti-E-8)</th>
<th>4B6C-2 (anti-E-1c)</th>
<th>4B5A-2 (anti-E-1d)</th>
<th>6A4D-1 (anti-E-7)</th>
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<tr>
<td>BH3479</td>
<td>160</td>
<td>1280</td>
<td>640</td>
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<tr>
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<td>10240</td>
<td>10240</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
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<tr>
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<td>5120</td>
<td>10240</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
</tr>
</tbody>
</table>

* Anti-MVE IMAF was prepared in female BALB/c mice following Tikasingh et al. (1966).
of the MVE E protein (Lee 1990; Guirakhoo et al., 1992); epitope E-1c has discontinuous determinants located at residues 126, 128 and 274, 276 and 277 (McMinn et al., 1995). The residues which define both epitopes are in domain II of E as deduced from the crystallographically determined structure of the tick-borne encephalitis virus (TBE) E protein (Rey et al., 1995).

The mutation in the BHv1 E protein which results in impaired fusion activity provides further evidence linking this function to the flavivirus E protein. The BHv1 E protein has isoleucine at position 277 compared to serine in the parental virus and asparagine in BHv2, both of which have identical fusion properties. In other flaviviruses there is evidence for a conformational change in E in the pH range which activates fusion (Guirakhoo et al., 1989; Roehrig et al., 1990). Exposure of TBE to mildly acidic pH altered binding affinities of MAb s (Guirakhoo et al., 1989) and led to reorganisation of the dimer form of the E protein into trimers (Allison et al., 1995). The substitution of a bulky hydrophobic amino acid (Ile) in the BHv1 E protein for the small polar amino acids (Ser, Asn) that occur in the same location in BH3479 and in BHv2 presumably exerts its effect by influencing the fusion-regulating structural change in the E protein. It is notable that E-277 is in a region with hinge-like characteristics in the structure of the TBE E protein (Rey et al., 1995). Furthermore, E-277 is spatially close to the highly conserved putative 'fusion' peptide (Roehrig et al., 1989).

The data presented in this study support the hypothesis that the mutation at residue 277 in E (Ser to Ile) causes delayed entry of BHv1 nucleocapsids into cells by interfering with fusion between the viral envelope and endosomal membrane during receptor-mediated endocytosis. Thus BHv1 nucleocapsids may enter the host-cell cytoplasm with low efficiency, resulting in a low rate of viral RNA synthesis early in the replication cycle. The marked attenuation of BHv1 neuroinvasiveness in mice (McMinn et al., 1995) may reflect the impact of this replication defect in retarding growth and spread from the site of infection to target tissues. This in turn may allow the host more time to mount a protective immune response.

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References


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