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Serologically defined linear epitopes in the envelope protein of dengue 2 (Jamaica strain 1409)

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Summary. Antisera from dengue patients and dengue virus infected rabbits recognized octapeptides corresponding to linear amino acid sequences in the envelope protein of dengue 2 (Jamaica 1409). Although no peptide was recognized by sera from all dengue infected hosts, two peptides (^{216}LPLPWLPG^{223} and ^{448}FSGVSWTM^{455}) were recognized by sera from all dengue 2 infected rabbits. One of these ^{448}FSGVSWTM^{455} was also recognized by sera from both the dengue 2 patients tested. No peptides were identified which reacted exclusively with all dengue 2 infected animals. Use of a mouse monoclonal antibody (1B7) enabled identification of two regions (^{50}AKQPATLR^{57} and ^{127}GKVVLPEN^{134}) and possibly a third (^{349}GRLITVNP^{356}) in the envelope protein of dengue 2 likely to be involved in haemagglutination inhibition and virus neutralization in vitro.

Introduction

Dengue viruses are a major cause of pediatric morbidity and mortality in the tropical regions of the world [13]. A vaccine and or a simple reliable test for the serodiagnosis of dengue infection could dramatically reduce this morbidity and mortality.

Although Sabin and Schlesinger produced an effective suckling mouse brain derived vaccine in 1945 [25], recent efforts to produce a vaccine acceptable for human use have been largely unsuccessful [1, 7, 22]. Vaccine development has also been slowed by the necessity to avoid the potentially fatal complication of antibody-mediated enhancement of virus infection [14, 15, 24]. Enhancement is reported to be dependent on properties of both antibody and epitopes on the virus [12, 15] and in experimental animals has been shown to be associated with anammestic infections with a second dengue serotype [14].

One dengue vaccine has avoided the problem of antibody mediated enhancement by employing a non-structural viral protein [26]. However, it provided only partial protection for animals challenged with live virus.

Serodiagnosis of dengue infections is complicated by the extensive serological cross reactivity between dengue serotypes and some other falviviruses [18]. Because many dengue-specific or dengue serotype-specific monoclonal antibodies appear to recognize conformational or non-linear epitopes [16] it is not yet clear whether any of the conserved linear amino acid sequences in the envelope protein of dengue viruses [6, 10, 11, 21, 31] are associated with conserved serological reactivity.

The aim of this study was to identify linear epitopes on the envelope protein of the dengue virion which are recognized by antibody and which could subsequently be evaluated for their role in neutralization and enhancement of infection, and as antigens for serodiagnosis.

Patients and methods

Antisera

Rabbit

Outbred laboratory rabbits were infected intravenously (iv) and intraperitoneally (ip) with the protype strains of dengue 1 (Hawaii), dengue 2 (New Guinea C), dengue 3 (H87) or dengue 4 (H241) and serum was obtained by bleeding from the marginal ear vein. Serum used in IgM assays was collected 7 days after an initial iv infection. Hyperimmune sera were obtained following 3–8 infections with virus.

Human

The convalescent sera used in this study were collected from Australian residents with primary clinical dengue infections. All sera from patients with dengue 1 infections were obtained during the 1981 outbreak of dengue 1 infection in North-Eastern Australia [19].

Neither acute nor convalescent sera from any of the patients in this study had haemagglutination inhibiting (HI) [4] antibody titres greater than 1 in 640. All acute phase sera contained IgM antibody [8] which reacted with dengue virus in an HI assay.

Mouse

Monoclonal antibody 1B7 was a gift from Dr. Mary K. Gentry, Walter Reed Army Institute of Medical Research. It neutralized and enhanced infection by dengue 2 and inhibited haemagglutination of gander erythrocytes by dengue viruses [16].

Epitope identification

This procedure has been described in detail elsewhere [9]. Briefly, the protocol was as follows. Octapeptides containing amino acid sequences corresponding to those in the E protein of dengue 2 (JAM 1409) [6] were synthesized on polyethylene rods using t-butyloxycarbonyl (BOC)-amino acids and beginning at the carboxy-terminal. The amino-terminal group of the final amino acid was acetylated to avoid the introduction of a positive charge adjacent to the peptide to be evaluated. Only an epitope at the amino-terminus of the native protein would contain such a charged main-chain amino group. Octapeptide sequences overlapped such that adjacent peptides had seven amino acids in common, e.g.,

the first three peptides corresponded to amino acid sequnences at the amino terminus of the dengue 2 envelope protein and were composed of the following sequences ^{1}MRCIGISN^{8}, ^{2}RCIGISNR^{9}, ^{3}CIGISNRD^{10}. (The one letter amino acid code has been used throughout. *A* alanine, *V* valine, *L* leucine, *I* isoleucine, *P* proline, *F* phenylalanine, *W* tryptophan, *M* methionine, *G* glycine, *S* serine, *T* threonine, *C* cysteine, *Y* tyrosine, *N* asparagine, *Q* glutamine, *D* aspartic acid, *E* glutamic acid, *K* lysine, *R* arginine, *H* histidine.)

Peptides coupled to these rods were incubated overnight (4 °C) with an appropriate dilution of serum (usually 1 in 1000). After washing peptide-coated pins four times in 0.5% V/V Tween 20 phosphate buffered saline pH 7.2 (PBS-tween), bound antibody was detected by addition of horse radish peroxidase-labelled species-specific or class-specific antibody (Kirkegaard and Perry Labs, U.S.A., or Sera-Lab, U.K.) for 1 hour at 25 °C. After a further four washes in PBS-tween, the rods were immersed in an 2,2-azinobis (3-ethylbenzthiazoline sulphuric acid) (ABTS, Boehringer Mannheim, Federal Republic of Germany) solution (50 mg ABTS, 300 µl H_2O_2 in 100 ml 0.1 M phosphate 0.08 M citrate buffer pH 4.0).

The horse radish peroxidase-ABTS reaction was stopped by removing the rods from the ABTS solution and the absorbance of the solution remaining in the wells of the microtitre plate read at a wavelength of 405 nm against a reference of 492 nm. ELISA absorbance values were transmitted to a microcomputer for subsequent analysis. The titre of an antibody-peptide reaction was defined as the dilution of antibody required to produce an absorbance value of 1.0 in the above ELISA procedure after subtraction of background absorbance values. The relationship between absorbance and titre was linear up to titres of 1 in 2000.

Before re-use, peptide-coated rods were freed of all antibody by immersion in an aqueous solution, prewarmed to 60 °C, containing 1% sodium dodecyl sulphate, 0.1% 2-mercaptoethanol, and 0.1 M sodium phosphate, pH 7.2. While in this solution, they were sonicated for 10 minutes at a power input of 1000 W for a bath volume of 5 litres. Rods were then immersed in water at 65 °C, followed by boiling methanol, and air dried.

Regional hydrophilicity

Hydrophobic and hydrophilic regions of the E protein of dengue 2 were identified using the programme derived by Hopp and Wood [17] based on hexapeptides.

Results

Serum from individual rabbits immunized with dengue 2 reacted with from 51 to 154 of the 488 overlapping octapeptides corresponding to linear amino acid sequences on the envelope (E) protein of dengue 2 (JAM 1409) (Fig. 1 c). Collectively, these sera reacted with 250 different octapeptides which could be divided into 6 discrete regions each separated from the next by at least 8 consecutive octapeptides which failed to react with antibody (^{1}MRC-LRK^{58}, ^{59}YCI-CRL^{287}, ^{288}RMD-LNW^{391}, ^{392}FKK-FGA^{442}, ^{446}AAF-SLS^{476}, ^{479}LVL-VQA^{495}). Two peptides (^{216}LPLPWLPG^{223} and ^{448}FSGVSWTM^{455}) reacted with serum from all 6 dengue 2 immunized animals. However, serum taken from two rabbits prior to any immunization also reacted with from 8–11 dengue 2 E protein octapeptides (Fig. 1 a). While most of these reactions were to isolated peptides and or of low titre, peptides in the region ^{362}DSPVNIEAEPPFGD^{375} reacted with serum from both rabbits and with titres in the range 300–500. The proportion of dengue infected rabbits responding to peptides from this region

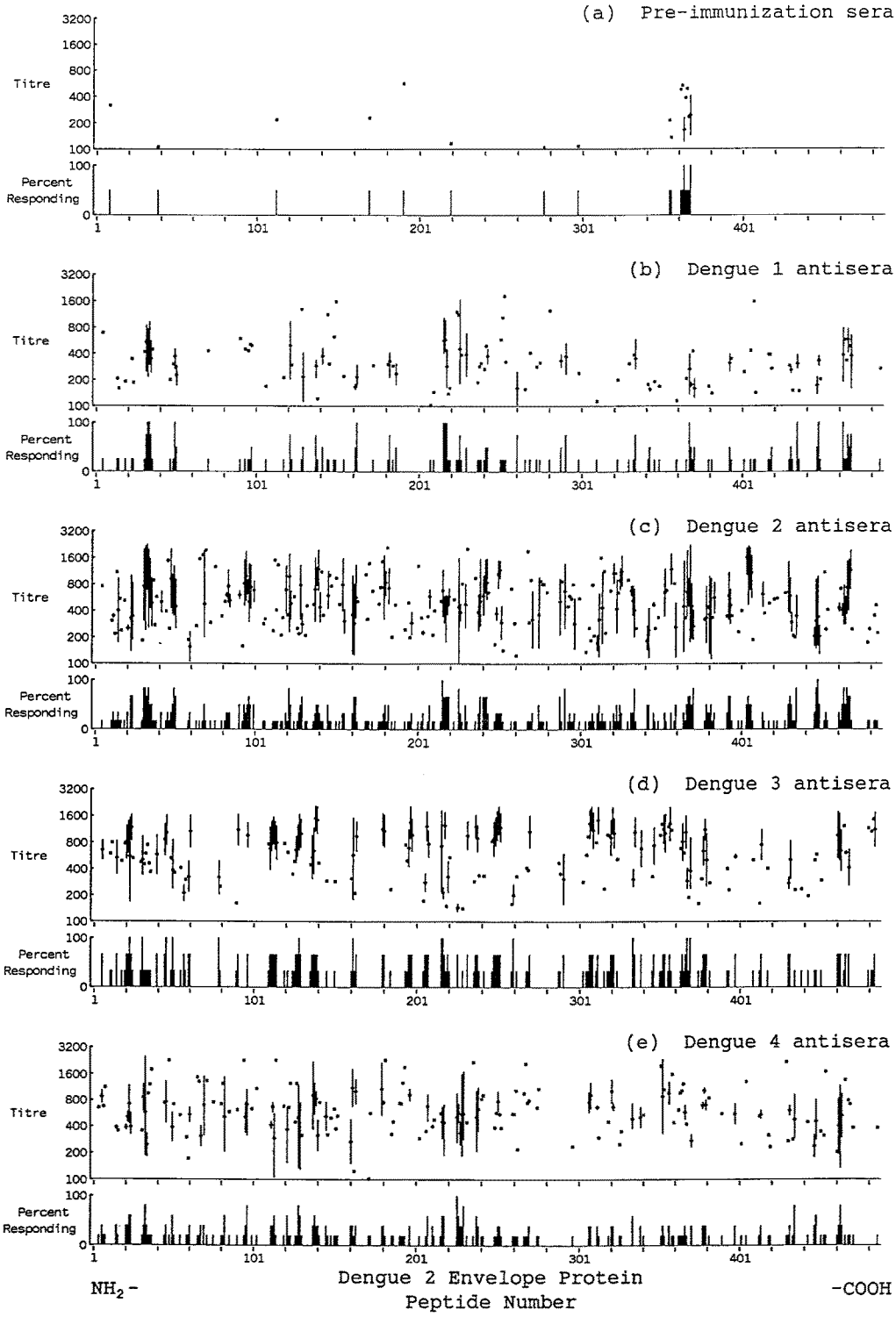

Fig. 1. Mean titre of serum from individual rabbits which reacted with overlapping octapeptides composed of amino acid sequences corresponding to those in the envelope protein of dengue 2. (Bars indicate the range of individual titres.) Dengue 2 peptides recognized by serum from **a** rabbits not infected with dengue virus, **b** four dengue 1 infected rabbits, **c** six dengue 2 infected rabbits, **d** three dengue 3 infected rabbits, **e** five dengue 4 infected rabbits

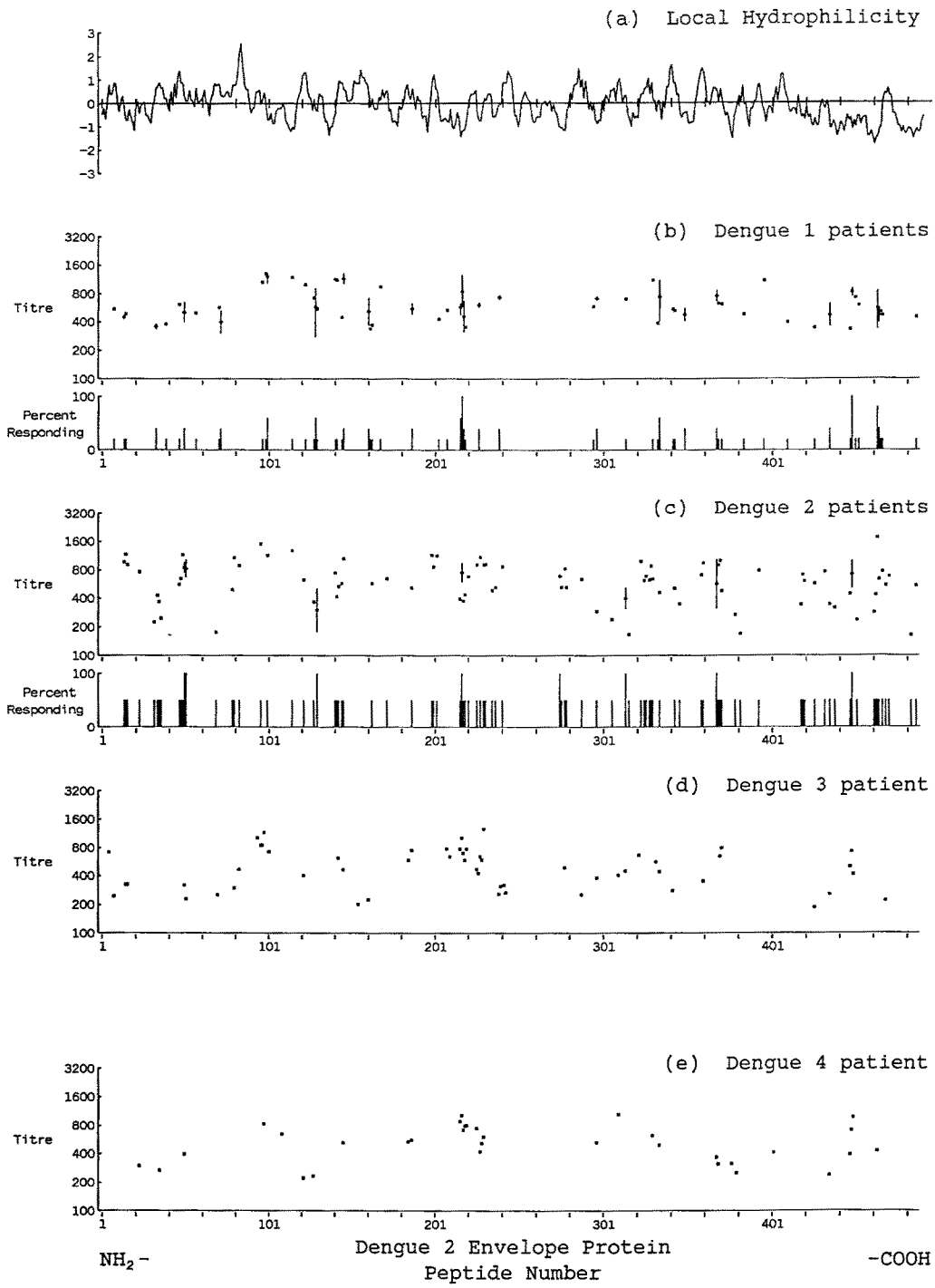

Fig. 2. Mean titre of serum from patients with primary, clinical, dengue infections which reacted with overlapping octapeptides composed of amino acid sequences corresponding to those in the envelope protein of dengue 2. (Bars indicate the range of individual titres.) **a** Regional hydrophilicity and hydrophobicity [17] in the E protein of dengue 2. Hydrophilicity is shown above the X-axis. **b–e** Dengue 2 peptides recognized by serum from **b** five dengue 1 patients, **c** two dengue 2 patients, **d** a dengue 3 patient, and **e** a dengue 4 patient

of the dengue 2 E protein and the titre of these reactions were similar to that of the sera from the non-immunized rabbits (Fig. 1 b–e).

The number of peptides recognized by serum from individual dengue 2 infected rabbits was not related to the number of times they were immunized. Both the rabbit recognizing the highest number of peptides (154/488) and that recognizing the lowest (51/488) had been immunized on 3 occasions.

Serum from the rabbit immunized most often (6 injections) recognized 61/488 octapeptides.

The titre of antibody produced against individual octapeptides also varied from animal to animal with the highest titre responses not always directed against peptides seen by sera from the majority of the rabbits.

Serum from dengue 1, 3, and 4 infected animals also reacted with octapeptides corresponding to amino acid sequences from the dengue 2 E protein (Fig. 1 b, d, e). Sera from dengue 1 infected rabbits reacted with 117 dengue 2 E protein octapeptides, sera from dengue 3 infected rabbits with 160 peptides and sera from dengue 4 infected rabbits with 174. In addition, 11 dengue 2 octapeptides were recognized by serum from all four dengue 1 infected rabbits, 13 by all three dengue 3 infected rabbits and 1 by all five dengue 4 infected rabbits.

As with the sera from dengue 2 infected rabbits, that from dengue 1, 3, and 4 infected animals reacted with regions of overlapping octapeptides spread throughout the E protein of dengue 2. However, all 66 octapeptides recognized exlusively by serum from one or more dengue 2 infected rabbit overlapped by at least one amino acid with an octapeptide recognized by antisera raised to a second dengue serotype.

Convalescent sera from patients with primary dengue 1, 2, 3, or 4 infections reacted with fewer dengue 2 E protein peptides than did sera from hyperimmune rabbits. Those peptides which reacted with the majority of sera from rabbits usually reacted with the majority of sera from patients (Fig. 2). This was the case with both homologous (dengue 2 antisera) and heterologous (dengue 1 antisera) reactions. However, human sera also reacted with a number of peptides not recognized by that from rabbits, e.g., peptide ^{172}EAELTGYG^{179} which was recognized by serum from a dengue 2 patient but failed to react with any sera from dengue 2 infected rabbits.

Unlike that from unimmunized rabbits, sera from two flavivirus HI antibody seronegative Queensland adults failed to react with any dengue 2 E protein octapeptides.

IgM antibody in serum collected from four rabbits seven days following a primary dengue 2 infection reacted with only 3 different peptides and at low titre (102–123). Serum from each rabbit reacted with only one peptide. Hyperimmune sera collected from the same four rabbits after multiple infections reacted with a greater number of peptides and at higher titre than the acute-phase IgM (Fig. 3 a). In addition, two of the four rabbits failed to produce IgG antibody which would combine with the peptides (^{217}PLPWLPGA^{224} and ^{275}GNLLFTGH^{282}) which were recognized initially by their IgM.

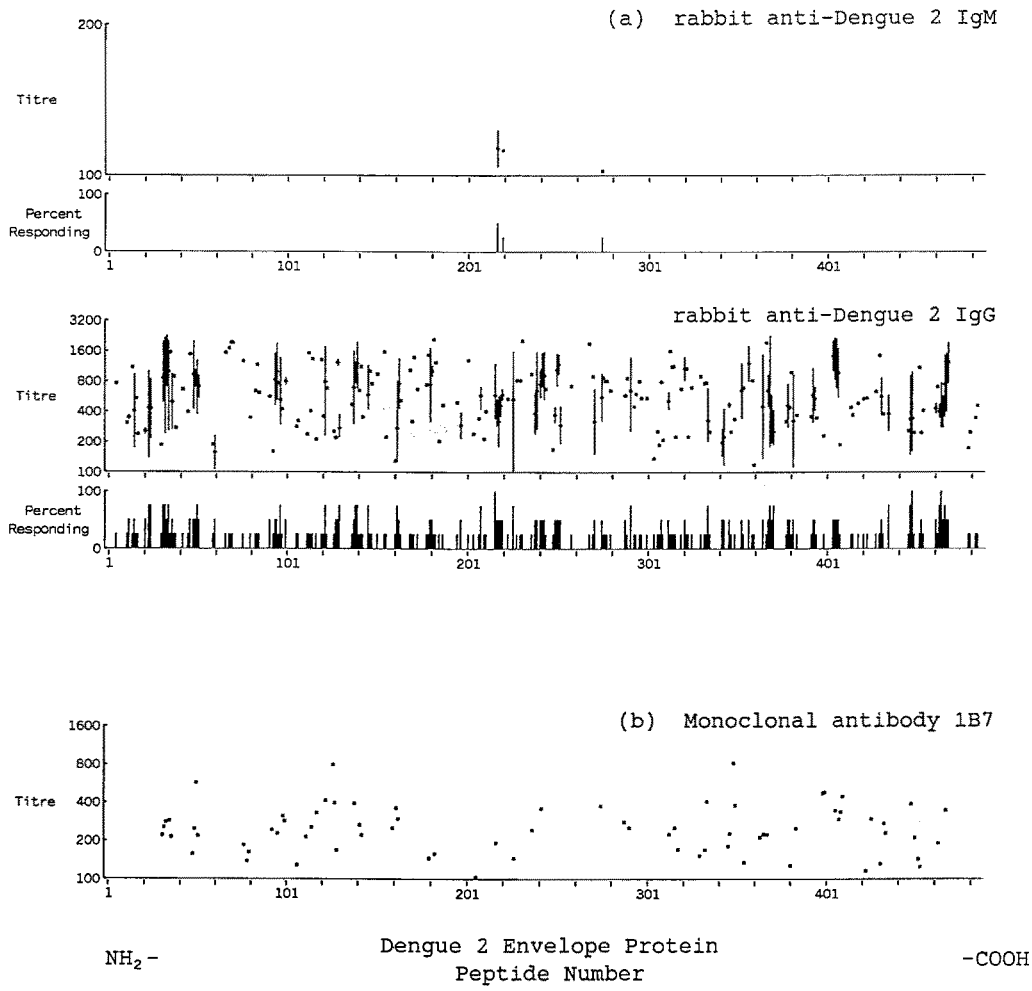

Fig. 3. a Comparison of octapeptides recognized by IgM antibody in serum taken from rabbits 7 days following a primary dengue 2 infection with those recognized by antibodies in serum from the same rabbits following hyperimmunization. (Mean titre ± range of individual values.) **b** Titre of a mouse anti-dengue 2 monoclonal antibody (1B7) which reacted with octapeptides composed of amino-acid sequences corresponding to those in the envelope protein of dengue 2

Mouse ascitic fluid containing monoclonal antibody 1B7 which inhibited agglutination of gander erythrocytes as well as enhancing and neutralizing infection by dengue virus [16] reacted with 74 dengue 2 octapeptides. However, the strongest of these reactions (to peptides ^{50}AKQPATLR^{57}, ^{127}GKVVPENL^{134} and ^{349}GRLITVNP^{356}) were at least twice the titre of that of any adjacent peptide-antibody combinations (Fig. 3 b).

Discussion

Although there was considerable animal to animal variation in the serological response of dengue 2 infected rabbits to octapeptides from the E protein of the virus, it would appear that antibody may be produced against almost the entire

protein including the C-terminal portion which is embedded in the lipid bilayer surrounding the nucleocapsid of the virion. This suggests that significant amounts of E protein or parts thereof may not be incorporated into virions and are released on lysis of infected cells and/or that disruption of the lipid bilayer of the virion may be an early step in the immunological processing of dengue viruses. Detection, in influenza virus infected mice, of class 1 MHC-restricted cytotoxic T-lymphocytes which reacted with the transmembrane segment of the influenza A haemagglutinin molecule [3], supports this and suggests that the membrane-associated portion of viral proteins may be more accessible immunologically than would be suggested by virus architecture.

In addition, while most of the immunologically active regions of the dengue 2 E protein (number of animals producing antibody against them, and the titre of that antibody) were hydrophilic [16], there were some notable exceptions. Hydrophobic peptides ^{216}LPLPWLPG^{223}, ^{334}KIPFEIMD^{341} and ^{435}ALHQVFGA^{442} were all recognized by sera from 5–6 of the six dengue 2 infected rabbits.

Although sera from dengue 2 (New Guinea C) infected rabbits reacted readily with the most variable region of the dengue 2 E protein [10] (^{126}EGKVVLPENLEYTIVI^{141}) and poorly with some highly conserved regions (^{98}DRGWGNGCGLFGKG^{111} or ^{416}GDTAWDF^{422}) we do not have sufficient data from animals infected with different dengue 2 strains to draw convincing conclusions about immunologically driven selective pressures on the amino acid sequence of the dengue 2 E protein. However, the observation that a large number of the animo acid changes observed in the E protein of different dengue 2 isolates [2] occur outside regions recognized by antibody from the majority of dengue 2 infected animals should caution against oversimplistic associations between immunological selective pressures and amino acid sequence variability.

Failure of many dengue serotype-specific monoclonal antibodies to react with detergent-denatured and β-mercaptoethanol reduced dengue structural proteins [16] (and unpublished observations) has led to a belief that serotype specific epitopes are all conformational or non-linear. However, in this study, 66 peptides were identified which reacted exclusively with serum from at least one dengue 2 infected rabbit. By combining a minimum of three peptides (e.g., peptides 148, 404, and 446), it would have been possible to detect antibody from all dengue 2 infected rabbits without any cross reactions with serum from dengue 1, 3, or 4 infected animals. However, the failure of any peptide to react with sera from all dengue infected hosts supports the concept that dengue group-specific epitopes may be conformational [16].

Cross-reactivity between dengue 2 peptides and sera from animals infected with *different* dengue serotypes did not relate entirely to amino acid homology between immunogens (Table 1). In some instances where amino acid sequences may be highly conserved between serotypes, expected patterns of serological cross reactivity were observed (e.g., peptides 216 and 217). Despite this, anomalies were observed. Homologous antisera reacted with peptide 217 less well

Table 1. Specificity of the serological response by rabbits to infection with dengue viruses

Infecting serotype	Peptide no.	Amino acid sequence* of		No. animals responding	Mean titre of positive responses
		infecting virus	peptide used for assay		
Dengue 1	50	VTNPAVLR	AKQPATLR	4/4	369
2		AKQPATLR		5/6	625
3				3/3	387
4		AKEVALLR		3/5	386
Dengue 1	98	DRGWGNGC	DRGWGNGC	2/4	495
2		DRGWGNGC		3/6	736
3		DRGWGMGC		0/3	
4				0/5	
Dengue 1	103	NGCGLFGK	NGCGLFGK	0/4	
2		NGCGLFGK		0/6	
3				0/3	
4		NGCGLFGK		1/5	1,081
Dengue 1	216	LPLPWTSG	LPLPWLPG	4/4	566
2		LPLPWLPG		6/6	687
3				3/3	734
4		LPLPWTAG		1/5	561
Dengue 1	217	PLPWTSGA	PLPWLPGA	4/4	582
2		PLPWLPGA		4/6	480
3				3/3	214
4		PLPWTAGA		3/5	452

* The amino acid sequence of the prototype strains of dengue 1, 3, and 4 are not yet available. The sequences used for the dengue 1, 2, and 4 infecting virus are for those found in [21, 6, 31], respectively

(4/6 rabbits responded; mean titre 480) than did sera from dengue 1 infected animals (4/4 responded; mean titre 582). In other instances of probable conserved amino acid sequence (e.g., peptides 98 and 103) there was very poor serological cross reactivity between serotypes. In the converse situation, where extensive variation between sequences might be expected (e.g., peptide 50), strong serological cross-reactivity was sometimes observed.

It is possible that these apparent anomalies in serological reactivity are due as much to the amino acid sequence flanking the epitope as to the epitope itself. Flanking sequences could influence the conformation of the epitope, the manner in which it is presented in the assembled virion and the manner in which it is processed by antigen presenting cells such as the macrophage. In addition, there are amino acids in many peptides which appear to be critical for antigen-antibody binding [9, 27], while others may be varied without significantly affecting the capacity of the peptide to combine with antibody.

Since IgM has been reported to neutralize but not to enhance dengue infections whereas IgG may do both [15], any epitopes seen only by IgM might warrant evaluation as potentially safe (enchancement free) subunit vaccines. However, we have been unable to identify any peptides seen exclusively by IgM. This study identified two peptides (^{217}PLPWLPGA^{224} and ^{275}GNLLFTGH^{282}) recognized by IgM but not IgG in serum from individual rabbits, but both these peptides reacted with IgG from several of the other dengue 2 infected animals.

There are several possible explanations for the failure to detect as many IgM-octapeptide reactions using serum collected seven days following a primary dengue 2 infection as IgG-octapeptide reactions in serum from hyperimmune animals.

1. The amount of IgM present in acute phase sera was likely to be less than the IgG present in hyperimmune sera.
2. The affinity of acute phase IgM for an epitope may have been less than that of hyperimmune IgG.
3. The majority of the IgM may have been directed against conformational rather than linear epitopes. In ELISAs employing sera from patients with other togavirus infections and Western blots of reduced and non-reduced envelope proteins of the homologous virus, IgM appeared to react only with the non-reduced proteins while IgG reacted with both reduced and non-reduced (unpubl. observations).

This study has also identified two regions of the dengue 2 E protein which may constitute an important conformational epitope. (Although the three dimensional structure of the dengue E protein is not known, the conservation of amino acid sequences, particularly cysteine residues, in the E protein of flaviruses suggests that structural features identified in West Nile virus, including disulphide bridges, may also occur in dengue viruses [23, 29, 30].) On this model, regions ^{47}KTEAKQPATLRKYC^{60} and ^{121}CKKNMEGKVVLPENLEY^{137} are likely to be adjacent by virtue of a disulphide bridge linking ^{60}cysteine and ^{121}cysteine. Peptides containing sequences found in these regions reacted with serum from 5–6 of the dengue 2 infected rabbits and both dengue 2 patients. Both regions also contain predicted T-cell epitopes [20]. In addition, two of the three peptides (^{50}AKQPATLR^{57}, and ^{127}GKVVLPEN^{134} and ^{349}GRLITVNP^{356}) recognized most readily by monoclonal antibody 1B7 are also found in these regions. Since the 1B7 monoclonal antibody reacts with intact virions in both radio-immunoassay [16] and enzyme linked immunosorbent assays (unpubl. observations), these three regions are likely to be on the surface of the virion. Together with the observation that the 1B7 antibody also enhanced dengue infection and causes haemagglutination inhibition, these data suggest a significant biological role for regions ^{121}CKKNMEGKVVLPENLEY^{137} and ^{47}KTEAKQPATLRKYC^{60} on the dengue 2 E protein. While it will probably require crystallographic data to confirm whether or not the sequence ^{349}GRLITVNP^{356} which was also recognized by the 1B7 monoclonal

antibody is in close enough proximity to this proposed epitope to be part of it, there are at least two other reports, supported by crystallography, in which antibody combined with three or more regions on an antigen molecule [5, 28].

While this study has identified linear and perhaps discontinuous, epitopes in the E protein of dengue 2 which are seen by antibody from most animals following a dengue 2 infection, the functional properties (immunogenicity, role in neutralization and enhancement) of peptides corresponding to these epitopes will need to be determined empirically. However, in the case of serodiagnostic assays these data suggest it may be possible to develop dengue serotype specific serological assays using as few as three octapeptides for each of the four dengue serotypes.

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