VIROLOGY 232, 281–290 (1997)
ARTICLE NO. VY978570

Changes in the Dengue Virus Major Envelope Protein on Passaging and Their Localization on the Three-Dimensional Structure of the Protein

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Received May 14, 1996; returned to author for revision June 13, 1996; accepted March 28, 1997

To help define the molecular events involved in dengue virus adaptation during serial passage *in vivo* and in cultured cells, we have sequenced the structural protein genes of three dengue type 3 isolates after intracerebral passage in mice and after passage in cultured monkey kidney (Vero) and *Aedes albopictus* (mosquito) cells. Passaging in each host selected for amino acid changes in the envelope protein E and occasionally in prM but not in the capsid protein. Most changes were first apparent within five passages. Nineteen of twenty mutations in the structural protein genes resulted in amino acid changes concentrated on 12 residues; 9 of the 12 amino acid changes were at residues which are conserved between the four dengue virus serotypes. Certain amino acid changes were repeatedly selected on passage in cell culture. In six independent Vero cell passage series, changes were observed in E at residues 191 (four times), 202 (twice), 266 and 268 (three times), and 291; change in prM was seen in two passage series at residue 26. Two independent passage series in mosquito cells each resulted in the loss of a conserved glycosylation site at Asn153 in E. Passage in mouse brain selected for mutations at E residues 18, 54, 277, 401, and 403. Residues which altered on passaging have been localized on the three-dimensional structure of the tick-borne encephalitis virus E protein soluble fragment (F. A. Rey, *et al.,* 1995, *Nature* 375, 291–298). Residues 54, 191, 202, 266, 268, and 277 map to a postulated "hinge" region between domains I and II which may be involved in fusion of flaviviruses with cell membranes. The oligosaccharide at Asn153 also appears to be involved in flavivirus fusion. Changes in the fusion characteristics of the passaged viruses were demonstrated.

INTRODUCTION

The dengue viruses are mosquito-transmitted flaviviruses annually responsible for epidemics involving up to 100 million cases of dengue fever and approximately 250,000 cases of dengue hemorrhagic fever/dengue shock syndrome (reviewed by Monath, 1994). Dengue viruses circulate as four serotypes (types 1–4), each of which shares with other flaviviruses a single-stranded, positive-sense RNA genome and a common genome organization and virion structure (Chambers *et al.,* 1990).

Although effective vaccines exist against the flaviviruses yellow fever virus (YF), Japanese encephalitis virus (JE), and tick-borne encephalitis virus (TBE), there is no widely accepted safe and effective vaccine against dengue viruses. In early studies on the generation of candidate dengue vaccines, attenuated virus was generated by serial intracranial passage in mice which led to a progressive loss in pathogenicity for humans and increased neurovirulence for mice (Sabin and Schlesinger, 1945; Hotta, 1952). Kawano *et al.* (1993) compared the virulence for mice of chimeras of mouse-adapted and non-mouse-adapted dengue type 4 virus (DEN-4) and concluded that the structural protein genes are prime determinants of mouse neurovirulence in mouse-adapted virus; apparently minor changes in the major dengue virus envelope protein (E) induced changes in mouse neurovirulence.

Most candidate dengue vaccines have been generated by passage in cell culture (Halstead *et al.,* 1984; Bhamarapravati *et al.,* 1987; Marchette *et al.,* 1990). Sequence changes accompanying cell culture passage point to an important role for the envelope protein in attenuation of YF (Hahn *et al.,* 1987), JE (Nitayaphan *et al.,* 1990), and Murray Valley encephalitis virus (MVE) (Lobigs *et al.,* 1990; McMinn *et al.,* 1995b). Hahn *et al.* (1987) compared the nucleotide and deduced amino acid sequences of the virulent Asibi strain of YF with that of the 17D vaccine strain derived from it by 240 passages in chick tissues. They found that 12 of 32 amino acid substitutions in the encoded proteins were in the E protein. Furthermore, a far greater proportion of nucleotide changes in the E gene led to amino acid substitutions (12/15) than in the rest of the coding region (20/53) suggesting a positive selection for change in E. After 10 passages of MVE in human adenocarcinoma (SW13) cells (Lobigs *et al.,* 1990) or monkey kidney cells (McMinn *et al.,* 1995b) the small number of amino acid substitutions which took place were also focused on the E protein.

Rey *et al.* (1995) have determined the three-dimen-

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0042-6822/97 $25.00

sional structure of a soluble fragment of the major TBE envelope protein; this structure can be taken as representative of the E proteins of flaviviruses generally and allows the localization of changes in E selected on passaging. The protein exists as a slightly bent elongated dimer; each monomer is composed of three domains: a dimerization/fusion domain (domain II), a central domain (I) which carries antigenic sites, and domain III which is probably involved in binding to the cell receptor. Rey *et al.* (1995) mapped virulence determinants for a number of different flaviviruses to the distal face of domain III, to the "hinge region" between domains I and II, and to the contact between the domain I/III interface and the *cd* loop of the opposite subunit. They proposed that changes in the last two regions modulate virulence by affecting the low-pH-mediated conformational transition and that cell attachment is likely to be altered by mutations on the distal face of domain III.

In this report we identify substitutions in the E protein of DEN-3 which are selected during serial passage in mouse brain and in cultured monkey kidney and mosquito cells. We examine the time course of appearance of these changes, localize them to particular regions of the 3D structure of the protein, and show that the majority of the changes in E are associated with changes in the fusion functions of the virus.

MATERIALS AND METHODS

Cells and virus

Aedes albopictus (C6/36) cells (Igarashi, 1978) were grown at 28° in Eagle's minimal essential medium (EMEM) plus nonessential amino acids and 8% fetal calf serum (FCS); African green monkey kidney (Vero) cells (ATCC, CCL81) were grown at 35° in medium 199 (M199) plus 5% FCS in 5% CO_2/95% air. Vero cells were used between passage levels 122 and 137.

DEN-3 isolates 1047, 1153, and 1239 were from human clinical cases in Java, Indonesia, between 1976 and 1978; they were from children with fatal encephalopathy (1047), fatal dengue shock syndrome (1153), and dengue fever (1239); 1047 and 1153 were from patients with serologic responses compatible with primary dengue infection (Gubler *et al.,* 1981; Sumarmo *et al.,* 1983). The isolates had been passaged once in *Ae. aegypti* mosquitoes, once in *Toxorhynchites amboinensis* mosquitoes, and three times in *Ae. albopictus* C6/36 cells. The parental working stocks used in passaging are referred to as Mos 5 stocks (Lee *et al.,* 1993). Virus was assayed by plaque formation on Vero cell monolayers in six-well Linbro trays; overlay medium contained M199, 1% agar, 0.02% DEAE dextran, 2% FCS, and 4% newborn calf serum. To stain cells, neutral red (0.02% in 0.7% agar solution) was added between 3 and 5 days p.i.

For preparation of plaque-purified virus stocks, DEN-3 plaques were picked from Vero cell monolayers within 16 hr of neutral red staining. Agar blocks were removed above selected plaques and cell monolayers were washed with 10 μl Hanks' balanced salt solution, pH 8.0 (HBSS), to collect virus. C6/36 cell monolayers in 35-mm dishes were infected with the plaque washing; culture fluids collected after 7 days at 28° were used for a second virus amplification in C6/36 cells.

Passaging of DEN-3 in mice

Uncloned Mos 5 stock (30 μl) was inoculated intracranially (ic) into five 1-day-old Swiss white mice; the inoculum contained 1.5×10^4 PFU (strains 1047 and 1153) or 1×10^5 PFU (strain 1239). Brains from two mice were collected after 7–8 days and pooled homogenates (a 10% suspension in phosphate-buffered saline, pH 7.4) were used undiluted for subsequent passaging. The remaining three mice were observed for morbidity and mortality over 21 days. Ten passages were performed [to yield passage (P) 10 virus]. Titers of 10^4–10^6 PFU/g wet weight were observed at early passage levels (P1–2 for 1047, P1–3 for 1239, and P1–5 for 1153); titers of 10^7 PFU/g or more were obtained at later passage levels. At P6–10, severe hindlimb paralysis leading to 100% mortality was observed in mice between 8 and 13 days p.i.

Passaging of DEN-3 in cell culture

Uncloned Mos 5 stock ($0.5–3 \times 10^5$ PFU) was used to infect Vero cell monolayers (7×10^5 cells in 35-mm plastic dishes); incubation was for 5 days or until cytopathic effects (CPE) were observed. Two independent Vero passage series (V1 and V2) were performed for each isolate. For 1047 and 1239, two independently amplified Mos 5 stocks were used for V1 and V2; 1153 was passaged using a single Mos 5 stock for both V1 and V2. Passaging was done in parallel on the same batch of cells for 1047V1, 1047V2, 1153V2, and 1239V2, and on a different batch of cells for 1153V1 and 1239V1. For P2–5, 100 μl of culture fluid (2×10^3–5×10^5 PFU) was used to infect cell monolayers; for P6–10, 20 μl (2×10^4–5×10^5 PFU) was used. After each passage, culture fluids and monolayers were stored at −70° for plaque titration and RNA extraction. Extracellular virus (EV) titers obtained at P6–10 ($0.5–2 \times 10^7$ PFU/ml) were 10- to 100-fold higher than those from earlier passage levels.

Different rates of plaque development were observed for parental and passaged virus. At P3 some large plaques (2–3 mm) were observed at 4 days p.i.; parental virus showed small plaques (0.5–1 mm) even at 6 days p.i. The proportion of large plaques increased with each passage. At P10, plaques were 0.5–3 mm in diameter at 4 days p.i. The proportion of large plaques (>2 mm) differed between each passage series.

Passaging in C6/36 cells (5×10^5 cells per 35-mm dish) was initiated with 5×10^4 PFU of parental 1153 or 1239. Ten serial passages were performed; incubation

was for 5–6 days using 20–100 μl (10^3–5×10^5 PFU) of culture fluid for infection. EV titers at each passage level were between 10^5 and 10^7 PFU/ml except for P3 and P4 ($1–5 \times 10^4$ PFU/ml). Between P4 and P10, CPE in infected C6/36 cell monolayers occurred at 5–6 days p.i. Virus from P5–10 produced small plaques (less than 0.5 mm) at 6 days p.i.; unpassaged virus plaques were 0.5–1 mm in diameter.

Sequence analysis of structural protein genes of passaged DEN-3

RNA was extracted from infected Vero cells, C6/36 cells, and mouse brain as described by Lee *et al.* (1992). For sequencing the prM and E genes, cDNA was generated by reverse transcription and polymerase chain reaction (RT-PCR) using primer sequences derived from the DEN-3 prototype strain H87 (Lee *et al.,* 1993). By using one phosphorylated primer during RT-PCR, followed by λ exonuclease digestion of the phosphorylated strand, single-stranded DNA was generated for sequencing with T7 DNA polymerase and deoxyadenosine 5′-[α-^{35}S]-thiotriphosphate (Lee *et al.,* 1992). The capsid protein (C) gene was amplified by RT-PCR using primer P1025 (5′-TTAGCCATGGTAGTCAC-3′; Lee *et al.,* 1993) for cDNA synthesis, and as upstream primer an oligonucleotide based on the sequence of the 5′-terminal 17 nucleotides of DEN-3 H87 strain (5′-AGTTGTTAGTCTACGTG-3′; Osatomi and Sumiyoshi, 1990) was used; the same primer was used in sequencing. All mutations were confirmed by sequencing cDNA from a second RT-PCR.

Preparation of ^{35}S-labeled DEN-3

C6/36 cells were infected with virus (m.o.i. $\approx$ 1) and from 63 hr p.i. incubated for 1 hr in serum-free EMEM containing no methionine; cells were then incubated in EMEM containing 4% FCS, 1/20th the normal concentration of methionine, and TRAN[^{35}S]-label (10 μCi/ml; ICN Radiochemicals) containing [^{35}S]cysteine and [^{35}S]-methionine. At 84 hr p.i. medium was harvested, clarified by centrifugation, and stored at −70°.

N-Glycosidase F digestion

Polyethylene glycol 6000 (Merck) was added to clarified ^{35}S-labeled, dengue-3-infected cell culture fluids (see above), to a concentration of 8%; the mixture was held at 4° for 2 hr. After centrifugation (1 hr, 10,000 *g*), virus from 1 ml of culture fluid (10^6–10^7 PFU) was resuspended in 20 μl of *N*-glycosidase F buffer (1% NP-40, 0.5% SDS, 1% 2-mercaptoethanol, and 50 m*M* sodium phosphate, pH 7.0). After heat denaturation (70°, 5 min), *N*-glycosidase F/endoglycosidase F (Boehringer Mannheim) was added (1 u/100 μl); incubation was at 37° for 12–16 hr. Analysis and detection of virion proteins was by SDS–PAGE and fluorography using Amplify (Amersham).

Fusion assays in *Ae. albopictus* (C6/36) cells

Fusion-from-within (FFWI) assays were performed in C6/36 cells following Randolph and Stollar (1990). Monolayers (5×10^4 cells) in 48-well plastic trays (Costar) were infected with virus (m.o.i. $\approx$ 5 PFU/cell) and incubated at 28° in EMEM plus nonessential amino acids and 10% FCS; cells were maintained at or above pH 7.6 by addition of 10 m*M* HEPES. At 4 days p.i., medium was removed and monolayers were rinsed with PBS. Fusion medium [0.5 ml; serum-free EMEM plus 20 m*M* HEPES (pH 6.8–8.0) or 20 m*M* MES (pH 5.6–6.6)] was added at 37°; cells were then incubated for 2 hr at 40°. Monolayers were examined by phase-contrast microscopy for syncytium formation and stained with a Diff-Quik Kit (Lab Aids) for counting cell nuclei. Fusion index (1 − [number of cells/number of nuclei]) was determined by counting five fields of 500 nuclei each. The threshold pH for fusion is defined as the pH at which the fusion index was 0.5. The proportion of infected cells was estimated by immunofluorescence assay on acetone/methanol (1/1)-fixed cell monolayers using PBS containing mouse anti-DEN-3 ascitic fluid (1:1000 dilution), followed by FITC-conjugated anti-mouse IgG (diluted 1:300 in PBS).

NH_4Cl treatment

Vero cell monolayers in 24-well trays were pretreated for 1 hr with freshly prepared medium containing NH_4Cl (0–50 m*M*) and adjusted to pH 8.0 with 20 m*M* HEPES. Cells were inoculated with virus (m.o.i. $\sim$ 0.5) and incubated at 37° for 1 hr. The inoculum was removed and cells washed ×3 with HBSS, pH 8; medium with or without NH_4Cl was then added. At 5 hr p.i., cells were washed once with HBSS and NH_4Cl-free medium was added. Culture fluids were collected at 24 hr p.i. and EV titers determined by plaque assay.

RESULTS

Changes in structural protein genes and in mouse neurovirulence on passaging DEN-3 in mice

Three isolates of DEN-3 virus (1047, 1153, and 1239) from human clinical cases (Lee *et al.,* 1993) were serially passaged 10 times in 1-day-old Swiss white mice by serial ic inoculation of 10^4–10^5 PFU. The isolates were not plaque purified and had been amplified five times in mosquitoes and mosquito cell culture. These parental viruses differed slightly from each other at the nucleotide and amino acid sequence levels and in their growth characteristics in cell culture (Lee *et al.,* 1993).

The structural protein genes (C, prM, and E) were sequenced using viral RNA extracted from the brains of mice infected with virus at different passage levels. RNA was reverse transcribed and appropriate regions amplified using PCR and DEN-3-specific primers to generate

TABLE 1

Envelope Protein Sequence Variation in DEN-3 Passaged in Mice and in Cell Culture

Passage	Variant stock[a]	Nucleotide change[b]	Amino acid change[c]	Presence of mutation (passage level)[d] P2	P4	P5	P6	P7	P8	P10
Mouse brain	1047MP10	G986 → U	Ala18 → Ser	−	−	−	ND	−	−	(+)
		C1095 → A	Ala54 → Glu	−	(+)	+	ND	+	+	+
	1153MP10	U1764 → C	Phe277 → Ser	−	(+)	+	+	+	+	+
	1239MP10	G2135 → A	Glu401 → Lys	−	−	(+)	ND	ND	+	+
		C2142 → U	Thr403 → Ile	−	−	(+)	ND	ND	+	+
		C1969 → U		−	−	(+)	ND	ND	+	+
Vero cell	1047V1P10	C1731 → U	Thr266 → Ile	−	−	−	ND	ND	ND	+
		U1737 → C	Ile268 → Thr	(+)	+	+	ND	ND	ND	+
	1047V2P10	U1505 → G	Phe191 → Val	−	(+)	(+)	ND	ND	ND	+
	1153V1P10	U1505 → C	Phe191 → Leu	−	(+)	+	ND	ND	ND	(+)
		A1806 → U	Glu291 → Val	−	−	−	ND	ND	ND	(+)
	1153V2P10	A512 → C	*Lys26* → *Gln*	−	−	−	ND	ND	ND	(+)
		U1505 → C	Phe191 → Leu	(+)	(+)	(+)	ND	ND	ND	−
		A1539 → G	Lys202 → Arg	−	−	(+)	ND	ND	ND	(+)
		G1736 → A	Ile268 → Val	−	−	−	ND	ND	ND	(+)
	1239V1P10	A513 → C	*Lys26* → *Thr*	−	−	−	ND	ND	ND	+
		U1737 → G	Ile268 → Ser	−	(+)	ND	ND	ND	ND	+
	1239V2P10	U1505 → C	Phe191 → Leu	(+)	(+)	(+)	ND	ND	ND	(+)
		A1539 → G	Lys202 → Arg	−	−	(+)	ND	ND	ND	(+)
Mosquito cell	1153CP5	A1397 → G	Thr155 → Ala	(+)	ND	+	ND	ND	ND	+
	1239CP5	C1398 → U	Thr155 → Met	(+)	ND	+	ND	ND	ND	+

[a] DEN-3 1047V1, 1047V2, 1153V2, and 1239V2 were passaged in Vero cells in parallel; 1153V1 and 1239V1 were passaged in a separate experiment.

[b] Numbering is from the 5′ terminus of DEN-3 genomic RNA (Osatomi and Sumiyoshi, 1990).

[c] Numbering is from the N-terminal residue of E (Lee *et al.,* 1993). Changes in prM (italics) are numbered from the N-terminal residue of prM.

[d] ND, not determined; −, change not detected; +, substantial change; (+), partial change.

single-stranded cDNA templates for sequencing (Lee *et al.,* 1993); parental virus was sequenced in parallel.

All three uncloned mouse-passaged P10 stocks showed mutations in the E gene; no changes were observed in the C or prM genes (Table 1). All mutations led to nonconservative amino acid changes in E except for one silent nucleotide change in isolate 1239. Five different amino acid changes were seen in the three MP10 stocks. For 1047MP10, changes were in the N-terminal region of E (Ala18 → Ser and Ala54 → Glu); for 1153MP10 there was a single change at E-277 (Phe → Ser) and for 1239MP10 there were changes in the C-terminal region of E (Glu401 → Lys, Thr403 → Ile) (Table 1). Parental amino acid residues at E-54 and E-403 are highly conserved between dengue viruses and other flaviviruses including TBE and YF.

To determine whether the multiple changes observed in individual uncloned stocks were linked in individual plaque isolates, we have sequenced the structural protein genes of three plaque isolates from each of the two passage series showing multiple mutations (Table 1). The data show that all three plaque isolates from stock 1239MP10 had the same three mutations, while all plaque isolates from 1047MP10 showed a single change: Ala54 → Glu; the Ala18 → Ser change was not present.

The neurovirulence of MP10 virus was examined by determining mortality following ic inoculation of 1-day-old Swiss white mice. MP10 virus was of markedly greater neurovirulence than parental virus: 80% mortality was observed when 10^3 PFU of each MP10 stock was inoculated; inoculation of 10^5 PFU of parental virus resulted in less than 50% mortality (data not shown). The data indicate an increase in the ic virulence of approximately 100-fold for MP10 virus. Growth in brain of 1047MP10 diverged markedly from that of parental 1047 after 2 days p.i., and titers of 2×10^8 and 5×10^5 PFU/g of brain, respectively, were reached at 8 days p.i. (Fig. 1). Similar results were obtained in experiments with parental 1239 and 1239MP10 (results not shown).

Mouse-passaged virus did not differ from parental virus in the rate of plaque development, in plaque size, or in the kinetics of development of cytopathic effects on Vero cell monolayers (data not shown).

Changes in structural protein genes on passaging DEN-3 in Vero cells

DEN-3 isolates 1047, 1153, and 1239 were passaged 10 times in Vero cells; 2 independent passage series (V1 and V2) were completed for each of the three isolates.

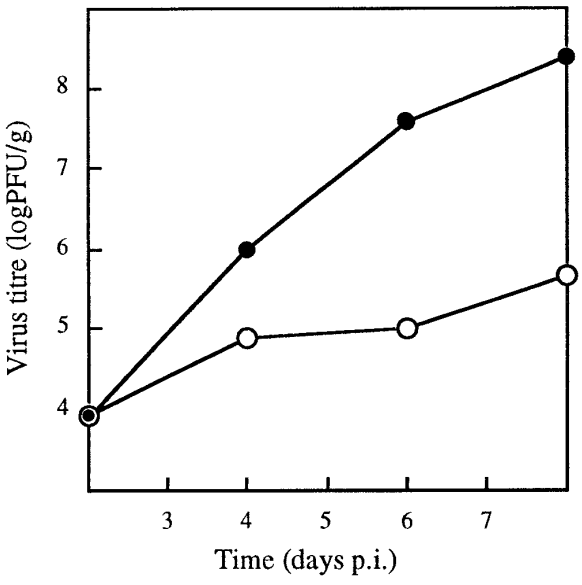

FIG. 1. Replication of unpassaged and mouse-passaged DEN-3 in mouse brain following ic inoculation. Groups of five 1-day-old Swiss white mice were inoculated ic with 10^3 PFU of unpassaged DEN-3 (parental 1047 isolate; open circles) or with 10^3 PFU of 1047MP10 (solid circles). At 2, 4, 6, and 8 days p.i., brains were harvested, weighed, and homogenized in PBS (pH 7.4) to yield a 10% suspension for infectivity titration by plaque assay on Vero cell monolayers.

Passaging was initiated using uncloned virus (see Materials and Methods). Complete sequencing of the C, prM, and E genes showed that each uncloned P10 stock contained one to three mutations in the prM and E genes but none in the C gene (Table 1). In 1047V1P10 mutations led to two nonconservative amino acid changes in E: Thr266 → Ile and Ile268 → Thr. In 1047V2P10, a single mutation changed Phe191 to Val. In 1153V1P10 and 1153V2P10 there were two E changes in each virus stock: Phe191 → Leu and Glu291 → Val were found in the former; Lys202 → Arg and Ile268 → Val were found in the latter. In 1153V2P10 there was also a change in prM at residue 26 (Lys → Gln). In 1239V1P10 two mutations changed prM26 (Lys → Thr) and E-268 (Ile → Ser). In 1239V2P10, two mutations changed E-191 (Phe → Leu) and E-202 (Lys → Arg). Repeat passaging of each isolate either in parallel (1047) or in different batches of Vero cells (1153 and 1239) selected for somewhat different patterns of mutations in the E gene in each instance (Table 1). However, we note that in each of the 6 independent passage series in Vero cells there were changes at either E-191 or E-268 in the uncloned P10 stock.

To determine whether the multiple changes observed in certain of the stocks (Table 1) were found together in individual viral genomes, we have sequenced viral RNA derived from individual plaques obtained from P10 uncloned stocks. Virus from plaques was amplified on C6/36 cells and RNA was sequenced through the region shown to be altered in the uncloned stocks. Virus was derived from two plaques each of 1047V1P10, 1153V1P10, and 1239V1P10; each derived stock showed the same two changes observed in the corresponding uncloned stock. For 1153V2P10, five of nine plaque isolates had one of the changes seen in the uncloned stock (E-202: Lys → Arg), and the remaining four plaques showed the other change (E-268: Ile → Val) together with the prM-26 change (Lys → Gln). For 1239V2P10, six of nine plaque isolates carried one change seen in the uncloned stock (E-202: Lys → Arg), two plaque isolates carried the other change (E-191: Phe → Leu), and one plaque isolate carried both changes. Changes at E-268 did not occur alone in the plaque-purified P10 stocks but were associated with changes at E-266 and prM-26. We conclude that during initial passage of dengue-3 in Vero cells, selection can be either for single or for multiple changes in the E gene sequence of individual viral genomes. A similar conclusion can be drawn from studies on mouse-passaged virus (see above).

Changes in the growth phenotype of Vero-passaged virus were observed. P10 virus from all three isolates produced plaques on Vero cells at 4 days p.i.; in growth experiments under liquid medium, cytopathic effects appeared in Vero cells at 3 days p.i. By comparison, with unpassaged virus plaques were not visible until 5–6 days p.i. and there was no CPE by 3 days p.i. Extracellular virus titers were 10 times higher for P10 virus than for parental virus at 24 hr p.i. (data not shown).

Changes in structural protein genes on passaging DEN-3 in *Ae. albopictus* cells

Mos 5 stocks of isolates 1153 and 1239 (see Materials and Methods) were passaged five times in *Ae. albopictus* cells to generate 1153CP5 and 1239CP5. No changes were observed in the sequence of the C or prM gene for 1153CP5 and 1239CP5. However 1153CP5 and 1239CP5 each had different mutations in the E gene resulting, in each case, in a change at Thr155 (to Ala and to Met respectively; Table 1). Both changes abolished the Asn-linked glycosylation site at E-153.

To determine whether the glycan content was reduced in the E proteins of 1153CP5 and 1239CP5, the proteins of ^{35}S-amino acid-labeled virus were examined by SDS–PAGE following digestion with *N*-glycosidase F. For undigested proteins, the E protein of 1153CP5 migrated faster (nominal MW 57 kDa) than the E proteins of 1153 and 1153V2P10 (58 kDa; Fig. 2). Following *N*-glycosidase digestion, the E proteins of the three viruses had similar increased mobilities (55 kDa). These results are consistent with the loss of a glycosylation site at Asn153 in both 1153CP5 and 1239CP5.

On Vero cells, the plaque size (0.5 mm diameter) of both 1153CP5 and 1239CP5 was smaller than parental virus (1 mm) at 7 days p.i. Both 1153CP5 and 1239CP5 induced extensive syncytium formation in C6/36 cells at 5–6 days p.i. under normal growth conditions; parental virus did not induce syncytium formation (see below).

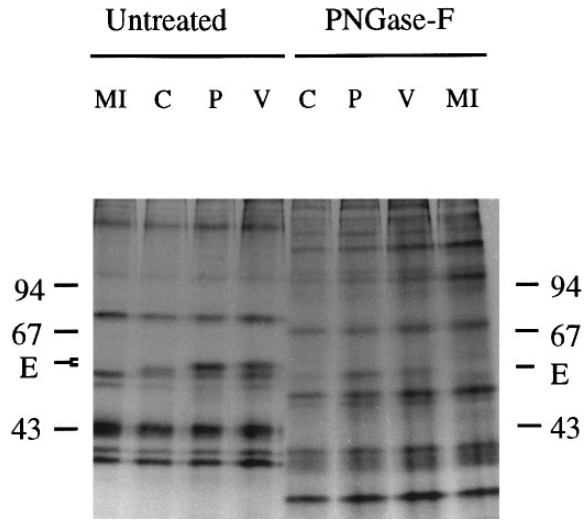

FIG. 2. *N*-Glycosidase F treatment of DEN-3 labeled with ^{35}S-amino acids. C6/36 cells infected with parental 1153 (P), C6/36-passaged 1153CP5 (C), and Vero-passaged 1153V2P10 (V) or mock-infected (MI) were labeled with TRAN[^{35}S]-Label (containing [^{35}S]methionine and [^{35}S]cysteine) from 64 to 84 hr p.i. Culture fluids were collected and clarified, and virus was precipitated with polyethylene glycol (8% final concentration) for 2 hr at 4°. *N*-Glycosidase F (PNGase-F) digestion (see Materials and Methods) was performed after heat denaturation of virion proteins (70° for 5 min in 0.5% SDS, 1% 2-mercaptoethanol). SDS–PAGE was in a 10% polyacrylamide gel for 1.5 hr at 150 V. The gel was fixed and stained in acetic acid/ethanol and Coomassie blue R250, respectively, and soaked in Amplify (Amersham) for 20 min before drying under vacuum and exposure to X-ray film. The positions of molecular weight standards of 43, 67, and 94 kDa are shown.

Time course of changes in the DEN-3 envelope protein gene on virus passage in mice and cell culture

On mouse passage, the mutations which led to changes at E-54, -277, -401, and -403 were all readily detected at P4 or P5; the mutation which changed E-18 in 1047MP was detectable at P10 but not at P7 or P8 (Table 1). On Vero cell passage, mutations which altered E-191, -202, and -268 were observed by P4. Mutations which altered prM-26 and E-266 and -291 were not detected at P5 but were present at P10. Among the four Vero-passage series showing more than one alteration in E, Phe191 was altered in three of the four. This mutation and the change at E-268 were detected earlier in the passage series than were the associated changes (Table 1), suggesting a stronger selective pressure for changes at E-191 and -268 than for changes at E-202, -266, or -291. Interestingly, in 1153V2, in which both residues E-191 and -268 were altered, the change Phe191 → Leu was detected early, but was not seen at P10 at which time mutations at Lys202 and Ile268 predominated. The most rapid and reproducible selection appeared to take place in *Ae. albopictus* cells; changes appeared early and selection was apparently complete by P5. Thus at P2 a proportion of the virus stock already contained the mutation at E-155. All parental working stocks had received three prior passages in C6/36 cells, and P2 therefore represented the fifth mosquito cell passage. We have previously reported (Lee *et al.,* 1993) that the mutations observed at P2 were not detected after passage in mosquitoes, i.e., before passaging in C6/36 cells (see Materials and Methods).

Localization of changes in passaged variants on the E protein 3D structure

We have localized 9 of the 11 changed amino acids in the DEN-3 E protein which altered on passaging on the three-dimensional structure of the soluble fragment (sE) of the TBE E protein (Rey *et al.,* 1995) (Fig. 3). (E-401 and -403 cannot be assigned positions as they are beyond the C-terminus of the sE fragment). Six of the nine changes map to the "hinge" region in domain II (Rey *et al.,* 1995). These changes are at E-54 and -277 in mouse-passaged virus and at E-191, -202, -266, and -268 in Vero-passaged virus. Three changes map outside the hinge region. These are at E-18 which maps to the loop between the Ao and the Bo beta-sheets in domain I and at E-291 which maps to the link between domains I and III (Fig. 3). The third change outside the hinge region was in domain I at E-155 in mosquito-cell-passaged virus.

The positions of amino acid substitutions in the E protein of three flavivirus vaccine strains, YF (17D), DEN-2 (16681-PDK-53), and JE (SA-14-14-2), compared to their respective parental viruses are also shown in Fig. 3. In 4 of 19 cases, the changes which accompanied passage and attenuation localized to the hinge region of the protein (see Discussion).

Fusion studies with passaged variants

Rey *et al.* (1995) suggested that both the hinge region of the TBE E protein and the N-linked oligosaccharide at E-154 may be involved in flavivirus fusion with cell membranes. Accordingly, we have compared the pH dependence of fusion by passaged and parental viruses in FFWI assays (Randolph and Stollar, 1990). C6/36 cells were infected (m.o.i. ~ 0.5) and at 4 days p.i. monolayers (100% infected as judged by immunofluorescence assay) were exposed to serum-free medium at pH 5.6–8.0.

The parental isolates 1047, 1153, and 1239 differ from each other in E at residues 65 and 298 (1047, 1153), 65 (1047, 1239), and 298 (1153, 1239). The fusion pH threshold for 1153 was at 7.2–7.4 and for 1047 and 1239 was at 7.4–7.6. Eight plaque isolates from Vero-passaged stocks were amplified in C6/36 cells and tested in FFWI experiments. The fusion pH threshold for seven of the eight which were altered at E-191 (Phe → Val), E-202 (Lys → Arg), or E-268 (Ile → Val or Ser) decreased by 0.6–1.0 pH units by comparison with the corresponding parental virus isolates (Table 2). Variant 1239V2P3c1 induced little decrease in fusion pH threshold but differs

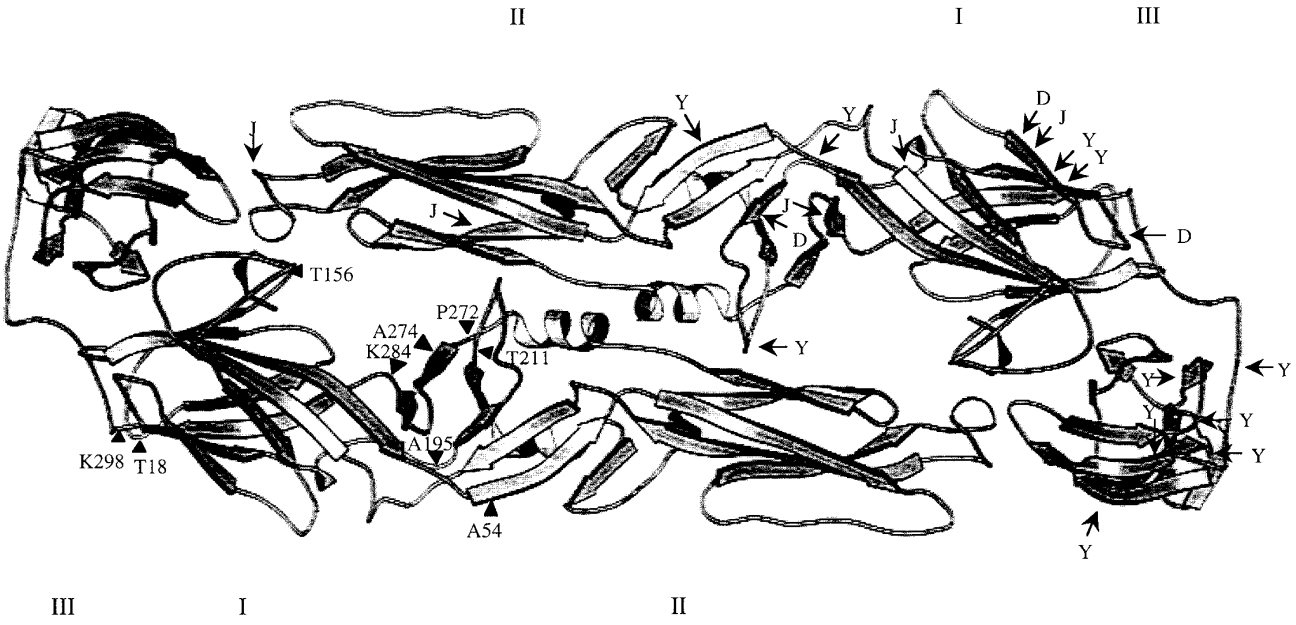

FIG. 3. Location of changes in the DEN-3 E protein on a three-dimensional representation of the TBE E protein. The locations of amino acid changes which occurred on passaging DEN-3 are mapped on the TBE E protein dimer structure (Rey *et al.,* 1995). Numbers in the bottom half of the dimer indicate the sites of amino acid changes in passaged variants of DEN-3 mapped to homologous residues in the TBE E protein. T18 in TBE E protein corresponds to A18 in DEN-3 E protein, A54 to A54, T156 to T155, A195 to F191, T211 to K202, P272 to T266, A274 to I268, K284 to F277, and K298 to E291 (single-letter amino acid code). Arrows in the top half of the dimer indicate the locations of differences between attenuated YF, DEN, and JE vaccine derivatives and the corresponding wild-type virus: vaccine 17D/YF Asibi (Y), vaccine DEN-2 16681-PDK-53/16681 (D), and vaccine SA-14-14-2/JE SA-14 (J).

in the amino acid substitutions at E-65 and E-191 compared to the other Vero-cell-passaged variants altered at E-191. Mouse-passaged virus also induced a marked decrease in fusion pH threshold in two of three plaque isolates; plaque-purified virus altered at E-277 and -401/403 showed a decrease in pH threshold of 0.7 and 0.6–0.8 pH units, respectively (Table 2). In contrast the two variants selected by passaging five times in C6/36 cells (resulting in the deletion of the N-linked glycosylation site) induced an increase in fusion threshold of 0.4 pH units by comparison with parental virus (Table 2).

The fusion properties of the cell-passaged variants were examined also in the presence of the lysosomotropic agent NH_4Cl. Low pH-dependent fusion between viral envelopes and endosomal membranes is inhibited by NH_4Cl which elevates intraendosomal pH (Gollins and Porterfield, 1986). Vero cells were infected with passaged or parental virus and incubated until 5 hr p.i. with or without NH_4Cl at concentrations up to 50 m*M.* Monolayers were washed and fresh growth medium was added at 5 hr p.i. Greater than 90% reductions in virus titer were observed at 24 hr p.i. for Vero-passaged DEN-3 (1153V2P10 and 1239V2P10) at NH_4Cl concentrations of 20 m*M* or higher (Fig. 4). The titers of parental and C6/36-passaged viruses were 15–40% lower at 20 m*M* NH_4Cl and 60–70% lower at 50 m*M* than in the absence of NH_4Cl. The increased sensitivity of these Vero-passaged viruses to elevated endosomal pH induced by NH_4Cl is consistent with their dependence on a lowered pH for fusion induction (see above).

DISCUSSION

The identification of changes associated with dengue virus adaptation to cells from nonhuman sources is likely to help identify the molecular determinants of viral attenuation consequent on passage in cell culture. To this end we have carried out limited serial passage of uncloned DEN-3 in mice, monkey kidney fibroblasts, and mosquito cells. Passaging in all three instances selected for changes predominantly in the envelope protein E. For the three independent DEN-3 isolates passaged intracerebrally in mice there were changes in E at residues 18 and 54 (isolate 1047), at residue 277 (isolate 1153), and at residues 401 and 403 (isolate 1239). The six independent passage series in Vero cells (two series with each of three isolates) generated changes at E-191 in four of the six passage series, at E-202 in two passage series, and at E-268 in three passage series. Changes occurred at prM-26 in two independent passage series. For the two isolates (1153 and 1239) passaged in cultured mosquito cells there was a change at E-155 in each, resulting in the loss of an Asn-linked glycosylation site at E-153. Thus passage of DEN-3 in cell culture consistently selected for particular amino acid changes, whereas on limited mouse passage, no change in E was found more than once. These observations may reflect the greater cell-

TABLE 2

Fusion pH Changes for DEN-3-Passaged Variants

Passage	Virus stock[a]	E protein change[b]	Change in fusion pH threshold[c]
Vero cell	1047V2P4c	Phe191 → Val	−1.0, −0.8
	1047V2P10c*	Phe191 → Val	−1.0, −0.8
	1239V2P3c1	Phe191 → Leu	−0.2, −0.2
	1153V2P10c1	Lys202 → Arg	−0.8, −0.7
	1239V2P3c2	Lys202 → Arg	−0.6
	1239V2P10c*	Lys202 → Arg	−0.6, −0.7, −0.8
	1153V2P10c2*	Ile268 → Val	−0.8, −0.7
	1239V1P10c	Ile268 → Ser	−0.6, −0.8, −0.8
Mouse brain	1047MP10c*	Ala54 → Glu	−0.2
	1153MP10c*	Phe277 → Ser	−0.7
	1239MP10c*	Glu401 → Lys	−0.6, −0.7, −0.8
		Thr403 → Ile	
C6/36 cell	1153CP5	Thr155 → Ala	+0.4, +0.4
	1239CP5	Thr155 → Met	+0.4, +0.4

[a] Plaque-purified Vero- and mouse-passaged virus stocks (twice amplified in C6/36 cells) were used; C6/36-passaged virus stocks were C6/36 cell culture fluids. Plaque-purified isolates have the suffix c.

[b] Numbering is from the N-terminal residue of E (Lee *et al.,* 1993).*Structural protein genes of plaque isolate were completely sequenced; other isolates were sequenced in regions altered in uncloned stocks.

[c] Determined in FFWI assays (see Materials and Methods) by comparison with parental virus in the same experiment; up to three experiments were performed and results are given for each.

type heterogeneity of mouse brain compared with cultured cells and a greater variety of selection pressures during intracerebral passage compared with cell culture passage.

Passaging generally led to substitutions at residues which are conserved between the four DEN serotypes, e.g., E-54, -191, -202, -266, -277, -401, and -403. E-268 is conserved in three serotypes; E-18 and -291 are relatively nonconserved between DEN serotypes. Two changes which took place on passaging in mice were at residues (E-54 and -403) which are conserved between the DEN serotypes, YF, and TBE. Considered together the data indicate the operation of strong selection pressures for the evolution of E during both cell culture passage and mouse passage. None of the changes in E were at residues which differ between the four DEN-3 subtypes identified by Lanciotti *et al.* (1994).

Five mutations of 20 were selected by P2 and 14 were evident by P5. Two changes which were selected relatively slowly (at E-18 and -291) corresponded to residues which are poorly conserved between serotypes; changes at these residues may exert only limited phenotypic effects which may account for the slow selection. In six of seven passage series where more than 1 mutation was selected in a single uncloned virus population, the mutations differed in their times of appearance. Changes at E-54, -155, -191, and -268 appeared early compared with changes at E-18, -202, -266, and -291 and at prM-26. The exception occurred in 1239MP10 where the 2 mutations leading to changes at E-401 and -403, together with a silent mutation, were all first detected at P5 (Table 1). All three were present in the same plaque isolate at P10, suggesting the possibility that these changes are codependent.

In three Vero passage series two changes were detected in the same plaque isolate at P10. These changes first appeared at different passage levels, indicating that sequential selection had occurred (changes at E-266 and -268 in 1047V1P10, at E-191 and -291 in 1153V1P10, and at prM-26 and E-268 in 1239V1P10). In two other Vero passage series (1153V2 and 1239V2), changes at E-191 and -202 appeared sequentially at the same relative times (Table 1), but these changes were mostly detected alone (13 of 18 plaques examined) in plaque-purified clones from P10, indicating a lack of dependence of one change on another. Plaques isolated from 1153V2P10 showed no mutation at E-191; instead either a single mutation at E-202 or double mutations at prM-26 and E-268 were seen in individual plaque isolates.

Six (residues 54, 191, 202, 266, 268, and 277) of the nine changes in the DEN-3 E protein, which can be positioned on the three-dimensional structure of E (Rey *et al.,* 1995), map to the hinge region in domain II (Fig. 3). This region is proposed as a focus for the low pH-mediated conformational change required for the surface exposure of the conserved hydrophobic *cd* fusion loop (E-

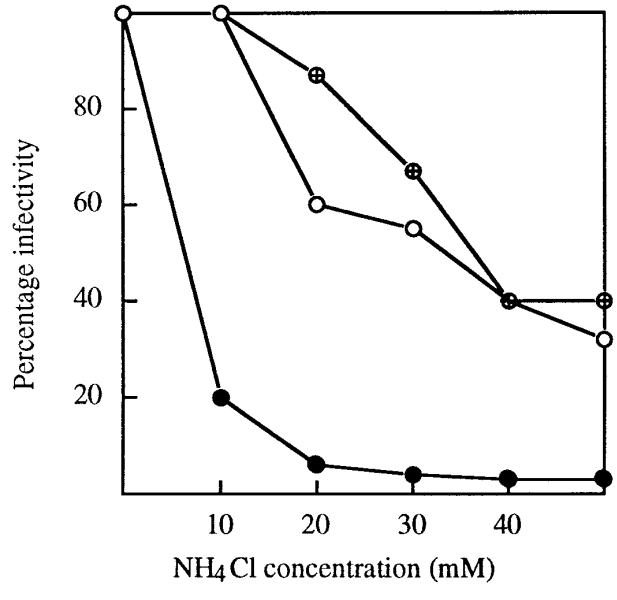

FIG. 4. Effect of NH_4Cl on replication of DEN-3 in Vero cells. DEN-3 virus stocks (parental 1153 ○; 1153CP5 ⊕; 1153V2P10 ●) were used to infect Vero cell monolayers pretreated for 1 hr with 0, 10, 20, 30, 40, or 50 m*M* NH_4Cl in growth medium (pH 8.0). The NH_4Cl concentration was maintained for 5 hr p.i. and incubation was continued in normal growth medium. EV titers at 24 hr p.i. were determined by plaque assay on Vero cell monolayers. Titers at each concentration of NH_4Cl were expressed as a percentage of titers in the absence of NH_4Cl.

98 to -113 in DEN-3) in domain II (Rey *et al.,* 1995). Residues E-401 and -403 localize to a relatively conserved putative α-helical region adjacent to the membrane-spanning anchor. This region is amphipathic in character and may be involved in the E trimer formation which also contributes to generating the fusogenic state of E (Stiasny *et al.,* 1996). These considerations suggested that many of the changes selected on passaging (with the probable exception of E-18 and -291) may result in alterations to fusion-related functions of the virus. Consistent with this, DEN-3 changed at E-191, -202, -268, -277, or -401 and -403 showed a marked decrease in fusion pH threshold in C6/36 cells. Furthermore Vero-passaged virus was much more sensitive to early inhibition of growth in Vero cells by NH_4Cl than parental virus, suggesting that the lower fusion pH threshold observed in FFWI assays in C6/36 cells reflected alteration in an early step in virus replication in Vero cells. An MVE mAb-resistant neutralization-escape variant altered at E-277 (MVE numbering) in the hinge region also shows an altered pH threshold in FFWI experiments and is unable to hemagglutinate (McMinn *et al.,* 1995a).

In the flavivirus E protein structure of Rey *et al.* (1995) the oligosaccharide at E-154 lies over the hydrophobic groove that receives the *cd* loop and most likely participates in stabilizing the E dimer. Consistent with this, mosquito cell-passaged DEN-3 lacking the corresponding glycosylation site has an increased pH threshold (by 0.4 units) in FFWI assays. The glycosylation site is abolished in DEN-2 variants selected in C6/36 cells in the presence of NH_4Cl and these variants show an elevated fusion pH threshold compared with parental virus (Guirakhoo *et al.,* 1993). Kawano *et al.* (1993) showed that the loss of the glycosylation site at E-153 enhanced the mouse virulence of DEN-4 virus, suggesting a direct connection between changes in fusion and virulence potential in mice.

We have mapped the position of E protein substitutions in vaccine strains of YF (Hahn *et al.,* 1987), DEN-2 (Blok *et al.,* 1992), and JE (Nitayaphan *et al.,* 1990) on the TBE E structure (Fig. 3). For YF 17D, which was generated by 240 passages mostly in chicken embryonic tissues, 6 of the 12 changes in E are in domain III; the rest are in domains I and II; only 2 changes were in the hinge region. For DEN-2 which was passaged 53 times in primary canine kidney cells, 2 of 3 changes are in domain I and 1 is in the hinge region. For JE which was passaged in primary hamster kidney cells and infant mice (subcutaneous inoculation), 2 changes are in domain I and 3 are in domain II (one in the hinge region). On passage of MVE 10 times in human adenocarcinoma (SW13) cells, changes at residue 390 were selected in five of six passage series (Lobigs *et al.,* 1990); residue 390 maps to the distal face of domain III, a region likely to be involved in cell attachment. Our data on DEN-3 show there to be a concentration of changes in the hinge region on passaging in monkey kidney cells and intracerebrally in mice. In mosquito cells, passaging led to a loss of glycan attachment at E-153. However, all three conditions of passaging appeared to modulate fusion, suggesting that the fusion of the virus envelope with cellular membranes is a point at which selection pressures can be exerted during the limited number of passaging steps employed in our study. It is not clear whether the focusing of changes in DEN-3 E in the hinge region reflects a rapid selection for changes in this region which on further passaging is followed by changes elsewhere in E (as seen in the sequenced vaccine strains) or whether other explanations for our data must be sought.

ACKNOWLEDGMENTS

This work was supported by a grant from the Australian Research Council. We thank Dr. Franz Heinz for the diagram of the TBE E protein.

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