IMMUNE RESPONSE TO INFECTION

Immune response to a Murray Valley encephalitis virus epitope expressed in the flagellin of an attenuated strain of *Salmonella*

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Recent developments in vaccine construction include the use of attenuated, avirulent strains of *Salmonella* as carriers of foreign antigens. These recombinant strains can elicit a heterologous immune response when injected into animals, demonstrating potential for their use in the construction of many vaccines. In the present study, a B-cell epitope of Murray Valley encephalitis virus (MVE) was identified and expressed in a *Salmonella* strain to evaluate its potential to induce a specific immune response to MVE. A synthetic oligonucleotide encoding the B-cell epitope (residues E_{201–224}) of the envelope protein of MVE was inserted into the cloned flagellin gene of the *Salmonella* strain. The construct was sequenced to ensure correct orientation of the epitope. Expression of the epitope was demonstrated by Western blot analysis and immunogold electron microscopy with monoclonal antibody specific to the epitope. Electron microscopy analysis revealed multiple copies of the epitope along the flagella. The recombinant *Salmonella* carrying the hybrid flagellin gene elicited an immune response to the MVE epitope in a mouse model. The MVE-specific antibodies partially neutralised the virus *in vitro*. The significance of this system for engineering vaccines for other medically important flaviviruses is discussed.

Introduction

Recent developments in vaccine construction include the use of attenuated, avirulent strains of *Salmonella* as vectors carrying foreign antigens which elicit protective immune responses [1, 2]. One important group of non-reverting *Salmonella* vaccine strains comprises the mutants dependent on aromatic compounds (*aro*[−]) [3]. The *aro*[−] mutants have a deletion in one of the genes of the aromatic amino acids biosynthesis pathway resulting in strains dependent on aromatic compounds that are available in limited amounts in host tissues. The insertion of a tetracycline resistance transposon, *Tn10*, into the *aroA* gene of *Salmonella* serotype Typhimurium produced *aroA* strain SL3261 [4, 5], a well-defined *aro*[−] mutant [6, 7]. The *aro*[−] mutants multiply only for a few generations in host tissues, but do persist in the spleen and liver for several weeks, inducing strong humoral and cellular immune responses [7, 8]. A further development has been the insertion of synthetic oligonucleotides, encoding foreign epitopes, into a cloned *Salmonella* flagellin gene

[3, 9]. A plasmid, pLS408, containing the H1-*d* flagellin gene of serotype Muenchen, has been constructed [10]. In this plasmid, a 48-bp deletion has been made in the hypervariable segment (region IV) of the flagellin gene. The remaining *EcoRV* site allows the blunt end insertion of oligonucleotides. T-cell epitopes have previously been expressed in the flagellin and the immune response to the epitopes examined in mice immunised with the recombinant *Salmonella* strain [11, 12]. A number of epitopes of viral and eukaryotic origin have also been expressed in *Salmonella* flagellin. In most cases, such recombinant *Salmonella* strains induced specific immune responses to the foreign epitopes [3, 13].

Murray Valley encephalitis virus (MVE) belongs to the flavivirus family of small enveloped, single-stranded RNA viruses [14–16]. MVE is responsible for endemic cases of encephalitis in tropical regions of Australia and Papua New Guinea, and occasional epidemics in south-eastern Australia [17, 18]. Other flaviviruses of major public health concern are the closely related Japanese encephalitis virus (JE), yellow fever virus (YF) and dengue viruses (DEN). Currently, an attenuated vaccine for YF [19], an inactivated

vaccine for tick-borne encephalitis virus (TBE) [20] and both attenuated [21] and inactivated [22] vaccines for JE are available [23]. Despite the success of the YF and TBE vaccines it is necessary to improve vaccines against other flaviviruses, particularly JE and DEN. A *Salmonella* strain is a promising vaccine vector for this purpose as it induces both cellular and humoral immune responses that may be necessary to confer protection against flavivirus infection.

The flavivirus virion contains three structural proteins: the capsid protein (C), the envelope protein (E) and the membrane protein (M), which is cleaved from the pre-membrane protein (prM) during maturation. The E protein [54 kD], located as horizontal dimers on the virion surface, is the carrier of the major antigenic determinants [24–26]. This protein is involved in receptor binding, fusion with cell membranes and is the target antigen for neutralising antibodies. The topology of the E protein of MVE and the location of B-cell epitopes on its surface have been investigated with the use of monoclonal antibodies (MAbs) [27]. The identification and use as a vaccine of a linear B-cell epitope consisting of residues 201–224 of the E protein of MVE are described. This epitope has the ability to induce neutralising antibodies against MVE *in vivo*. The MVE E_{201–224} B-cell epitope was expressed in an attenuated *Salmonella* strain as part of a chimeric flagellin gene and the recombinant vaccine was used to induce a specific immune response to MVE in immunised mice.

Materials and methods

Bacterial strains, plasmids and media

The bacterial strains used in this study are shown in Table 1. *Salmonella* strains were grown at 37°C on Luria-Bertani medium (LB) (Oxoid) supplemented with appropriate antibiotics. The pEX plasmids and the host *Escherichia coli* strain MC1061/pCI857 were as described by Stanley and Luzio [28]. The latter carries the plasmid pCI857 encoding kanamycin resistance and the *ts* cl repressor gene of bacteriophage λ . *E. coli* strain MC1061/pCI857 was grown at 30°C in LB medium [29] supplemented with ampicillin 100 mg/L and kanamycin 40 mg/L (Sigma).

Plasmid p2/1/22 [30] was from an MVE genomic library [31]. It comprised the 1.9-kb plasmid vector

pMT21 (H. V. Huang, Department of Molecular Microbiology, Washington University School of Medicine, St Louis, Missouri, USA) which codes for ampicillin resistance, and the 5' terminal 5.4 kb of the MVE genome coding for C, prM, E and the non-structural proteins; NS1, NS2A, NS2B and half NS3; the complete sequence of p2/1/22 has been established [30].

DNA techniques

Small preparations of plasmid DNA were made by the alkaline lysis method of Birnboim and Doly [32]. Ligations were performed as described by Maniatis *et al.* [29]. Transformation procedures were performed as by Dower *et al.* [33]. The Sequenase Version 2.0 sequencing kit (United States Biochemical) was used for sequencing by the method of Sanger *et al.* [34].

Construction of expression plasmids for mapping the E-8 epitope

Plasmid pE22–272 (Fig. 1) contained a *Taq*I fragment (0.75 kb), which coded for MVE E protein from Asp22 to Val272, from the MVE cDNA clone p2/1/22 [30]. This fragment was first ligated to *Acc*I-digested M13 mp8 DNA, then cleaved at the *Bam*HI and *Pst*I sites which flank the *Acc*I site in M13 mp8 to produce a 0.8-kb E gene fragment with *Bam*HI and *Pst*I cohesive ends for ligation to *Bam*HI- and *Pst*I-digested pEX plasmids. Plasmid pE146–429 (Fig. 1) contained a partially digested *Sau*3A fragment (0.85 kb) coding for E protein from Ser146 to Gly429. This plasmid was obtained from a library of expression plasmids generated by ligating p2/1/22, partially digested with *Sau*3A, to *Bam*HI-digested pEX plasmids. pE146–429 was identified by immunoscreening with polyclonal anti-MVE antisera and sequence analysis.

A series of deletion plasmids which expressed E fragments of 24–183 amino acids were generated from pE22–272 and pE146–429 (Fig. 1). Construction of these utilised the *Stu*I, *Nco*I and *Pst*I sites in the E gene (corresponding to residues 201, 224 and 328, respectively) and the *Sma*I and *Pst*I sites in pEX plasmids which flank the 5' and 3' ends of E gene inserts, respectively. Thus, fragments from the 5' and 3' ends of the E gene inserts were deleted by restriction enzyme digestion as shown in Fig. 1 and the linearised plasmids isolated on low-melting-

Table 1. *E. coli* and *Salmonella* strains

Strain	Genotype	Source
<i>E. coli</i>		
CL447	<i>hag</i>	B. Stocker
<i>Salmonella</i> Dublin		
SL5928	<i>flhC::Tn10, ΔaroA</i>	B. Stocker
SL5930	SL5928/pLS408	B. Stocker
B5127	SL5928/pNV291/MVE E _{201–224}	This study
B5128	SL5928/pNV291/MVE E _{201–224} wrong orientation	This study

Fig. 1. Construction of expression plasmids for mapping the E-8 epitope. pE22-272 (A) and pE146-429 (B) were used to generate a series of deletion plasmids that expressed E fragments of 24-183 amino acids.

temperature agarose gel, blunt-ended by T4 DNA polymerase and self-ligated. Recombinant pEX expression plasmids were transformed into *E. coli* strain MC1061 + pc1857 [28], incubated at 30°C in the presence of ampicillin and kanamycin and selected by immunoscreening with polyclonal anti-MVE antisera. The correct constructs were confirmed by immunoreactivity, restriction endonuclease analysis of plasmid DNA and size of the E- β -Gal fusion proteins.

Insertion of the MVE E₂₀₁₋₂₂₄ B-cell epitope coding sequence into the salmonella flagellin gene

A synthetic double-stranded oligonucleotide that encodes the 24 amino acid MVE E₂₀₁₋₂₂₄ B-cell epitope was designed according to *Salmonella* codon usage (Fig. 2). This construct included a single *Nru*I restriction site. An Asp codon was added to the 3' end of the sequence so that when the oligonucleotide

was inserted into the *Eco*RV site of plasmid pLS408, the site was retained.

The complementary oligonucleotides were annealed and blunt-end ligated to phosphatase-treated, *Eco*RV-digested DNA of plasmid pLS408. A flagellin-deficient *E. coli* strain, CL447 (Table 1), was transformed with the ligated product and transformants were selected on ampicillin plates. Plasmid DNA from transformants was extracted and screened for the presence of the insert by *Nru*I and *Eco*RI double restriction digests.

Motility tests

Motility tests were performed on 60 ml of semi-solid agar (Difco) 0.6% w/v, 30 ml of LB broth and 90 ml of gelatin 16% w/v. Colonies were spotted on to individual semi-solid agar plates and incubated at 37°C

Fig. 2. Structure of the MVE E₂₀₁₋₂₂₄ epitope-flagellin chimeric gene. Plasmid pLS408 is a pUC19 derivative carrying a 3.8-kb *EcoRI* fragment that includes the flagellin structural gene *H1-d* (*fliC*) from serotype Muenchen. Arrow indicates the direction of transcription of the *fliC*. The nucleotide sequence encoding the MVE E₂₀₁₋₂₂₄ epitope which was inserted in the hypervariable region (shaded segment) is indicated below. The additional amino acid is underlined and the *NruI* restriction site is italicised.

for 16 h. The degree of motility was measured as the distance the colony spread.

Immunoblotting

Whole-cell lysates were tested for their reactivity with antibodies by immunoblotting after SDS-PAGE. Bacteria (5×10^7 cfu) were boiled in sample buffer and separated on a polyacrylamide 10% w/v gel. For immunoblotting, the gel containing separated proteins was transferred electrophoretically to nitrocellulose membrane [35]. Primary antibodies used to probe Western blots included a mouse monoclonal anti-E8 antibody, specific to the epitope, a rabbit polyvalent H antibody specific to the flagellin antigen *d* of *Salmonella* and polyclonal anti-MVE hyperimmune ascitic fluid (HIAF). Immune complexes were detected with HRP-labelled anti-mouse or anti-rabbit antibodies for the ECL Western blotting detection system (Amersham Corporation).

Immunogold labelling and electron microscopy

Salmonella strains were negatively stained with uranyl acetate 0.5%, by the method of Ho *et al.* [36], for electron microscopy. For immunogold labelling, the primary antibody, monoclonal anti-E8, was diluted 1 in 400 in PBS with bovine serum albumin (BSA) 0.4% w/v. Sheep anti-mouse gold conjugate, diluted 1 in 9 was used as secondary antibody. The grids were viewed at magnifications of between 30 000 and 100 000 with a Hitachi H600 transmission electron microscope.

Purification of fusion proteins

E. coli cells containing pEX or recombinant plasmids were grown at 30°C in LB medium containing ampicillin 100 mg/L and kanamycin 40 mg/L until stationary phase was reached. Cells were diluted in fresh medium to an OD of 0.05 at 630 nm. Incubation was continued at 30°C for 1 h and at 42°C for 2 h. Cells were collected by centrifugation at 8000 rpm for 10 min, resuspended in NaCl 0.9% w/v to 1/20th the

original volume and lysed by three cycles of freezing in liquid nitrogen and thawing at 30°C. Lysozyme was added to 125 mg/L and the extract was held on ice for 20 min followed by another cycle of freezing and thawing. The lysates were then sonicated for 30 s and centrifuged for 15 min at 4°C. The resulting pellets, which were enriched in fusion proteins, were resuspended in 8 M urea (Ultrapure, Sigma) in 10 mM Tris-HCl (pH 7.4). After clarification by centrifugation, fusion protein solutions were dialysed overnight against PBS at 4°C.

Inoculation of mice

Fusion proteins (10 µg each in 50 µl of PBS) were mixed with equal volumes of Freund's incomplete adjuvant (FIA, Difco), emulsified by passing through a syringe several times and injected intraperitoneally (i.p.) into groups of five outbred Swiss mice aged 6–8 weeks. Booster injections were given 4 weeks and 9 weeks after the initial injection.

Bacterial clones for immunisation were grown overnight at 37°C in LB containing ampicillin 50 mg/L. These overnight cultures were subcultured (1 in 20) and grown to log phase. Cells were washed twice and resuspended in phosphate-buffered saline. Groups of four BALB/c mice were inoculated i.p. with 200 µl of 10^6 cfu on days 1 and 4. Blood was taken from the retro-orbital plexus before immunisation and then at weekly intervals. Sera were collected from microfuged blood and stored at -20°C.

Enzyme-linked immunosorbent assay (ELISA)

Fusion protein was purified from *E. coli* expressing the recombinant pEX plasmid (pE₂₀₁₋₂₂₄). This plasmid carries the MVE E₂₀₁₋₂₂₄ epitope fused to the *E. coli* β-gal gene. Reactivity of immune sera with MVE virus or fusion protein was examined by ELISA. Colour development was in 100 µl of citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na₂HPO₄), H₂O₂ 0.0008% and *o*-phenylene diamine dihydrochloride (OPD). The

reaction was stopped by the addition of 50 μ l of H₂SO₄ 25% v/v to each well. Plates were read at 490 nm with an automated plate reader (Deltasoft). Controls comprised sera collected from mice before vaccination.

Plaque reduction neutralisation tests

Plaque reduction neutralisation tests (PRNT) were used to determine the neutralisation efficiency of the anti-MVE mouse sera. Vero cells were grown as monolayers in six-well Linbro plates. Sera were serially diluted two-fold in 150 μ l of Hank's Balanced Salts Solution (HBSS), pH 8, and incubated at 37°C in a CO₂ incubator for 1 h with 200 pfu of MVE-1-51 in 100 μ l of HBSS. Samples (100 μ l) of these mixtures were used to infect a cell monolayer, in duplicate. These were incubated for 1 h in the CO₂ incubator with occasional gentle shaking. A 3-ml agar overlay was added to the infected cells in each well and the plates were incubated for 3 days in a CO₂ incubator. The cells were then stained with neutral red 0.01% in HBSS and examined for plaque formation. Control assays were performed with mouse sera taken before vaccination and with polyclonal antibody to MVE.

Results

Mapping epitope E-8 of MVE by bacterial expression cloning

The epitope E-8 is one of five topologically related epitopes (E-1a, E-1c, E-1d, E5 and E-8) defined by type-specific, neutralising, and passively protective MVE MAbs [27, 37, 38]. Only MAb E-8 reacted with MVE E protein in Western blots of both unreduced and β -mercaptoethanol-reduced virus. Thus it was feasible to attempt mapping of the E-8 epitope by bacterial expression of E protein fragments. Two fragments of MVE E protein, Asp-22–Val-272, encoded in a *Taq*I restriction fragment, and Ser-146–Gly-429, encoded in a partial *Sau*3A restriction fragment, were first expressed in *E. coli* as fusion proteins (E22–272 and E146–429) with truncated β -galactosidase (β -gal; *c.* 100 kDa). The two E fragments together represented *c.* 80% of the complete E sequence (501 amino acids). The construction of the pEX-derived expression plasmids is summarised in Fig. 1.

Reactivity of MAb 6B4A-10 (anti-E-8) with β -mercaptoethanol-reduced bacterial lysates containing E22–272 and E146–429 fusion proteins was demonstrated in Western blots (Fig. 3). Anti-E-8 reacted with unreduced E22–272 and E146–429 with equivalent intensity (data not shown). Thus, on the basis of these results, E-8 is located in E protein between Ser-146 and Val-272.

To define the E-8 epitope further, expression plasmids pE22–272 and pE146–429 were deleted by restriction endonucleases to generate seven subclones expressing

Fig. 3. Reactivity of anti-E-8 MAb with fusion proteins comprising MVE E protein fragments of 24–284 amino acids. Whole bacterial lysates were separated on SDS-PAGE (7.5%) and analysed by Western blotting with mouse MAbs diluted 1 in 500; molecular masses (kDa) are shown on the right.

E protein fragments from 24 to 183 amino acids in length (Fig. 1). The corresponding fusion proteins were of the expected sizes and all reacted with polyclonal anti-MVE hyperimmune ascitic fluid in Western blots (results not shown). Of these fusion proteins, E146–328, E201–328, E146–224 and E201–224 reacted with anti-E-8 in Western blots, while E225–272, E225–328 and E327–429 did not (Fig. 3). However, fusion proteins E201–224 and E146–224 were consistently less reactive than E201–328 and E146–328. As the E fragment Tyr-201–Pro-224 was present in all of the reactive fusion proteins and not in the unreactive fusion proteins, the dominant determinant of epitope E-8 is located in the 24 amino acids from Tyr-201 to Pro-224 of the MVE E protein. The marked reduction in reactivity of anti-E-8 with fusion protein lacking the sequence from Trp-225 to Gln-328 indicates that part or all of this sequence may be required for authentic presentation of epitope E-8.

Partially purified fusion protein E201–224 (*c.* 95% purity) was tested for ability to elicit anti-MVE antibodies in mice. The resulting antisera reacted with E201–224 β -gal fusion protein and MVE in ELISA (maximum titres of 51 200 and 400, respectively). However, these antisera lacked any significant neutralisation activity against MVE. Use of a larger fusion protein E22–272 for immunisation gave similar results.

Identification of recombinant *Salmonella* carrying the chimeric flagellin gene containing the MVE E_{201–224} epitope

Following insertion of the MVE E_{201–224} B-cell epitope into the cloned flagellin gene of the *Salmonella* strain, three clones carrying the recombinant plasmid were identified. The inserts in these plasmids carrying the chimeric flagellin gene were sequenced with two 18-bp

primers located upstream from the insertion point. A plasmid, pNV291, was identified that had a complete copy of the 25 amino acid encoding insert in the correct orientation. The flagellin-deficient, *aroA* vaccine strain, serotype Dublin SL5928 (Table 1), was transformed with the recombinant plasmid by electroporation and transformants were selected on ampicillin plates. The strain carrying plasmid pNV291 was designated B5127.

Expression of the chimeric flagellin genes

The expression of the E_{201–224} B-cell epitope as part of the flagellin protein by the recombinant strain B5127 was demonstrated by Western blotting of whole cell lysates with the E8 MAb and a polyclonal antibody to the flagellin antigen *d*. A protein migrating with an apparent molecular mass of 53 kDa in strain B5127, carrying the E_{201–224} epitope of MVE, was bound by MAb E8 (Fig. 4a). As expected, the MAb did not bind to proteins from strains SL5930 (carrying pLS408; wild-type flagellin) and SL5928 (flagellin deficient), indicating that there was no reactivity of the anti-E8 antibody with any of the salmonella proteins.

The polyclonal antibody specific for the flagellin antigen *d* also bound the 53-kDa protein of strain B5127, and a protein of 51 kDa in strain SL5930 (Fig. 4b). This indicated that the chimeric flagellin was expressed and contained the E_{201–224} epitope.

Topological location of the MVE E_{201–224} epitope on the flagella of B5127

The topology of the B-cell epitope with respect to the flagellum filament in the *Salmonella* recombinant was

detected by immunogold electron microscopy with the mouse MAb, anti-E8, and a gold-labelled goat anti-mouse IgG as the secondary antibody (Fig. 5). The MAb bound along the length of the B5127 flagella, indicating that the epitope was present in multiple copies on the surface of the flagella filaments. The anti-E8 antibody did not bind to flagella of SL5930 (a control strain) which carries pLS408, encoding a wild-type flagellin.

Motility and electron microscopy of recombinant strains

Motility tests revealed that the recombinant strain B5127 was non-motile on semi-solid agar. The morphology of the flagella on recombinant strains was observed by electron microscopic examination of negatively stained specimens. Strain B5127 (pNV291) showed some distinguishable differences in flagella morphology compared with wild-type flagella. Cells of strain B5127 had lower numbers of flagella and these were truncated and were often found separated from the bacterial cell. Thus, the chimeric flagella were morphologically and functionally abnormal compared with wild-type flagella. These results suggest that certain sequences inserted into the hypervariable region of the salmonella flagellin gene may impart significant differences in flagella morphology and function.

*Immune response in mice to attenuated *Salmonella* expressing chimeric flagellin containing the MVE E_{201–224} epitope*

To determine if the recombinant *Salmonella* strain carrying chimeric flagella containing the MVE B-cell epitope could induce an MVE specific immune

Fig. 4. Western blot showing expression of the MVE E_{201–224} chimeric flagellin protein. Whole bacterial cell lysates of strain B5127 were analysed by Western blotting with either: **a**, a mouse MAb to the MVE E_{201–224} epitope or **b**, polyclonal antiserum to flagella antigen *d*. The arrow indicates the increased apparent molecular mass of the B5127 flagellin by the addition of the epitope.

Fig. 5. Location of the MVE E₂₀₁₋₂₂₄ epitope on the surface of the B5127 flagella filament. **a**, the strain B5127 expressing the chimeric flagellin, $\times 80\,000$; **b**, the wild-type flagellin expressing strain, SL5930, $\times 60\,000$. The presence of gold particles (10 nm) on the surface of the chimeric flagellin represents the expression of the MVE E₂₀₁₋₂₂₄ epitope.

response *in vivo*, mice were inoculated i.p. with the recombinant Dublin strain, B5127, or strain SL5930 (control strain). Sera from weekly bleeds were tested for MVE-specific antibodies by ELISA, with the fusion protein containing the epitope and purified MVE as detecting antigens.

Antibody responses are shown in Table 2. Reactivity against the fusion protein was detected in all mice by 2 weeks after the initial inoculation of the recombinant *Salmonella* strain. The titres increased steadily up

Table 2. Production of MVE specific antibodies by four mice immunised with strain B5127 (MVE E₂₀₁₋₂₂₄ chimeric flagellin)

Week after immunization	ELISA titre with	
	fusion protein 10 μ g/ml	whole MVE virus 10 ⁸ pfu/ml
0	<1000	<25
2	4000	100
3	8000	100
4	16 000	200
5	32 000	200
6	32 000	400
8	16 000	200
10	16 000	200
12	16 000	50
16	8000	25

ELISA titres are the highest dilution of serum that yielded three times the absorbance value at 490 nm of SL5930 control sera.

to a maximum at week 6. No MVE antibody activity was detected in mice inoculated with the control strain SL5930. The antibody titres against whole MVE virus followed a similar pattern but were much lower than those detected against the fusion protein. The peak antibody titre was *c.* 32 000 against fusion protein and *c.* 400 against the virus.

MVE neutralisation by the anti-epitope E₂₀₁₋₂₂₄ antisera

It was of interest to determine whether the sera that reacted with MVE in an ELISA neutralised MVE virus infectivity. The results of neutralisation assays (PRNT) with sera obtained 6 weeks after inoculation are represented in Fig. 6. All four mouse antisera neutralised 60–70% of virus at a dilution of 1 in 5. Three antisera neutralised > 40% of virus at a dilution of 1 in 20. Only one antiserum neutralised > 40% of virus at a dilution of 1 in 80. Although a significant neutralising antibody response was obtained in all mice, complete neutralisation of virus was not obtained with any of the antisera.

Discussion

The experiments in this study were designed to identify a linear B-cell epitope from the E protein of MVE and to examine the ability of this epitope to induce a humoral immune response *in vivo*, when inserted into the central region of a salmonella flagellin gene and administered as a live vaccine.

To define neutralisation epitopes in the MVE E protein, E gene fragments fused to the β -galactosidase coding region were subjected to a series of deletions, and the resulting fusion proteins were characterised and examined for their reactivities with neutralising MAbs to map the epitopes. The bacterially expressed β -galactosidase fusion proteins allowed the E-8 epitope to be mapped to a sequence of 24 amino

Fig. 6. Virus neutralising activity of sera from mice immunised with strain B5127. Sera from week 6 bleeds were used for PRNT. The percentage neutralisation was taken as the percentage of plaque reduction by sera from mice vaccinated with strain B5127 relative to reduction by sera from mice immunised with strain SL5930. **a**, individual results of sera at different dilutions from four mice immunised with strain B5127. **b**, average results of sera at different dilutions from four mice immunised with strain B5127.

acids from residues 201 to 224. The data are consistent with the results of Guirakhoo *et al.* [38] that placed E-8 between residues 200 and 327 on the basis of MAb reactivity with E fragments derived by proteolysis. By homology with the E protein of West Nile (WN) virus [39], E-8 is presumably in a loop formed by the disulphide bond Cys190–Cys288. As reduction of the disulphide bonds had little effect on E-8, it appears to be a linear epitope. Fig. 7 shows the sequence of the MVE E protein from Tyr-201 to Pro-224 aligned with that of other flaviviruses. The sequence contains two hydrophobic regions (at the N- and C-termini) and a hydrophilic central region

	201	224
MVE	YYVMTIGTKHFLVHREWFNDLLLP	
JE	F...V.S.S.....H..A..	
WN	...SV.E.S.....M..N..	
SLE	...F.VKE.SW...N.D..H..N..	
YF	S.IAEME.ESWI.D.Q.AQ..T..	
DEN-1	MVLL.MEK.SW...KQ..L..P..	
DEN-2	MVLLQMED.AW....Q..L..P..	
DEN-4	MIL.KMKK.TW...KQ..LN.P..	
TBE	DKTVEHLPTAQ...D.....A..	
	↑	
	A5	

Fig. 7. Amino-acid sequence of the MVE E protein from Tyr 201 to Pro 224. The MVE sequence [30] is shown on the top line with an underlined segment representing a strongly hydrophilic region. Aligned sequences below are for Japanese encephalitis virus (JE) [44], West Nile virus (WN) [45], St Louis encephalitis virus (SLE) [46], yellow fever virus (YF 17D) [47], dengue 1 virus (DEN-1) [48], DEN-2 [49], DEN-4 [50] and tick-borne encephalitis virus (TBE) [42]. The single letter amino-acid code is used; dots represent amino-acid residues identical to MVE at similar positions. The arrow indicates the A5 neutralisation determinant of TBE.

(underlined in Fig. 7). As the E-8 MAb cross-reacts in ELISA with JE, SLE and WN and also in protection assays in mice with JE and SLE, the corresponding epitope is likely to show considerable homology between members of the MVE serocomplex. The sequence homology in the region 201–224 between MVE and JE, WN or SLE is 75, 75 and 58% respectively. Homology is considerably higher at the C-terminal end than overall. As cross-reaction occurs with SLE (which shows *c.* 70% homology in the C-terminal 13 amino acids and only 45% in the N-terminal section), it seems likely that the major binding determinants of E-8 are located in the C-terminal half (residues 212–224). The sequence of amino acids in this region should provide good targets for further mapping of E-8 by peptide analysis.

From competitive binding studies of anti-E-8 with other MAbs against MVE [27], it has been shown that E-8 overlaps with epitopes E-1a, E-1c, E-1d and E-5, and is spatially distinct from epitopes E-3, E-4b, E-6 and E-7. These data suggest that a cluster of epitopes (E-1a, E-1c, E-1d, E-5 and E-8), most of which induce neutralising antibodies, form a domain in the E protein of MVE [41]. The epitope E-8 is in close proximity to the positions of the A4 and A5 neutralisation determinants of TBE, which have been mapped to residues 233 and 207 of the gE sheet of domain II, respectively, on the three-dimensional structure of the TBE E protein [40–42]. Domain II has been shown to comprise two discontinuous segments (residues 50–125, residues 200–250) in the disulphide-rich regions of TBE E protein, and is active in inducing neutralising and haemagglutination-inhibiting antibodies [41]. Significantly, the cluster of MVE neutralisation epitopes, including E-8 and E-1c [27], map to the same domain, suggesting conservation of

structure as well as function across the family of flaviviruses.

As the antisera induced by the fusion protein E201–224 lacked significant MVE neutralising ability, it was of interest to express the epitope E-8 in a *Salmonella* strain in an attempt to induce a neutralising antibody response in mice. Also, because epitope E-8 is encompassed by a relatively short linear peptide sequence it was suitable for expression in the flagellin of an attenuated *Salmonella* strain. The chimeric flagellin expressed by the Dublin recombinant strain was demonstrated to contain the E_{201–224} sequence. This epitope was situated in a region of the flagellin sequence that specifies the flagellar serotype and, therefore, is considered to be relatively tolerant of amino-acid deletions and substitutions [43]. Also, the epitope is relatively small when compared with the 500 amino-acid length of the native flagellin. For these reasons, the resulting chimeric flagellin was expected to form normal flagella. Expression of the chimeric flagellin by the recombinant strain was confirmed by Western blotting of whole cell lysates and the location of the epitope as part of the flagellar filament was demonstrated by immunogold electron microscopy. Electron microscopy showed that the hybrid flagellin had been exported to the bacterial surface and assembled into flagella-like filaments. However, the ultrastructural appearance of the flagella was abnormal. The flagella were truncated and present in very small numbers on the recombinant *Salmonella* strain carrying the MVE B-cell epitope. It was not surprising then that bacteria expressing the chimeric flagella were non-motile in semi-solid medium. In spite of the functional abnormality of the flagella, the chimera generated a significant humoral immune response *in vivo*. These data suggest that motility and immunogenicity are not necessarily associated, particularly when the vaccine is administered intraperitoneally. It may be possible to restore motility in the non-motile recombinant strain by transforming the strain with a plasmid encoding normal flagellin. This in turn may enhance the potential for its use as an orally administered vaccine.

Mice immunised with the recombinant bacteria generated specific antibodies that reacted with the MVE E-8 epitope- β -galactosidase fusion protein and the intact MVE virus. The MVE specific immune response increased until 6 weeks after the initial immunisation. However, the MVE specific antibodies only partially neutralised the infectivity of the virus *in vitro*. Incomplete neutralisation may be an inherent property of the E-8 epitope as in our hands the anti-E-8 mAb did not completely neutralise MVE *in vitro* (unpublished data). Because anti-E-8 antibody cross-reacts with MVE, SLE, JE and WN, this epitope may be of use in generating a common vaccine for a significant group of flaviviruses. It would be interesting to test the protective effect of the recombinant

Salmonella strain carrying the MVE E-8 epitope in an animal model challenged with these flaviviruses.

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