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Identification of epitopes on the envelope (E) protein of dengue 2 and dengue 3 viruses using monoclonal antibodies

Brief Report

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Summary. Of a panel of forty-six anti-dengue 3 monoclonal antibodies (MAbs) only three neutralised infection of BHK cells by dengue 3 virus. Attempts to select neutralisation escape mutants (n.e.m.) with two of these antibodies failed. The n.e.m. population selected in the presence of the third neutralising antibody, 1H9, had a nucleotide change at position 1157 of the E protein gene resulting in a non-conservative amino acid change at E386 for a Lys to an Asn. A dengue 2 n.e.m. was selected with the flavivirus crossreactive IgG monoclonal antibody 4G2, had deduced amino acid changes at E169 (Ser to Pro) and E275 (Gly to Arg). This dengue 2 n.e.m. population produced smaller plaques in BHK cells than the parental virus, decreased fusion activity (FFWI) and had lost the ability to agglutinate gander erythrocyes at pH 6.0 to 6.7.

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Dengue viruses (serotypes 1–4) are small, enveloped, positive-sense RNA arboviruses, that are members of the genus *Flavivirus* in the family *Flaviviridae* [42]. They cause disease in humans varying from mild (dengue fever, DF) to potentially fatal (dengue haemorrhagic fever, DHF; dengue shock syndrome, DSS). It has been estimated that two-fifths of the world's population is at risk of infection; that an estimated 50 million infections occur annually with an estimated 500,000 cases of DHF annually [43].

A safe, effective and inexpensive vaccine against dengue infection is required. The ideal vaccine would protect against all four dengue serotypes and provide long-lasting immunity against dengue infection [3, 13, 32]. The vaccine should also be able to produce greater than 95% seroconversion, and be able to be administered to children between the ages of 6 months and one year [12]. Importantly, the vaccine should not sensitise vaccinees to DHF/DSS [12].

There would seem to be two broad approaches to the issue of dengue vaccination. The first, which is in progress, is to produce four separate vaccines, one against each dengue serotype [18, 44]. The second is to develop a chimeric vaccine containing determinants capable of eliciting protective immunity against multiple serotypes [2].

In order to produce a safe, effective chimeric vaccine against dengue infection, the determinants on the surface of the virion of each dengue serotype responsible for inducing neutralising need to be identified. While linear immunodominant serological determinants on the envelope (E) protein of dengue virions have been identified [1, 20, 32, 37], only two neutralising epitopes have been identified [15, 27, 41]. This study was undertaken to identify the epitopes involved in neutralisation of dengue viruses that could be incorporated into a chimeric vaccine.

The viruses used in this study were as follows: Dengue 1 (Hawaii), dengue 2 (NGC), dengue 3 (H87, 602); and dengue 4 (H241) were obtained from Yale Arbovirus Reference Centre. Murray Valley Encephalitis (MVE; MRM66), Kunjin (KUN; MRM 16) and Edge Hill (EH, C281) were maintained since isolation. Dengue 3 virus, strain PRS 225489 isolated in Burma in 1982, was obtained from CDC, Fort Collins, USA.

Monoclonal antibodies were produced by standard procedures [45] using dengue 3 virus (strains H87 and 602) immunised BALB/c mice. The 4G2 hybridoma cells were provided by the Walter Reed Army Institute for Research [10]. The culture supernatants from the hybridomas were screened for the presence of anti-dengue 3 MAb by indirect enzyme linked immunosorbent assay (ELISA) and indirect immunofluorescence assay (IFA) [22]. Patterns of crossreactivity between dengue serotypes and between selected flaviviruses were determined by indirect immunofluorescence employing infected BHK or C6/36 cells, in haemagglutination inhibition (HI) assays [9]. Virus neutralisation assays were performed using BHK suspension cells based on the method of Morens et al. [33] and a neutralisation index was calculated [22]. Viral proteins recognised by the MAbs were identified using western blots of lysates of virus infected BHK cells [22]. Isotyping was performed using a commercial Ouchterlony assay according to the manufacturer's instructions (The Binding Site, UK). The competitive binding ELISA was based on the procedure described by Jianmin et al. [22].

Neutralisation escape mutants were selected by growing dengue 2 (NGC) virus and dengue 3 (PRS 225489) virus in BHK cells in a modification of a procedure by Holzmann et al. [16]. The selection of n.e.m. of dengue 3 virus, strain PRS 225489 was attempted with the MAbs 1H9, 11D5, 17F3-D8 and 4G2. This strain was used because it produced clear and reproducible plaques in BHK cells. The selection of a dengue 2 n.e.m. was attempted with 4G2. The starting viral inoculum was referred to as Vo. Briefly, monoclonal antibody (MAb) (heat inactivated mouse ascitic fluid) was diluted at 1/5, 1/20 and 1/40 in RPMI 1640 (Gibco, USA) containing 2% v/v FCS and incubated with an equal volume of virus diluted 10^{-1} to 10^{-6} in RPMI 1640- 2% v/v FCS for 2 h at 4 °C in a 24 well plate (NUNC, Denmark). Suspensions of BHK cells (0.5 mL; at 2.5×10^6 cells/mL)

in RPMI 1640 with 5% v/v FCS were added to each well of the 24 well plate and incubated for a further 2 hours. The tissue culture supernatant (t.c.s.) was then removed from the BHK cells and replaced with tissue culture medium containing MAb (1 ml), at the same concentration as was originally in the well and the plates were incubated at 37 °C for 7 days. The plates were stained with 0.5% crystal violet/25% formaldehyde in PBS for 30 min. The virus was passaged in the presence of the selecting MAb until there was no difference between the virus grown in the presence and absence of selecting antibody (i.e. the neutralisation index was < 1.0). The n.e.m. virus (200 μl) was infected in C6/36 cells [19] in the presence of 10% v/v MAb in RPMI 1640 for 5 days, then replaced with fresh medium and incubated for a further 2 days at 28 °C. The virus was harvested, FCS to 30% v/v added and the virus was stored at −70 °C. Stocks of "passage control" virus were prepared in a similar manner except they were grown in the absence of any selecting MAbs, to enable detection of any changes due to repeated passage in cell culture.

Sequencing of the starting virus, termed Vo, the passage control virus (p.c.) and the neutralisation escape mutant virus (n.e.m.) was performed following amplification of the cDNA. Oligonucleotide primers used for the amplification of cDNA and for sequencing of dengue 3 virus were those described by Lanciotti et al. [24]. Other dengue 3 primers were designed with the use of the OLIGO 5.0 analysis program (National Biosciences). (P988 5′ TACGTGGGTCGAAGTGGTG 3′; P1331 5′ AGAACCTCAAATACACCGTCATCAT 3′; CP1584 5′ GGTAGAG GTAAGACAAAGAAC 3′). The oligonucleotide numbers refer to the nucleotide number in the dengue 3 genome. The oligonucleotide primers used to amplify and sequence dengue 2 were those described by Lewis et al. [26]. RNA extracted from virus using guanidine isothiocyanate [24] was reverse transcribed using random primers (Pharmacia, USA) and Superscript reverse transcriptase (Gibco, USA). The DNA was amplified using the following cycling conditions: one cycle at 94 °C for 4 min for initial denaturation of the cDNA, followed by 35 cycles of amplification at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 4 min, then one cycle at 72 °C for 10 min. The products were purified using BresaClean Kits (Bresatech, Australia) and directly sequenced with an automated ABI DNA sequencer following manufacturer's protocols (PE Biosystems, USA).

In order to establish if the changes identified in the dengue 2 n.e.m. following sequencing of the E protein resulted in phenotypic changes in the virus, the haemagglutination properties [8]; and the fusion properties (by fusion from within (FFWI) [25]) were investigated.

Forty-six hybridomas (23 IgM and 23 IgG) were produced from three fusions; two with dengue 3 (H87) and one with dengue 3 (602) virus primed mice. Only one out of twenty-three IgM monoclonal antibodies and two out of twenty-three IgG monoclonal antibodies neutralised and/or inhibited agglutination of gander erythrocytes by dengue 3 virus (Table 1). The three monoclonal antibodies, 17F3-D8, IgG2a; 11D5, IgG1; 1H9, IgM which neutralised dengue 3 virus and also inhibited agglutination of gander erythrocyte cells by the virus, reacted with the E protein in western blots (data not shown). In competitive binding ELISAs

Table 1. Neutralisation and haemagglutination inhibition titre of monoclonal antibodies that neutralised dengue 3 virus

MAb	Isotype	IFA	Blot	Neutralisation index						Haemagglutination inhibition titre				
				DEN1	DEN2	DEN3	DEN4	MVE	KUN	DEN1	DEN2	DEN3	DEN4	MVE
17F3-D8	IgG2a	Flavi[a]	E	<1.0	1.0	1.1	1.0	<1.0	<1.0	1280	1280	2560	640	320
11D5	IgG1	DEN 3	E	<1.0	<1.0	1.1	<1.0	<1.0	<1.0	40	160	1280	80	80
1H9	IgM	DEN 3	E	<1.0	<1.0	1.3	<1.0	<1.0	<1.0	40	40	640	40	40

[a]MVE, KUN, EH, DEN 1–4

the dengue 3 specific neutralising MAbs 11D5 and 1H9 competed with each other for sites on the virion (data not shown).

A dengue 3 n.e.m. population was succcessfully selected using the dengue 3 specific IgM MAb 1H9 in a total of five passages. Dengue 3 virus which escaped neutralisation by antibodies 11D5, 17F3-D8 or 4G2 could not be detected. Passaging of dengue 3 virus in BHK cells in the absence of a selecting MAb resulted in a viral population with five nucleotide substitutions (Table 2). The nucleotide substitutions at position 1038 (103 of the E protein gene) resulted in a conservative amino acid replacement of a valine in the parental virus to Ala in the passage control. A nucleotide substitution at position 2340 (1405 of the E protein gene) also resulted in a conservative replacement in the deduced amino acid residue of an Arg in the parental viral population with Lys in the

Table 2. Summary of changes in the nucleotide and deduced amino acid sequences of the E protein in dengue 3 virus (starting viral stock, Vo), the passage control (p.c.) and neutralisation escape mutant (n.e.m.) 1H9

Nucleotide number [a]	Virus preparation	Nucleotide change	Amino acid change from Vo
1038 (103)	Vo	T	
	p.c.	C	E35 Val → Ala
	n.e.m.	C	E35 Val → Ala
1109 (174)	Vo	T	
	p.c.	C	No change
	n.e.m.	T	
1648 (713)	Vo	C	
	p.c.	T	No change
	n.e.m.	C	
1669 (734)	Vo	A	
	p.c.	A	
	n.e.m.	G	No change
1681 (746)	Vo	C	
	p.c.	C	
	n.e.m.	T	No change
2092 (1157)	Vo	A	
	p.c.	A	
	n.e.m.	T	E386 Lys → Asn
2245 (1310)	Vo	G	
	p.c.	A	No change
	n.e.m.	A	No change
2340 (1405)	Vo	G	
	p.c.	A	E486 Arg → Lys
	n.e.m.	A	E486 Arg → Lys

[a]Numbering from the 5′ end of the genome [34]. Numbers in parenthesis indicate the number of this nucleotide in the E protein gene

passage control. The neutralisation escape mutant virus population had six nucleotide substitutions, with three of these changes also being present in the passage control population. Three nucleotide substitutions were silent and did not result in amino acid changes (Table 2). Only one of these changes, at position 2092 (1157 of the E protein gene) resulted in a unique change from Lys to Asn at residue E386.

A dengue 2 n.e.m. population was also successfully selected using the flavivirus crossreactive MAb 4G2 in six passages. Two nucleotide changes (1442,1760) both of which resulted in significant amino acid changes (E169 Ser → Pro; E275 Gly → Arg) were detected in the E protein gene of dengue 2-4G2 neutralisation escape mutant virus (Table 3). The passage control virus showed neither of these changes but contained a unique change from Phe in parental virus and the n.e.m. to Lys in the passage control at E43. Several differences were also detected between the nucleotide and deduced amino acid sequence of the parental strain of dengue 2 and published sequences of the same strain of virus. However, none of these differences occurred at sites that changed under the selective pressure of the antibody 4G2 (E169, E275).

While both the wild type (Vo) and passage control dengue 2 viruses agglutinated gander erythrocytes at the pH optima of 6.2–6.4, while the neutralisation escape mutant virus failed to agglutinate erythrocytes at any pH between 6.0 and 6.7. The wild-type dengue 2 virus produced extensive fusion of the C6/36 cell monolayer (syncytia) throughout the range of pH tested in the FFWI experiment (pH 5.6 to 7.0). So extensive was the fusion produced that within the field of view a definite cell boundary could not be seen. In an attempt to quantitate the degree

Table 3. Comparison of the deduced amino acid sequences of the E protein of dengue 2 (NGC) Vo, the passage control and the 4G2 mutant virus

Nucleotide number [a]	Virus preparation	Nucleotide change	Amino acid change from Vo
1064 (127)	Vo	T	
	p.c.	C	E43 Phe → Lys
	n.e.m.	T	
1442 (505)	Vo	T	
	p.c.	T	
	n.e.m.	C	E169 Ser → Pro
1760 (823)	Vo	G	
	p.c.	G	
	n.e.m.	A	E275 Gly → Arg
2120 (1086)	Vo	T	
	p.c.	T	
	n.e.m.	C	No change

[a] Numbering from the 5′ end of the genome. Numbers in parenthesis indicate the number of this nucleotide in the E protein gene

Table 4. Comparison of the fusion activity in C6/36 cells infected with dengue 2 wild-type virus and n.e.m. 4G2 virus following treatment at varied pH (FFWI)

pH	DEN 2 (Vo)		DEN 2 n.e.m.		
	Average number of cells	Average number of nuclei	Average number of cells	Average number of multinucleated cells	% fused cells
5.6	1[a]	42	62	1.8	2.9
5.8	1	67	77	1.7	2.2
6.0	1	75	84	1.8	2.2
6.2	1	80	97	1.5	1.6
6.4	1	67	79	0.5	0.6
6.6	1	59	90	0.7	0.7
6.8	1	66	99	0.5	0.5
7.0	1	63	92	0.5	0.5

[a]Complete fusion of the monolayer produced

of fusion produced by the wild-type virus, the nuclei in a set area (defined by an eyepiece graticule) were counted in six fields (Table 4). The fusion appeared to peak at pH 6.2 with an average of 80 nuclei per unit field.

By comparison, the n.e.m. virus produced substantially less fusion. In order to assess the fusion from within produced by the n.e.m. population, six areas were counted and the total number of cells, together with the number of multinucleated cells were recorded. The highest degree of fusion observed by the mutant virus in the C6/36 cells was produced following treatment of the cells with media at pH 5.6.

This is the first report of a neutralisation escape mutant of dengue 3 virus. The change in the E protein of dengue 3 virus at position E386 (Lys to Asn) occurred on the lateral surface of domain III of the flavivirus E protein structure described by Rey et al. [36]. The changes in the dengue 2 E protein at E169 (Ser to Pro) and E275 (Gly to Arg) were adjacent to the hinge region between domains I and II.

In contrast to results reported for a number of other flaviviruses (including dengue 2 virus) [6, 10, 22, 23, 38], the majority of monoclonal antibodies derived from dengue 3 virus infected mice, did not neutralise dengue infection in vitro. Two of the three MAbs that did neutralise, did so at titres that were too low to permit selection of mutants. A possible explanation for the lack of production of neutralising antibodies against dengue 3 virus may be the restricted MHC background of the inbred BALB/c mice. Roehrig and his coworkers [37] suggested that some of the responses to peptides were limited by the restricted genetic background of BALB/c mice. Alternatively, the lack of neutralising antibodies may have been due to the failure of the dengue 3 virus to replicate in mice and the use of the short immunisation schedules to obtain IgM antibodies.

These findings may support the ADE concept of immunopathology of dengue infections in humans. If most of the antibodies produced against dengue 3 virus are non-neutralising and cross-reactive they might enhance infection by the other

dengue serotypes. Epidemiological data indicating DSS is more common in a sequence of infections ending in dengue 2 than in one ending in dengue 3 is compatible with these data [14, 40]. The high level of non-neutralising antibodies produced in these mice following a dengue 3 infection may be of concern in the development of a dengue vaccine.

Although a dengue 3 virus neutralisation escape mutant was selected with the monoclonal antibody 1H9, the failure to select neutralisation escape mutants with antibodies 11D5, 17F3 and 4G2 may have been due to a variety of factors such as a relatively homogeneous wild-type virus population, a non-viable mutant population, or to low affinity of the neutralising antibody. To demonstrate that the nucleotide changes observed in the escape mutants were not simply due to changes induced by cell culture adaptation a passage control virus was passaged in parallel.

Assuming that the three dimensional structure of the dengue 3 virus E protein resembles that of the TBE E protein [36], the change at position 386 of the E protein from a Lys in the wildtype to an Asn in the n.e.m., would be adjacent to the proposed receptor binding site (the *F-G* loop on lateral surface of domain III; [36]). Changes in this domain have been associated with changes in neurovirulence in other flaviviruses such as TBE, MVE and LI [17, 21, 28, 29].

Two nucleotide changes at positions 505 and 823 in the E protein gene of the dengue 2 virus n.e.m. resulted in amino acid changes at positions 169 and 275 of the E protein. The two amino acid changes; at position E169 from a Ser in the wild-type and p.c. to a Pro in the suspected n.e.m. and at residue E275 from a Gly in the wild-type and p.c. to a Arg in the n.e.m. were non-conservative substitutions. The substitution at residue 275 would have been accompanied by localised changes in the charge, from no charge to a positive charge and may have altered the interaction between the virus and the cell [4]. The changes corresponded to the *Go* sheet (aa 169–176) and the hinge region of the klD_o sheets (k = aa 275–277, l = aa 279–283 and D_o = aa 39–52) in the crystallised TBE E protein structure respectively [36]. The change at amino acid 275 corresponded to the region that has been proposed to project outwards to enable the virus to gain access to the host cell via the viral receptor [36]. This finding was recently supported by Falconar [9] with the binding of MAb 4G2 being localised to a peptide corresponding to E274–283. Amino acid changes from wild-type virus sequences have also been reported in similar regions of the E protein, for a TBE n.e.m. (E171) [11, 17], a MVE n.e.m. (E274, E276 and E277) [31] and a JE n.e.m. (E270) [6].

The changes in the E protein of the dengue 2 virus mutant population resulted in a virus population which failed to agglutinate gander erythrocytes, which produced smaller plaques than the passage control virus in BHK cells and which produced less syncytia in monolayers of C6/36 cells (FFWI). A change at this site has previously been reported to affect haemagglutination [31] and the fusion ability of flaviviruses [25, 30]. Previously, Randolph and Stollar [35] and Summers et al. [39] had demonstrated that the MAb 4G2 inhibited cell fusion from without (FFWO). Summers et al. [39] proposed that antibodies like 4G2 may inhibit fusion by steric interference or by inhibiting attachment of the virus

to the host cell and therefore preventing fusion and entry of the virus into the cell. Alternatively, the changes in the hinge region of the E protein may produce conformational changes in the laterally exposed cell receptor site [7] and may result in the receptor site being inaccessible to the cell surface and the virus being unable to bind to cells [25].

In this report, we described the identification of an unrecognized epitope on the E protein of dengue 3 virus by a dengue 3 specific IgM monoclonal antibody (MAb) and changes at two sites in the E protein of dengue 2 virus associated with escape from neutralisation by the flavivirus cross-reactive IgG MAb, 4G2.

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