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Murray Valley Encephalitis Virus Envelope Protein Antigenic Variants with Altered Hemagglutination Properties and Reduced Neuroinvasiveness in Mice

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Neutralization escape variants of Murray Valley encephalitis virus were selected using a type-specific, neutralizing, and passively protective anti-envelope protein (E) monoclonal antibody (4B6C-2) which defines epitope E-1c. Nucleotide sequence analysis revealed single nucleotide changes in the E genes of 15 variants resulting in nonconservative amino acid substitutions in all cases. One variant had a three-nucleotide deletion in the E gene which resulted in loss of serine at residue 277. Changes were clustered into two separate regions of the E polypeptide (residues 126–128 and 274–277), indicating that E-1c is a discontinuous epitope. One variant (BHv1), altered at residue 277 (Ser → Ile), failed to hemagglutinate across the pH range 5.5–7.5, in contrast to parental virus and the other escape variants which hemagglutinated at an optimal pH of 6.6. BHv1 was also of reduced neuroinvasiveness in 21-day-old mice following intraperitoneal inoculation compared to the other viruses. Parental virus and the neutralization escape variants grew equally well in both vertebrate and invertebrate cell cultures, indicating that the reduced neuroinvasiveness of BHv1 was not due to a major abnormality of replication.

INTRODUCTION

Murray Valley encephalitis virus (MVE) is a member of the family Flaviviridae, a group of small, lipid-enveloped plus-stranded RNA viruses (Westaway *et al.*, 1985), many of which are important human or animal pathogens (Monath, 1986). MVE is one of several well-characterized flaviviruses including Japanese encephalitis (JE), kunjin, St. Louis encephalitis (SLE), and West Nile viruses grouped in the JE serocomplex on the basis of antigenic and biological properties. MVE is most closely related genetically and in clinical effects to the more prevalent Japanese encephalitis virus, which is of major public health significance over a wide area of Asia from Japan to India. The known distribution of MVE is confined to certain regions of Australia and Papua New Guinea, where it is responsible for endemic cases of encephalitis in tropical regions and occasional epidemics in southeastern Australia (French, 1973; Doherty, 1977).

The major virion envelope protein (E) of flaviviruses is the dominant antigen in eliciting neutralizing antibodies and protective immune responses (Heinz and Roehrig, 1990). It also has hemagglutination activity and is the putative viral cell receptor-binding protein and mediator of membrane fusion in cell entry (Chambers *et al.*, 1990). In recent years the antigenic structure of the E protein of several flaviviruses has been analyzed through the use of monoclonal antibodies (mAbs). Topological epitope maps derived from competitive binding studies have indicated that most epitopes cluster into three distinct antigenic domains, A, B, and C (Heinz *et al.*, 1983). This information together with the recognition that all 12 cysteine residues are conserved (Nowak and Wengler, 1987) and that hydrophilicity profiles are very similar led to the proposal of a structural model of the flavivirus E protein (Mandl *et al.*, 1989).

Neutralization escape variants selected with mAbs have been characterized as single-amino-acid mutants which identify dominant amino acid determinants of neutralization epitopes of a number of flaviviruses: yellow fever virus (Lobigs *et al.*, 1987), JE (Mason *et al.*, 1989; Cecilia and Gould, 1991), tick-borne encephalitis virus (TBE; Holzmann *et al.*, 1989), louping ill virus (Jiang *et al.*, 1993), and dengue virus type 2 (Lin *et al.*, 1994). Some of these mutations have been associated with effects on other functions of the E protein. Certain neutralization escape variants of TBE (Holzmann *et al.*, 1990) and JE (Cecilia and Gould, 1991) show attenuation of virulence in mice suggesting that the corresponding neutralization epitopes may also function as virulence determinants. Cecilia and Gould (1991) also observed escape variants of JE that had lost the hemagglutination function.

In this communication, we describe the structural and

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functional analysis of antigenic epitopes of the MVE E protein using nine E-specific mAbs. Both MVE and SLE share an epitope (E-1c) which elicits mAbs with very high neutralization, hemagglutination-inhibition, and passive-protection activity compared to other E-specific mAbs (Hawkes *et al.,* 1988; Roehrig *et al.,* 1983; Mathews and Roehrig, 1984). Amino acid determinants of epitope E-1c of MVE have been identified by the selection of neutralization escape variants and sequencing of their structural protein genes. The mouse virulence, hemagglutination properties, and growth kinetics in cell culture of the E-1c escape variants were examined.

MATERIALS AND METHODS

Virus strains and cells

MVE-1-51, the prototype strain of MVE, had been passaged twice in chick embryo and 15 times in suckling mouse brain (smb) before use (French, 1952; Berge, 1975). MVE-BH3479 was isolated from a pool of *Culex annulirostris* mosquitoes and had been passaged once in smb (Marshall *et al.,* 1982). Working stocks were 10% smb homogenates in Hanks' balanced salt solution, pH 8.0 (HBSS).

African green monkey kidney (Vero) cells (ATCC, CCL 81) were grown at 37° in Medium 199 supplemented with lactalbumin hydrolysate and 8% newborn bovine serum in 5% CO_2/95% air. Vero cells were used between passage levels 122 and 137. *Aedes albopictus* (C6/36) cells (Igarashi, 1978) were grown at 28° in Eagle's basal medium plus nonessential amino acids and 8% fetal calf serum. C6/36 cells were used between passage levels 122 and 132.

Plaque assay

Virus was assayed by plaque formation on Vero cell monolayers grown in 6-well plastic trays (tissue culture grade; Linbro Scientific Inc., U.S.A.). Monolayers were stained 2 days postinoculation (pi) with 1 ml of agar stain (0.7% agar, 0.01% neutral red in water) and plaques were counted after overnight incubation. Infectivity titers are expressed as plaque-forming units (PFU) per milliliter.

Polyclonal and monoclonal antibodies

Anti-MVE-1-51 hyperimmune ascitic fluids (HIAFs) were prepared in adult Swiss white mice following Tikasingh *et al.* (1966). Briefly, mice were immunized by intraperitoneal (ip) inoculation on Day 1 with 10^6 PFU of heat-inactivated (56°, 45 min) MVE-1-51 (smb) in Freund's complete adjuvant (FCA; Gibco Life Technologies Inc., NY). Booster immunizations (10^5 PFU of live MVE in FCA) were given ip on Days 7, 14, 21, and 28. On Day 25, mice were inoculated ip with $\sim 10^6$ Sarcoma 180 cells. Ascitic fluid was tapped on Day 35, centrifuged at 4500 rpm for 15 min to remove Sarcoma 180 cells, and heat-inactivated (56°, 45 min). Ascitic fluid was also collected from uninfected mice as a source of nonimmune mouse ascitic fluid (NMAF).

MAbs 4B6C-2 and 4B5A-2 define the E-1c and E-1d epitopes which are specific to the MVE E protein, mAbs 4B3B-6 and 4B6B-10 define the E-5 and E-6 epitopes and cross-react with the MVE and JE E proteins and mAb 4A1B-9 defines the E-4b epitope and cross-reacts with all flaviviruses; all five mAbs were raised against MVE and neutralize MVE infectivity (Hawkes *et al.,* 1988). MAb 4B6A-2 defines the E-1a epitope and is specific for the MVE E protein but does not neutralize MVE infectivity (Hawkes *et al.,* 1988). MAb 2B5B-3, which was raised against SLE, defines the E-3 epitope and cross-reacts with all members of the JE serocomplex, but neutralizes only MVE (Roehrig *et al.,* 1983). MAb 6B4A-10, which was raised against JE, defines the E-8 epitope and cross-reacts with MVE, JE, and SLE (Guirakhoo *et al.,* 1992). MAb 6A4D-1 was derived from the same anti-JE fusion which yielded 6B4A-10. This mAb defines epitope E-7 and cross-reacts with MVE and JE. The anti-E-7 and anti-E-8 mAbs also neutralize MVE.

Enzyme-linked immunosorbent assay (ELISA)

Ninety-six-well microtiter plates were coated with purified MVE antigen at a concentration of 4 μg/well in borate-buffered saline by overnight incubation at room temperature. Plates were rinsed (×3) with phosphate-buffered saline, pH 7.4, (PBS) containing 5% nonfat milk powder and incubated for 2 hr (37°) with anti-E mAbs (above) diluted 1:100 in PBS–5% milk. After rinsing (×6) with PBS/1% Tween 20, bound antibody was detected with goat anti-mouse IgG conjugated to horseradish peroxidase (Silenus, Melbourne) diluted 1:500 in PBS–1% milk (30 min at room temperature). Color development was with 2,2′-azinobis(3-ethylbenzthiazoline-sulfonic acid) (ABTS; 4 mg/ml) for 10 min at room temperature. Optical density (OD) was measured at 410 nm using an automated plate reader (Titertek Multiskan, Flow Laboratories, CA). Titers were taken as the highest dilution which gave OD readings greater than two times higher than OD values for NMAF-treated control wells.

Competitive binding assay

Competitive binding assays (CBAs) followed Roehrig *et al.* (1982). Briefly, anti-E mAbs were purified by chromatography on protein A–Sepharose columns and standardized to a concentration of 2 mg/ml. Alkaline phosphatase conjugates of each mAb were prepared by glutaraldehyde condensation (Voller *et al.,* 1976). Purified MVE-1-51 was used as antigen and was adsorbed to 96-well microtiter plates (4 μg/well) by overnight incubation at room temperature. From a starting concentration of 1 mg/ml, 50-μl aliquots of twofold dilutions of unconjugated mAb were mixed with 50-μl aliquots of antibody–enzyme

conjugate (at one-half maximal binding concentration determined by end-point ELISA titration) and the mixtures were allowed to equilibrate in the wells of the antigen-coated plates for 2 hr at 37°. Plates were rinsed, 100 μl of ABTS (4 mg/ml) was added per well, and the reaction was stopped after 30 min with 50 μl of 3 *M* NaOH. The OD at 410 nm was read in an automated plate reader. Competition was considered positive if the heterologous mAb competition curve was similar to the homologous mAb competition curve, and by comparison with positive and negative control anti-MVE-1-51 HIAF and NMAF.

Plaque reduction neutralization test (PRNT)

PRNTs were performed by incubating 50–100 PFU of virus in 100 μl of HBSS with serial dilutions of either HIAF or mAb (in HBSS) at 37° for 1 hr. The mixtures (200 μl) were adsorbed to Vero cells for 1 hr, the inoculum removed, and the monolayer washed with PBS and overlaid with 1% agar containing M199/LAH, 0.2% diethylaminoethyl-dextran, and 8% newborn calf serum. At 2 days pi, the monolayers were stained with 0.01% neutral red and plaques counted. Neutralization titers were expressed as the maximum dilution of antibody that yielded a 50% reduction compared to control samples which had been incubated in the presence of NMAF.

Selection of neutralization escape variants

MVE-1-51 (smb) and BH3479 (smb) were plaque-purified on Vero cells. Isolated plaques were amplified by a single passage on Vero cells until a cytopathic effect was observed; supernatants were titrated by plaque assay ($\sim 10^7$ PFU/ml). Plaque-purified MVE-1-51 or BH3479 (10^6 PFU) was incubated for 1 hr at 37° with mAb 4B6C-2 (E-1c) (Hawkes *et al.*, 1988) at a concentration 100-fold greater than the 90% neutralization titer for the parental virus. The virus–mAb mix was adsorbed to Vero cell monolayers for 1 hr at 37°, the inoculum removed, and cells overlaid for plaque development. Neutralization-resistant virus was subjected to a second plaque purification in the presence of mAb 4B6C-2. Candidate mutants were amplified on C6/36 cells; extracellular titers were $\sim 10^8$ PFU/ml at 4 days pi.

Serial passage of MVE in mice

Groups of 50 21-day-old Swiss outbred mice were inoculated into the left footpad (fp) with $\sim 10^3$ PFU of BH3479 or a neutralization escape variant of MVE (BHv1) that did not cause encephalitis in 21-day-old mice after fp inoculation. The brains of mice with symptoms of encephalitis were dissected intact, weighed, snap-frozen on dry ice, and stored at −80°. They were subsequently dispersed in a Dounce homogenizer and prepared as 10% suspensions in HBSS containing 2% bovine serum albumin; virus titration was by Vero plaque assay. Viruses originating from BH3479 or BHv1 were passed serially four times in mice.

Sequence analysis

Vero cell monolayers in 35-mm plastic dishes (Nunc, Roskilde, Denmark) were infected (m.o.i. ≈ 1) and incubated at 37° for 48 hr. Monolayers were washed in PBS and infected-cell RNA was extracted with sodium *p*-aminosalicylate and phenol-cresol, followed by a second phenol-cresol extraction; nucleic acids were precipitated with ethanol and high-molecular-weight single-stranded (ss) RNA was precipitated by addition of 4 *M* LiCl to a final concentration of 2 *M* (Shine and Dalgarno, 1973). This was followed by ethanol precipitation and resuspension of RNA in 10 μl TE buffer (5 m*M* Tris–HCl, 0.5 m*M* EDTA, pH 7.4).

MVE genome segments in infected-cell RNA extracts were amplified for sequencing by reverse transcription and polymerase chain reaction (RT–PCR) following Lee *et al.* (1992). First-strand cDNA synthesis was with two HPLC-purified oligonucleotide primers (Bresatec, Adelaide, Australia) of minus polarity (P1 — ACTTCATATTTGATG; P2 — GGTTCGTTGACCGATTTC) and avian myeloblastosis virus (AMV) reverse transcriptase (Pharmacia LKB, Uppsala, Sweden); cDNAs corresponding to MVE genome nucleotides 3 to 1375 and 948 to 2588 (Dalgarno *et al.*, 1986) were amplified by PCR. Four oligonucleotides were used in PCR: two primers of minus polarity (P1/P2) and two primers of plus polarity (P3 — ACGTTCATCTGCGTGAGC; P4 — GCTCCTCGTTGCTCCTGC); cDNA was amplified using *Taq* DNA polymerase (Pharmacia LKB) and primer pair P1/P3 or P2/P4 with 30 cycles at 95° (1 min), 45° (1.5 min), and 72° (2 min) in a Hybaid thermal reactor. To generate ssDNA for sequencing, one of the primers used in RT–PCR was phosphorylated; amplified cDNA was treated with λ-exonuclease (Gibco-BRL, Gaithersburg, MD) for 1 hr at 37° to digest the phosphorylated cDNA strand (Higuchi and Ochman, 1989). ssDNA was extracted using phenol, phenol–chloroform, and chloroform, precipitated with ethanol, and resuspended in 10 μl of TE buffer. This preparation provided sufficient ssDNA for four sets of sequencing reactions.

DNA was sequenced by the dideoxynucleotide method (Sanger *et al.*, 1977) using T7 DNA polymerase (Pharmacia LKB) and [^{35}S]dATP (Amersham, UK; Biggin *et al.*, 1983). Eight minus-strand primers were used to completely sequence the E, M/prM, and C genes. The sequences of the oligonucleotide primers are available upon request to the corresponding author (R.C.W.). The deletion within the E gene of MVE-BHv3 was confirmed by dideoxynucleotide sequencing of BHv3-infected-cell RNA, using a minus-strand oligonucleotide primer and AMV reverse transcriptase (Fichot and Girard, 1990). Reaction products were electrophoresed on 6% acrylamide, gradient buffer gels (Biggin *et al.*, 1983) under denaturing

conditions (7 *M* urea) at 40 mA for 1.5–3.5 hr. Gels were fixed in 5% acetic acid–5% methanol (20 min), dried under reduced pressure, and exposed overnight to Fuji RX100 film for autoradiography.

Hemagglutination

Hemagglutination assays followed Clark and Casals (1958). Virus-infected C6/36 cell supernatants were used as a source of hemagglutinin (HA). Goose erythrocytes were collected into Alsever's solution, washed (×3) in PBS, resuspended as a 10% solution (v/v) in PBS, and stored at 4°; the erythrocyte preparation was used for up to 7 days after collection. HA was serially diluted in borate-buffered saline, pH 9, in 96-well microtiter plates (Costar, Cambridge, MA). Titers were determined as the reciprocal of the highest dilution which produced complete hemagglutination. Hemagglutination was assayed over the pH range 5.6 to 7.2; peak titer for BH3479 was at 6.5–6.7.

Virulence

Virulence assays followed Monath *et al.* (1980). Swiss mice (at 21 days old) were divided into groups of 5 and inoculated with 10-fold dilutions (10^{-1} to 10^{-9}) of virus in HBSS either intracerebrally (ic) for examination of neurovirulence or ip for examination of neuroinvasiveness. Mice were observed daily for 17 days for signs of illness and death; 50% lethal doses (LD_{50}s) were calculated (Reed and Muench, 1938). Mice were kept on a deep liter of sawdust and given food and water *ad libitum.* Experiments were performed in accordance with the guidelines of the ANU Animal Ethics Committee.

Viral growth kinetics

For examination of growth kinetics, Vero or C6/36 cells were infected (m.o.i. ≈ 10) by adsorption of virus to cells for 1 hr at 37° (Vero) or 28° (C6/36). Cells were washed with PBS, and growth medium was added and replaced at 6, 12, 18, 24, 30, 36, and 48 hr pi. One hour after replacement, growth samples were collected from duplicate cultures and titrated by plaque assay on Vero cells; titers were expressed as PFU/ml/hr.

RESULTS

Mapping of epitopes on the MVE E protein

Nine mAbs, which define MVE E protein epitopes on the basis of antigenic cross-reactivity and serological activity in neutralization and hemagglutination assays (Hawkes *et al.,* 1988), were used in competitive binding assays to examine the topological arrangement of the epitopes. Three independent antigenic domains were identified (Table 1). The first domain comprised five epitopes (E-1a, E-1c, E-1d, E-5, and E-8), which are defined by type-specific, neutralizing, and passively protective mAbs. The second domain encompassed three epitopes (E-3, E-4b, and E-6), identified by flavivirus cross-reactive mAbs which expressed high hemagglutination-inhibiting activity and low neutralizing activity. The third domain was defined by a single epitope (E-7), identified by a mAb which expresses both neutralizing and passive-protection activity.

Selection of neutralization-resistant variants of MVE

Neutralization escape variants were selected from plaque-purified parental populations of BH3479 and MVE-1-51. BH3479 (Marshall *et al.,* 1982) is a low-passage mosquito isolate; MVE-1-51 (Berge, 1975) is the prototype and has been extensively passaged in mouse brain. Variants were selected with mAb 4B6C-2, which defines epitope E-1c on the E protein of MVE; mAb 4B6C-2 is type-specific, neutralizing, and passively protects susceptible mice against a normally lethal ip challenge with virulent MVE (Hawkes *et al.,* 1988). The seven neutralization escape variants of MVE-1-51 and nine variants of BH3479 were at least 10^3-fold more resistant to mAb 4B6C-2 in PRNTs than parental virus and did not bind the mAb as judged by ELISA (data not shown). Attempts to select neutralization escape variants with the other anti-E mAbs have been unsuccessful to date. These mAbs have lower neutralizing titers and are not fully neutralizing at the higher virus concentrations used for variant selection compared to PRNTs.

The cross-reactivities of four BH3479-derived escape variants with anti-E mAbs specific for epitopes E-1d, E-5, E-7, and E-8 were examined by PRNT to determine whether structural changes in epitope E-1c altered the neutralization activity within other epitopes (Table 2). MAb 4B6C-2, of high neutralization titer against BH3479, failed to neutralize the E-1c escape variants at the lowest dilution tested. The escape variants showed increased sensitivity to neutralization with mAbs specific for epitopes E-1d, E-5, and E-8 in PRNTs; the increased sensitivity to neutralization ranged from 2- to 57-fold and was observed only for mAbs which competed with mAb 4B6C-2 in CBAs (Table 1). MAb 6A4D-1, which defines epitope E-7 and did not compete with mAb 4B6C-2 in CBAs, was of a low neutralizing titer which did not differentiate between parental virus and the escape variants. Presumably the E proteins of the escape variants have undergone a conformational change or destabilization restricted to the region of the topologically related neutralization epitopes. The mutations may have generated a locally less stable structure such that binding of the E-1d, E-5, or E-8 mAbs more readily led to destabilization and consequent increased sensitivity to neutralization.

TABLE 1

Competitive Binding Assay Mapping of MVE E Protein Epitopes Using Monoclonal Antibodies

	Competition reaction with alkaline phosphatase-conjugated mAb[a]								
Competitor	E-1d	E-1a	E-1c	E-8	E-5	E-6	E-3	E-4b	E-7
Anti-E-1d	+	+	+	+	−	−	−	−	−
Anti-E-1a	+	+	+	+	+	−	−	−	−
Anti-E-1c	+	+	+	+	+	−	−	−	−
Anti-E-8	+	+	+	+	+	−	−	−	−
Anti-E-5	−	+	+	+	+	−	−	−	−
Anti-E-6	−	−	−	−	−	+	+	−	−
Anti-E-3	−	−	−	−	−	+	+	+	−
Anti-E-4b	−	−	−	−	−	+	+	+	−
Anti-E-7	−	−	−	−	−	−	−	−	+

[a] Competition was considered positive (+) if the heterologous mAb competition curve was similar to the competition curve of the homologous mAb (Roehrig *et al.*, 1982); mAb pairs which did not compete in CBAs are shown as (−).

Nucleotide sequence analysis of neutralization escape variants

The C, prM, and E genes and the 5′ untranslated regions of the parental viruses and the escape variants were sequenced and compared. All of the escape variants differed from parental viruses in the E gene (Table 3). Seven MVE-1-51-derived escape variants had single-point mutations in the E gene leading to changes at amino acid residue 126, 128, 274, 276, or 277 in the E protein. Of nine BH3479-derived variants, eight had single-point mutations leading to changes at residue 128 or 277 in E. BHv5 had an amino acid change in prM at residue 76 (Val → Ala) in addition to the change at E-128 (Arg → Ser). BHv4 (unchanged in prM) was identical to BHv5 in E and was equally resistant to mAb 4B6C-2 in PRNTs (data not shown), suggesting that the prM change was not implicated in mAb selection; none of the other escape variants were altered in prM.

Sequencing of cDNA encompassing the E gene of BHv3 suggested that the codon for Ser-277 had been deleted. The deletion was confirmed by: (1) direct sequencing of infected-cell RNA using virus-specific primers (data not shown) and (2) by comparison of *AluI* restriction digest profiles of [^{35}S]dATP-labeled double-stranded cDNA (amplified by RT–PCR) encompassing the E genes of BH3479 and BHv3 after electrophoresis in 6% acrylamide gels. A predicted ~280-bp cDNA fragment (containing the E-277 codon) derived from BH3479 was approximately three nucleotides larger than a similar fragment derived from BHv3 cDNA (data not shown). The predicted sizes of the *AluI* digest products and the observed fragment mobilities are consistent with deletion of three nucleotides from the BHv3 RNA.

In summary, resistance to neutralization of MVE by mAb 4B6C-2 was associated with changes at five amino acid residues (E-126, -128, -274, -276, -277) clustered into two regions of the primary structure of the E protein.

TABLE 2

Neutralization of MVE BH3479 and Escape Variants with Anti-E Protein Monoclonal Antibodies

	Neutralization titers[a] of mAbs				
MVE variant	4B6C-2[b] (E-1c)	4B5A-2[b] (E-1d)	4B3B-6[b] (E-5)	6B4A-10[b] (E-8)	6A4D-1[b] (E-7)
BH3479	74,000	320	48	<20	48
BHv1	<20	960	1024	115	60
BHv2	<20	920	1250	120	48
BHv3	<20	770	1540	832	78
BHv5	<20	640	960	88	40

[a] Neutralization titers are reciprocals of mAb dilutions resulting in 50% neutralization in PRNTs; all PRNTs were performed in duplicate.

[b] MAbs used in PRNTs were selected and characterized as under Materials and Methods.

Hemagglutination of parental virus and escape variants

As 4B6C-2 not only is a neutralizing mAb but also is hemagglutination-inhibiting, it was of interest to determine whether the neutralization escape variants were affected in hemagglutination activity. MVE-BH3479 and the escape variants (BHv1, BHv2, BHv3, and BHv4) were screened for hemagglutination activity in the pH range 5.6 to 7.4; the optimum pH for hemagglutination was 6.6 (data not shown). Hemagglutination titers of the BH3479-derived viruses at pH 6.6 are shown in Table 4. BHv1 was unable to hemagglutinate at the lowest dilution tested (1:2) and over the entire pH range examined (data not shown). By contrast, the hemagglutination titer for BH3479 was 1:160. The hemagglutination titer for BHv3 was 1:40, fourfold lower than that of the parental virus.

TABLE 3

Nucleotide and Deduced Amino Acid Changes in Antigenic Variants of MVE-1-51 and BH3479

Variant[a,b]	Mutation[c]	Amino acid change[d]
1-51v1	UUU → GUU (1789)	Phe 274 → Val (E)
1-51v2	AGC → AGA (1797)	Ser 276 → Arg (E)
1-51v3	AGC → AUC (1799)	Ser 277 → Ile (E)
1-51v4	AGC → AAC (1799)	Ser 277 → Asn (E)
1-51v5	GCG → GAG (1346)	Ala 126 → Glu (E)
1-51v6	AGA → AGU (1353)	Arg 128 → Ser (E)
1-51v7	AGA → AAA (1352)	Arg 128 → Lys (E)
BHv1 (4)[e]	AGC → AUC (1799)	Ser 277 → Ile (E)
BHv2 (2)[e]	AGC → AAC (1799)	Ser 277 → Asn (E)
BHv3	CAG deletion (1797–1799)	Ser 277 deleted (E)
BHv4	AGA → AGC (1353)	Arg 128 → Ser (E)
BHv5	AGA → AGU (1353)	Arg 128 → Ser (E)
	GUA → GCA (693)	Val 76 → Ala (prM)

[a] Variants were selected from MVE-1-51 or from BH3479, using the type-specific anti-MVE E mAb 4B6C-2 (Hawkes *et al.*, 1988).

[b] Parental viruses were sequenced in parallel. The nucleotide sequences of MVE-1-51 (Dalgarno *et al.*, 1986; Lee *et al.*, 1990) and the BH3479 E gene (Lobigs *et al.*, 1988) have been established. The C and prM genes of BH3479 were sequenced and compared to MVE-1-51. There were five silent nucleotide changes in C at positions 172 (C → U), 204 (C → U), 215 (A → G), 315 (G → A), and 465 (U → C). Substitution at 419 (A → G) resulted in a Glu108 → Gly change in the C protein. There were six silent nucleotide changes in prM at positions 513 (U → C), 515 (C → U), 537 (C → U), 626 (C → U), 747 (C → U), and 813 (G → A).

[c] Numbering is from the 5′-terminal nucleotide of MVE RNA (Dalgarno *et al.*, 1986).

[d] Numbering is from the N-terminal amino acid of E or prM proteins (Dalgarno *et al.*, 1986).

[e] Number in parentheses indicates the number of times this mutation was observed in an independent clone; other variants were selected only once.

BHv2 and BHv5 each gave hemagglutination titers of 1:320. The lower hemagglutination titers of BHv1 and BHv3 were not due to the presence of lower concentrations of infectious virus or E protein in the virus stocks as the BH3479 and escape variants examined had very similar titers of infectious virus by plaque assay (Table 4) and similar concentrations of E protein by ELISA using anti-E mAb 6A4D-1 (Table 4). Thus, BHv1(E-277 Ile) had no detectable hemagglutination activity and BHv3(E-277 deleted) was of reduced activity compared to BH3479(E-128 Arg, E-277 Ser), BHv2(E-277 Asn), and BHv5(E-128 Ser).

Virulence of parental virus and escape variants in mice

As very small amounts of mAb 4B6C-2 (epitope E-1c specific) passively protect mice from challenge with virulent MVE (Hawkes *et al.*, 1988), it was of interest to assess the effect of mutations in epitope E-1c on neuroinvasiveness and neurovirulence. The criteria for neuroinvasiveness and neurovirulence were LD_{50} virus titers after ip or ic injection, respectively. Neuroinvasiveness and neurovirulence of MVE-1-51, BH3479, and the escape variants are shown in Table 5. All viruses examined were of high NV (ic LD_{50} 0.1–1.8 PFU), indicating that mutations in epitope E-1c had no effect on the ability of virus to replicate in mouse brain. MVE-1-51 and BH3479 were both of high neuroinvasiveness (ip LD_{50} 0.5 PFU) as previously reported (Lobigs *et al.*, 1988). The neutralization escape variants 1-51v3 and BHv1 (E-277, Ser → Ile) were of low neuroinvasiveness, with ip LD_{50} of each exceeding 10^6 PFU. Variants 1-51v1 (E-274, Phe → Val), 1-51v2 (E-276, Ser → Arg), 1-51v3 and BHv2 (E-277, Ser → Asn), BHv3 (E-277, Ser deleted), 1-51v5 (E-126, Ala → Glu), and 1-51v6 and BHv5 (E-128, Arg → Ser) were similar in LD_{50} to the parental viruses; all of these viruses were of high neuroinvasiveness. Thus, the Ser → Ile mutation at E-277 of BHv1 and 1-51v3 was specifically associated with a major change in the ability of virus to invade the central nervous system from a peripheral site of inoculation. Other mutations at E-277 (Ser → Asn; Ser deleted) were not associated with markedly decreased neuroinvasiveness.

Although BHv1 was not fully attenuated for 21-day-old mice by peripheral inoculation, it was able to protect mice from challenge with virulent MVE. Mice (17 days old) inoculated ip with 10^5 PFU of BHv1 and challenged ip 13 days later (at 30 days old) with 100 LD_{50} of BH3479 were fully protected against infection, whereas mock-immunized mice showed 80% mortality when challenged at the same age (Fig. 1). BHv1-immunized mice were also protected from ip challenge with BHv5 (data not shown). The immunizing dose of BHv1 caused 5% mortality in the mice. In a more severe test of immunity there was no protection against ic challenge with BH3479 but there was partial protection (30% reduction in mortality) against the immunizing virus (BHv1).

TABLE 4

Hemagglutination by MVE BH3479 and Antigenic Variants[a]

Virus	Hemagglutination titer[b]	Infectivity titer[c]	ELISA titer[d]
BH3479	320	7.9	1280
BHv1	<2	8.0	1280
BHv2	320	8.3	640
BHv3	40	8.2	1280
BHv5	320	8.1	640

[a] The hemagglutinin used in the assay was MVE-infected C6/36 cell superanatants. Hemagglutination assays followed Clark and Casals (1958). Similar results were obtained in duplicate assays.

[b] Reciprocal of the highest dilution of virus causing complete agglutination of goose erythrocytes at pH 6.6.

[c] Infectivity titers determined by plaque formation on Vero cells; titers are expressed as $\log_{10}$ PFU/ml.

[d] Reciprocal of the highest dilution producing a signal:noise ratio of >2; mAb 6A4D-1 was used to bind E protein.

TABLE 5

Virulence of MVE-1-51, BH3479, and Antigenic Variants in 21-Day-Old Mice[a]

Virus	Amino acid change in E[b]	ic LD_{50} (PFU)[c]	NV[d]	ip LD_{50} (PFU)[c]	NI[e]
MVE-1-51	—	0.1	H	0.3	H
1-51v1	Phe-274 → Val	0.5	H	0.8	H
1-51v2	Ser-276 → Arg	0.2	H	10.0	H
1-51v3	Ser-277 → Ile	1.4	H	2×10^6	L
1-51v4	Ser-277 → Asn	0.2	H	1.8	H
1-51v5	Ala-126 → Glu	0.1	H	0.1	H
1-51v6	Arg-128 → Ser	0.2	H	2.8	H
BH3479	—	0.4	H	0.5	H
BHv1	Ser-277 → Ile	1.0	H	$>10^7$	L
BHv2	Ser-277 → Asn	1.4	H	2.1	H
BHv3	Ser-277 deleted	1.2	H	4.6	H
BHv5	Arg-128 → Ser	1.8	H	6.0	H

[a] Virulence assays followed Monath *et al.* (1980); all assays were in duplicate.
[b] See Table 3.
[c] LD_{50} values are expressed as Vero PFU and were determined by the method of Reed and Muench (1938).
[d] Neurovirulence (NV), high (H) < 2 PFU, low (L) > 20 PFU.
[e] Neuroinvasiveness (NI), high (H) < 10 PFU, low (L) > 10^3 PFU.

Growth kinetics of parental virus and escape variants in cell culture

To investigate whether the decreased neuroinvasiveness of BHv1 in mice correlated with restricted growth in cell culture, the growth kinetics of BH3479, BHv1, BHv2, BHv3, and BHv5 were compared in Vero and C6/36 cells. Interestingly, the rate of production of BHv1 in Vero cells was 20 and 5× lower than that for the other viruses at 12 and 18 hr pi, respectively (Fig. 2). This result was reproducible in all three experiments. The growth kinetics of BHv1 were identical to those of the other viruses at 24–36 hr pi. The rates of virus production for the escape variants were very similar to those for BH3479 in C6/36 cells up to 48 hr pi (data not shown).

Isolation and characterization of virulent revertants

Neuroinvasive revertants of BHv1 were selected by four serial passages in 21-day-old Swiss mice. Groups

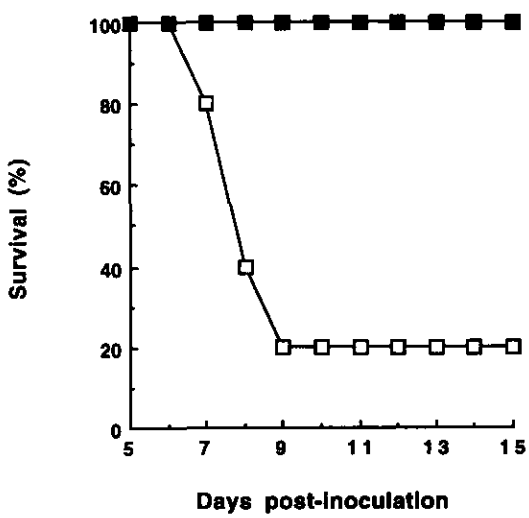

FIG. 1. Protection of 30-day-old Swiss mice from ip challenge with BH3479. Groups of 40 17-day-old mice were mock-inoculated or inoculated with 10^5 PFU of BHv1. Thirteen days after inoculation mice were challenged ip with 100 PFU of BH3479. Mice were observed for 21 days after challenge for signs of illness and death. BHv1-inoculated, ■; mock-inoculated, □.

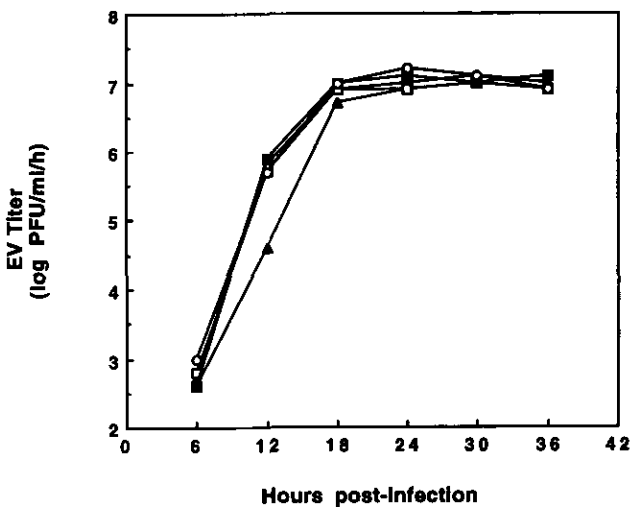

FIG. 2. Kinetics of extracellular virus (EV) synthesis in BH3479-, BHv1-, BHv2-, BHv3-, and BHv5-infected Vero cells. Vero cell monolayers were infected (m.o.i. ≈ 10) and incubated at 37°. At the indicated times, cell growth medium was removed and replaced with fresh medium for 1 hr prior to sampling. EV released into the medium in 1 hr was titrated by plaque formation on Vero cells; titer is expressed as PFU/ml/hr. Titers are plotted at the midpoint of each collection period. All assays were performed in duplicate. BH3479, △; BHv1, ▲; BHv2, □; BHv3, ■; BHv5, ○.

TABLE 6

Neuroinvasive Revertants Derived from MVE BHv1 by Serial Passage in Mice[a]

Virus	Mutation[b]	Amino acid change in E[c]	Hemagglutination titer[d]	ip LD_{50} (PFU)[e]
BH3479[f]	—	—	320	0.5
BHv1	AGC → AUC (1799)	Ser-277 → Ile	<2	$>10^7$
BHv1R1[f]	AUC → AGC (1799)	Ile-277 → Ser	640	2.2
BHv1R2[f]	AUC → AGC (1799)	Ile-277 → Ser	640	2.7
BHv1R3[f]	AUC → AGC (1799)	Ile-277 → Ser	320	1.9

[a] BHv1 was selected from BH3479 using the type-specific anti-MVE E mAb 4B6C-2; BHv1R1, BHv1R2, and BHv1R3 were selected from BHv1 by serial passage in 21-day-old Swiss mice.

[b] Numbering is from the 5′-terminal nucleotide of the MVE RNA (Dalgarno *et al.*, 1986).

[c] Numbering is from the N-terminal amino acid of E (Dalgarno *et al.*, 1986).

[d] Hemagglutination assays followed Clark and Casals (1958); titer is the reciprocal of the highest dilution causing complete agglutination of goose erythrocytes at pH 6.6. MVE infectivity titers (determined by Vero cell plaque assay) and E protein concentrations (determined by ELISA with mAb 6A4D-1) were essentially identical for all virus stocks (data not shown).

[e] Virulence assays followed Monath *et al.* (1980); LD_{50} values determined by the method of Reed and Muench (1938).

[f] Mouse brain virus stocks after four serial passages in 21-day-old Swiss mice (see Materials and Methods).

of 50 mice were inoculated into the left footpad, and virus was harvested from brains of mice which developed encephalitis. Brains containing the highest virus titers (by plaque assay on Vero cells) were used for the subsequent passage. During the first three passages of BHv1, 2–3 mice in each group of 50 developed encephalitis with an average survival of 9.7 (±1.7) days pi compared to 7.8 (±1.4) days pi for BH3479. At passage level 4 a biphasic pattern of encephalitis onset was observed after inoculation of the BHv1 passage 3 virus — 3 mice developed encephalitis with an average survival of 7.3 (±1.3) days pi and 2 mice developed encephalitis with an average survival of 9.4 (±2.5) days pi; BH3479-infected mice had an average survival of 7.9 (±1.6) days at passage level 4.

Virus populations within the brains of BHv1-infected mice which developed early onset encephalitis at passage level 4 were examined by virulence assay. LD_{50}s after ip inoculation (1.9–2.7 PFU) were of a magnitude similar to that for BH3479 (0.5 PFU) at passage level 4 (Table 6), indicating that the three virus stocks (designated BHv1R1, BHv1R2, and BHv1R3; R, revertant) had reverted to full neuroinvasiveness. No changes in neurovirulence were observed in BH3479- or in BHv1-derived virus stocks during mouse passage (data not shown).

The three revertant virus populations had regained hemagglutination activity at pH 6.5–6.7 (Table 6) and also full sensitivity to neutralization by mAb 4B6C-2 in PRNT assays (data not shown). Nucleotide sequencing of RT–PCR-amplified E gene cDNA of the three revertant virus populations showed reversion to the parental virus sequence at nucleotide position 1799 (U → G), resulting in reversion to the BH3479 deduced amino acid sequence at residue 277 (Ile → Ser) (Table 6); no other mutations were observed in the E genes of the revertants. Nucleotide sequencing of the E genes of three BHv1 passage level 3 stocks and of two BHv1 (late onset encephalitis) virus stocks from passage level 4 revealed no change at nucleotide 1799 compared to BHv1 and thus no alteration to the deduced amino acid sequence (data not shown).

DISCUSSION

The immunodominant determinants of a major neutralization epitope (E-1c) of MVE have been identified by selection of neutralization escape variants using an E-1c-specific mAb (4B6C-2). The escape variants had a nucleotide change coding for single amino acid substitution at residue 126, 128, 274, 276, or 277 in E and were fully resistant to neutralization by the selecting mAb. This is a remarkably clear indication that epitope E-1c is formed by two clusters of amino acid residues widely separated in the primary sequence of E; i.e., E-1c is a discontinuous epitope. Both regions are hydrophilic and thus likely to be located on the surface of E. Presumably the native conformation of E brings the 126–128 and 274–277 sequence regions into apposition in such a way that they may be bound by the paratope of the E-1c-specific mAb. Alternatively it is conceivable that only one of the two regions binds to the paratope and that mutations at the other locus disrupt the conformation of the epitope from a distance. The complex three-dimensional structure of epitope E-1c is supported by the abolition of mAb binding to E under reducing conditions (Lee, 1990) and the poor reactivity of E-1c-specific mAb with synthetic peptides which incorporate either residues 126–128 or 274–277 (Roehrig *et al.*, 1989).

According to the two-dimensional model of the TBE E protein (Mandl *et al.*, 1989), the determinants of epitope E-1c are in the A domain, putatively a highly folded region of the polypeptide stabilized by five disulfide bonds. The

determinants of a number of flavivirus epitopes have been identified in domain A but the identified determinants of MVE E-1c provide new and direct evidence for inclusion of the C-terminal region of the peptide loop formed by Cys-186/Cys-290 disulfide bond in domain A.

The E-1c-specific mAb (4B6C-2) is type-specific for MVE. The amino acid sequences in the regions of the E protein associated with epitope E-1c (126–128 and 274–277) are poorly conserved between viruses of the JE serocomplex, including MVE-1-51 (Dalgarno *et al.,* 1986), JE (Sumiyoshi *et al.,* 1987), WN (Wengler *et al.,* 1985), and SLE (Trent *et al.,* 1987), consistent with the type specificity of mAb 4B6C-2. It is notable that the epitope determinants at residues 126 and 128 are near the most highly conserved region (E 98–111) of the flavivirus E protein which has the characteristics of a "fusion peptide" (Roehrig *et al.,* 1989) and may be involved in cell entry of virus. The failure of mAb 4B6C-2 to bind to the escape variants is of particular interest because of the possibility that binding of antibody may neutralize by preventing attachment to cells. Considered together, the data suggest that epitope E-1c not only may be important as a binding site for neutralizing antibody but also may be involved in virus entry.

The E-1c escape variants showed increased sensitivity to neutralization by mAbs which competed with mAb 4B6C-2 in CBAs, suggesting that the amino acid substitutions in epitope E-1c also caused conformational change in epitopes E-1d, E-5, and E-8. MAb 6A4D-1 (anti-E-7) neutralized the E-1c escape variants and parental virus with similar titers in PRNTs, suggesting that the conformation of epitope E-7 was unchanged by substitutions in E-1c. The CBAs showed that the nine epitope-specific mAbs cluster into three noninterfering topologically independent antigenic sites on the MVE E protein. E-1c, E-1d, E-5, and E-8 cluster in a single domain identified as domain A by the location of E-1c. Thus, conformational change induced in E-1c escape variants leading to increased sensitivity to neutralization by the other mAbs appeared to be restricted to the overlapping epitopes in domain A. The increase in sensitivity to neutralization of E-1c escape variants is unexpected. It suggests that the mutations introduced in E-1c have produced subtle modifications in the conformation of E which enhance mAb binding and/or neutralization at the overlapping epitopes. The topological mapping of the MVE epitopes is in agreement with previous studies which have demonstrated that epitopes defined by mAbs against flavivirus E proteins tend to cluster into nonoverlapping immunodominant antigenic sites (Heinz *et al.,* 1983; Kimura-Kuroda and Yasui, 1983; Henchal *et al.,* 1985).

The E-277 (Ser → Ile) mutation not only affected neutralization of MVE by the E-1c-specific mAb, but also abolished pH-dependent hemagglutination of goose erythrocytes and mouse virulence (see below). Substitution of Ile for Ser introduced a hydrophobic residue into a hydrophilic region which may perturb the local structure of E or may induce a more extensive reorganization of domain structure in order to stably accommodate the hydrophobic residue. A number of interpretations of the consequences may be suggested. Ile-277 may directly prevent binding of E to the erythrocyte surface by disrupting the receptor-binding site at slightly acidic pH, or Ile-277 may prevent the low pH-dependent conformational change in E required for hemagglutination and membrane fusion. More indirectly, this mutation may have transmitted conformational change to a distant hemagglutination site on E. Gross change in the structure of E in response to the Ile substitution seems unlikely as binding and neutralization by the other mAbs were not abolished. The other changes at residue 277 (Asn, deletion of Ser) do not lead to significant alteration of the hydrophilicity of the region and did not lead to major change in hemagglutination activity. Cecilia and Gould (1991) selected neutralization escape variants of JE using four anti-E mAbs and these variants lacked hemagglutination activity despite efficient replication in cell culture, suggesting that conformational change in the E proteins of escape variants may frequently lead to a decreased ability of virus to interact with erythrocyte membranes.

None of the mutations in the E proteins of the E-1c escape variants were associated with altered neurovirulence compared to parental virus; thus these mutations do not appear to modify the ability of MVE to replicate in the central nervous system (CNS). Although at least five amino acid determinants were found to affect the structure of epitope E-1c (see above), change at only one position (E-277) was associated with attenuation of neuroinvasiveness of MVE in weanling mice. Furthermore, of the three observed mutations at E-277 only a Ser → Ile change altered neuroinvasiveness. Interestingly, prior inoculation of the BHv1 variant (of low neuroinvasiveness) induced a protective immune response in mice, suggesting that this virus replicated in peripheral tissues despite an inability to invade the central nervous system. The low neuroinvasiveness of BHv1 was not associated with a major alteration of replicative ability in cell culture as the BH3479-derived escape variants grew equally well in Vero and in C6/36 cells compared to parental virus. However, growth of BHv1 in Vero cells was below that of BH3479 during the early phase of infection (12–18 hr pi), suggesting a defect in some early event in replication by the mutant virus. As BHv1 is also the only mutant lacking hemagglutination activity, it is possible that the mutation affects interaction of mutant virus with the cell surface or with endosomal membranes. These effects are indirect indicators of attachment or cell entry capacity and other explanations of the data such as a reduced rate of assembly or release of virions from infected cells are possible.

Other studies have also documented single-amino-acid changes in the E protein of neutralization escape

variants associated with loss of neuroinvasiveness in mice. Cecilia and Gould (1991) selected a JE variant (altered at Ile 270) of low neuroinvasiveness for weanling mice associated with lack of hemagglutination activity. Hasegawa *et al.* (1992) selected a JE variant (altered at Gln 52) of low neuroinvasiveness for weanling mice associated with an apparent defect in binding to the Vero cell surface. Holzmann *et al.* (1990) selected a TBE variant (altered at Tyr 384) of reduced neuroinvasiveness for weanling mice. Lobigs *et al.* (1990) selected MVE variants by serial passage in SW13 cells. These variants were of decreased neuroinvasiveness for weanling mice and were consistently altered at residue E-390. Considered together, these studies suggest that at least three neuroinvasiveness determinants may be located on the flavivirus E protein: in the region of E-270–277 of MVE/JE (associated with altered hemagglutination activity), in the region of E-384–390 of TBE/MVE, and in the region of E-52 of JE (possibly associated with altered receptor-binding activity).

Selection of neuroinvasive revertants during serial passage in 21-day-old Swiss mice provided further confirmation of the role of residue Ser-277 in E as a determinant of neuroinvasiveness. The three neuroinvasive BHv1-derived revertant viruses had mutated to the BH3479 virus sequence at residue 277 (Ile → Ser) in E, suggesting that strong selection pressure may be exerted on this residue during passage in a vertebrate host; no other mutations were observed in the E genes of the revertants. This finding further reduces the possibility that attenuation of BHv1 is due to an undetected mutation(s) in other regions of the virus genome. The revertants also regained hemagglutination activity at acidic pH, suggesting that the function assayed as hemagglutination is closely linked to the ability to invade the CNS. Thus the kinetics of interaction of MVE virus with cell membranes (during entry of virus into cells) may determine the ability of virus to replicate within vertebrate host tissues to a level sufficient to result in CNS invasion and encephalitis before the immune response provides protection. These hypotheses are currently being tested by examining the binding and entry of MVE virus into a variety of cell lines *in vitro* and by examination of the tissue distribution of MVE virus in weanling mice after peripheral inoculation.

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