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Enhancement of the Antibody Response to Flavivirus B-Cell Epitopes by Using Homologous or Heterologous T-Cell Epitopes

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We have been investigating the T-helper (T_h)-cell response to the flavivirus envelope (E) glycoprotein. In our studies with Murray Valley encephalitis (MVE) virus, we previously identified synthetic peptides capable of priming T_h lymphocytes for an in vitro antivirus proliferative response (J. H. Mathews, J. E. Allan, J. T. Roehrig, J. R. Brubaker, and A. R. Hunt, J. Virol. 65:5141–5148, 1991). We have now characterized in vivo T_h-cell priming activity of one of these peptides (MVE 17, amino acids 356 to 376) and an analogous peptide derived from the E-glycoprotein sequence of the dengue (DEN) 2, Jamaica strain (DEN 17, amino acids 352 to 368). This DEN peptide also primed the T_h-cell compartment in BALB/c mice, as measured by in vitro proliferation and interleukin production. The failure of some MVE and DEN virus synthetic peptides to elicit an antibody response in BALB/c mice could be overcome if a T_h-cell epitope-containing peptide was included in the immunization mixture. A more detailed analysis of the structural interactions between T_h-cell and B-cell epitope donor peptides revealed that the peptides must be linked to observe the enhanced antibody response. Blockage or deletion of the free cysteine residue on either peptide abrogated the antibody response. The most efficient T-B-cell epitope interaction occurred when the peptides were colinearly synthesized. These T_h-cell-stimulating peptides were also functional with the heterologous B-cell epitope-containing peptides. The T_h-cell epitope on DEN 17 was more potent than the T_h-cell epitope on MVE 17.

Flaviviruses are composed of a single-strand, positive-sense RNA enclosed in a spherical nucleocapsid. The virus has a lipid envelope that has been modified by the insertion of multiple copies of two virus-encoded proteins: the matrix (M) protein (10 kDa) and the envelope (E) glycoprotein (55 to 60 kDa) (14). The E glycoprotein encodes the important biological functions of attachment to cellular receptor, membrane fusion, and elicitation of virus-neutralizing antibodies. It is also elicits the most protective immune response (14).

Flavivirus vaccine development has been enigmatic. Only two live attenuated vaccines, against yellow fever and Japanese encephalitis, have been successfully developed (4). The hypothesis that dengue (DEN) virus, a major cause of human morbidity and mortality, might contain enhancing epitopes that prime individuals for severe disease complications makes the design of a univalent whole-virus vaccine difficult (4). DEN virus is therefore a likely candidate for subunit vaccine development. Inclusion of only type-specific protective epitopes in a DEN virus vaccine might limit severe disease complications. Subunit vaccines, however, usually require multiple injections to achieve adequate protection, and individuals may be refractory to immunization. These limitations could be due to the absence of or inadequate processing of T-helper (T_h)-cell epitopes necessary for a strong antibody response.

Our laboratory and others have been attempting to further define the B- and T-cell epitopes present on the flavivirus E glycoprotein, using synthetic peptides or expressed viral proteins (1, 16, 17, 19, 33, 36). We have prepared and analyzed a number of synthetic peptides derived from the amino acid sequences of the E glycoprotein of both Murray Valley encephalitis (MVE) and DEN 2 viruses (21, 29, 30). Some of these peptides contained T_h-cell epitopes predicted by the Rothbard motif or AMPHI algorithm as described by Margalit et al. (20, 31, 32). Using the MVE virus peptides, we have identified several peptides capable of priming inbred mice for an in vitro T_h-cell proliferative response to a homologous peptide (21).

It has now been demonstrated for a number of viruses that T_h-cell epitopes can be modeled by using synthetic peptides and that active T_h-cell epitopes can modulate the antibody response to B-cell epitopes (2, 3, 5–11, 13, 18, 24–28, 35). These observations are important in vaccine design and mean that effective vaccines will most likely require expression of both B- and T-cell epitopes (for a review, see reference 23). In this report, we identify biologically active flavivirus T_h-cell epitopes active with both heterologous and homologous B-cell epitopes. As with studies of other viruses, the flavivirus derived T_h-cell epitopes must be linked to the B-cell epitope to be effective. This linkage can apparently occur passively by simple mixing of peptides which contain free cysteine residues.

MATERIALS AND METHODS

Peptides. All of the sequences and antibody responses in BALB/c and outbred NIH-Swiss mice to the DEN virus peptides 1-2 (amino acids [aa] 1 to 30), 3-8/2 (aa 49 to 60 and 121 to 140), 4-6 (aa 72 to 91 and 93 to 105), 79 (aa 79 to 99), 5-7 (aa 90 to 104 and 106 to 120), 04 (aa 121 to 140), 142-1 (aa 165 to 172), 208 (aa 208 to 219), 67 (aa 255 to 274), and 17 (aa 352 to 368) and the BALB/c antibody responses to MVE virus peptides 03 (aa 77 to 97), 04 (aa 122 to 141), and 17 (aa 356 to 376) have been previously published (21, 29, 30). The newly synthesized or modified peptides used for determining the structural requirements of T_h-cell help are shown in Table 1. Peptides were synthesized on an Applied Biosys-

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TABLE 1. Sequences of new DEN synthetic peptides used in this study

Peptide	Sequence	% Coupling efficiency[a]
04alk	NH_2-CKKNMEGKVVLPENLEYTIV-COOH (Cys alkylated: $COCH_2$)	99.5
17(-cys)	NH_2-ITVNPIVTEKDSPVNIE-COOH	99.5
04-17	NH_2-KKNMEGKVVLPENLEYTIVITVNPIVTEKDSPVNIE-COOH	99.3

[a] Average coupling efficiency as determined by the quantitative ninhydrin test (34).

tems 430A peptide synthesizer, using standard *tert*-butyloxycarbonyl chemistry (Applied Biosystems, Foster City, Calif.). Peptides were cleaved from resin with anhydrous hydrofluoric acid with suitable scavengers and monitered for purity by reverse-phase high-performance liquid chromatography (Multiple Peptide Systems, San Diego, Calif.). All peptides except peptides 04alk, 17(-cys), and 04-17 had an encoded terminal cysteine residue, or a carboxy-terminal cysteine residue was added to facilitate carrier coupling. Peptide 17(-cys) was prepared by removing part of the peptide 04-17 resin during synthesis after completion of the carboxy-terminal peptide 17 sequence. As determined from previous studies showing that the most efficient B-cell epitope orientation is on the amino terminus of colinear peptides, the amino-to-carboxyl order of colinear synthesis of the DEN virus peptides was 04 to 17 (3, 8, 9, 28).

Peptide alkylation. To block the possible dimerization of DEN 04 in mixing experiments with DEN 17, the amino-terminal cysteine of DEN 04 was alkylated with iodoacetamide to produce DEN 04alk (15). Briefly, 10 mg of DEN 04 was dissolved in 16.6 ml of 0.25 M Tris buffer (pH 9.0) containing 6 M guanidine-HCl and 60 μl of 2-mercaptoethanol (Fisher Biotech, Fair Lawn, N.J.). After incubation at 37°C for 4 h, 60 mg of iodoacetamide (Sigma Chemical Co., St. Louis, Mo.) was added, and the solution was incubated for 1 h at 37°C. Alkylated peptide was dialyzed against 0.1 M Tris (pH 9.0)–0.1 M Tris (pH 7.0) and finally deionized water. Following dialysis, the peptide solution was lyophilized. The yield of DEN 04alk was 45%. To determine the percent alkylation of DEN 04alk, peptides DEN 04 and DEN 04alk were derivatized with Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid); Pierce, Rockford, Ill.]. Derivatized product was removed from free reagent by chromatography on a Sephadex G-10 column (Pharmacia LKB Biotechnology, Piscataway, N.J.) and monitoring A_{412}. The peak at the front was collected and lyophilized. The yield of Ellman's derivatized product varied from 25 to 41%. Percent derivatization, which was a direct measurement of the efficiency of alkylation, was determined by comparing reduction of Ellman's derivatized peptides DEN 04 and DEN 04alk with dithiothreitol (Research Organics, Inc., Cleveland, Ohio). The produced yellow color was monitored at 412 nm. Concentration of released product was calculated by using a molar extinction coefficient of 1.36×10^4 cm^{-1} M^{-1}.

Immunization of animals. All animals (BALB/c, *H-2^d*, or outbred NIH-Swiss mice; Harlan Sprague-Dawley, Indianapolis, Ind.) were immunized with two subcutaneous 0.2-ml injections of 50 μg of peptide mixed 1:1 in Freund's incomplete adjuvant at days 0 and 14. At day 28, animals were bled and the antipeptide titers were determined by enzyme-linked immunosorbent assay (ELISA). As noted in the tables, some animals received a third immunization. These animals were rebled on day 35. Animals immunized with mixtures were administered 50 μg of each peptide. Animals immunized with the DEN 04-17 copolymer were immunized with 100 μg of peptide to simulate the molar dosage of the DEN 04-DEN 17 mixture.

ELISA. An ELISA was used to determine antipeptide and antivirus responses (29, 30). Because all peptides elicited virus-reactive antibody, the antibody response to peptide mixtures was measured only against the individual peptides to differentiate the antigenic contributions from each peptide. Antiviral ELISA titers were determined by using purified DEN 2 Jamaica at 1 to 2 μg per well.

In vitro proliferation assays. The procedures for the in vitro proliferation assay have been published previously (21, 22). Briefly, BALB/c mice were administered two subcutaneous inoculations of 50 μg of peptide in Freund's incomplete adjuvant. After 28 days, spleens were harvested, single-cell suspensions were prepared, and T-cell populations were enriched by nylon wool chromatography following lysis of erythrocytes with ammonium chloride buffer. Cells were stimulated in culture with peptide or purified virus prepared as previously described (21). All samples were run in triplicate. Virus-primed lymphocytes were included as controls and were stimulated in culture with peptide or virus. Proliferation was monitored by pulsing T cells with 1 μCi of $[^3H]$thymidine ($[^3H]$TdR; 6.7 Ci/mmol; E. I. DuPont de Nemours & Co., Wilmington, Del.) per well on day 4 for 18 h. Cells were harvested as previously described (21). A stimulation index ratio (SIR) was calculated by dividing the stimulation index derived by using T cells from peptide-immunized mice cultured in the presence of homologous peptide by the stimulation index of T cells from unimmunized mice incubated with the same peptide. Calculation of the stimulation index has been published previously (21, 22). An SIR of 2.0 or larger ($P < 0.05$) was considered significant.

IL-2 production analysis. The in vitro analysis for the production of interleukin-2 (IL-2) by proliferating cells was a modification of the previously published procedures (12, 21). In this study, IL-2-dependent HT-2 cells replaced CTLL-2 cells. Briefly, 10,000 HT-2 cells were incubated 18 h with triplicate culture supernatant samples derived from in vitro proliferation assays and then pulsed with 1 μCi of $[^3H]$TdR per well 6 h prior to harvesting. The amount of $[^3H]$TdR incorporation was compared with that in control cultures with no conditioned media or in cultures grown with supernatants of control peptide proliferations.

RESULTS

T-cell help provided by an MVE peptide previously shown to have T_h-cell activity in vitro. We previously determined that MVE 17 had in vitro T_h-cell activity (21). Because MVE 17 was able to associate with class II major histocompatibility complex proteins from three haplotypes (*H-2^b*, *H-2^d*, and *H-2^k*) of inbred mice (21), we determined how effective this

TABLE 2. Homologous T-cell help provided by MVE 17 in peptide mixing experiments in BALB/c mice

Antiserum	No. of immunizations	Titer[a]		
		17	03	04
03	2	ND	2.2 (0.2)[b]	ND
04	2	ND	ND	1.5 (0.2)[b]
17	2	3.1 (0)	1.3 (0)	1.3 (0)
	3	3.7 (0)	ND	ND
17 + 03 + 04	2	2.8 (0)	1.9 (0)	1.9 (0)
	3	4.3 (0)	3.1 (0)	3.0 (0.1)

[a] Geometric mean ELISA titer on the homologous peptide ($\log_{10}$) and standard deviations (in parentheses) for antisera raised against MVE virus peptides or mixtures. Serum pools of six animals were tested in triplicate unless otherwise noted. Starting serum dilution was 1:20 for all samples. ND, not determined.

[b] Data from reference 29 for comparison. Geometric mean ELISA titers for four animals.

TABLE 4. Reconstitution of the BALB/c antibody response to DEN peptides by inclusion of DEN 17

Peptide	Titer[a]		
	BALB/c	NIH-Swiss	BALB/c + DEN 17
1-2	1.3 (0.0)	2.1 (0.9)	2.2 (0.4)
3-8/2	1.9 (0.3)	2.7 (0.8)	2.8 (0.7)
4-6	2.2 (0.4)	2.8 (0.7)	2.8 (0.5)
04	1.6 (0.6)	2.8 (0.5)	2.9 (0.6)
79	1.7 (0.7)	1.6 (0.7)	1.9 (0.5)
5-7	1.4 (0.1)	1.8 (0.8)	1.9 (0.3)
142-1	1.3 (0.0)	1.3 (0.0)	1.6 (0.3)
208	1.8 (0.2)	1.3 (0.1)	1.7 (0.1)
67	1.5 (0.3)	1.3 (0.0)	1.4 (0.1)
17	4.1 (0.5)	1.3 (0.0)	3.4 (0.0)

[a] Geometric mean ELISA titer on the homologous peptide ($\log_{10}$) and standard deviation (in parentheses) (n = 10 for BALB/c and NIH-Swiss mice; n = 6 for BALB/c mice immunized with peptide mixtures). Starting dilution was 1:20 for all samples. Antibody responses in BALB/c and NIH-Swiss mice are taken from reference 30 for comparison with the response with the DEN 17 mixture in BALB/c mice.

peptide was at helping the in vivo antibody response to a mixture of two peptides (MVE 03 and MVE 04) that did not independently induce an antibody or in vitro T_h-cell proliferative response in BALB/c (*H-2*^{d}) mice (21). MVE 03 but not MVE 04 induced an antibody response in outbred NIH-Swiss mice (29). MVE 17 was effective in significantly enhancing antibody ($P < 0.05$) to MVE 04 after two inoculations and to MVE 03 after three inoculations (Table 2).

In vitro T-cell activation by DEN 17. Because both MVE 17 and DEN 17 induced an antibody response in BALB/c mice and MVE 17 demonstrated in vitro T_h-cell proliferation activity, the T_h-cell proliferative response to DEN 17 was evaluated (Table 3). BALB/c splenic T_h-lymphocytes could be primed for an in vitro blastogenic response, in a dose-dependent manner, to DEN 17 following stimulation with a homologous peptide compared with control cells from unimmunized animals. The average SIR for DEN 17 priming and in vitro proliferation with DEN 17 from four independent experiments was 2.8. Lymphocytes from DEN 17-primed mice also proliferated in vitro when stimulated with DEN virus (SIR = 2.0). In vitro proliferation mediated by DEN 17 was also accompanied by IL-2 production, which is an important indicator of T_h-cell activity.

In contrast to the results for DEN 17, DEN 04, which was nonimmunogenic in BALB/c mice, was also unable to prime an in vitro lymphoblastogenic response. The mean SIR for three independent tests was 1.0 for both peptide and virus stimulation of peptide-primed lymphocytes. These cultures were also negative for the production of IL-2.

Antibody response to individual or mixed DEN peptides in different mouse strains. We noticed previously that some DEN peptides were poorly immunogenic in BALB/c mice (30). We also observed that some of these poorly immunogenic peptides could, however, elicit an antibody response in outbred NIH-Swiss mice (30) (Table 4). In many cases, peptides that were nonimmunogenic in BALB/c mice were immunogenic in outbred mice. We interpreted these results to mean that the BALB/c T_h-cell response to the nonimmunogenic peptides was genetically restricted. We were interested in determining whether DEN 17 was similar to MVE 17 and could also function in vivo as a T_h-cell epitope donor and reconstitute the BALB/c antibody response to the nonreactive peptides. By pooling all seronegative peptides and then mixing them with DEN 17, the BALB/c antibody response to DEN 1-2, 3-8/2, 4-6, and 04 could be enhanced to a level statistically indistinguishable ($P < 0.05$) from that seen in outbred mice (Table 4). The enhanced antibody response in BALB/c mice to seronegative peptides was also observed if peptides were mixed individually with DEN 17 prior to

TABLE 3. T_h-cell activity of DEN 17 as measured by in vitro lymphoblastogenesis assay and IL-2 production

Antigen[a]	In vitro dose (μg)	[^{3}H]TdR incorporation (10^3) after immunization with[b]:				
		DEN virus, LBT	DEN 17		Unimmunized	
			LBT	IL-2	LBT	IL-2
None	0	3.2 (0.4)	1.9 (0.3)	0.3 (0.1)	1.1 (0.2)	0.2[c]
DEN virus	1	19.6 (0.9)	4.8 (1.5)[d]	1.2 (0.2)	1.9 (0.2)	0.3 (0.7)
	5	27.4 (5.0)	6.8 (0.6)[d]	1.5 (0.06)	2.8 (0.5)	0.3 (0.05)
DEN 17	0.1	ND	2.6 (0.5)	0.5 (0.1)	0.9 (0.04)	0.3 (0.08)
	1.0	5.5 (1.6)	4.3 (0.3)[d]	3.6 (0.8)	1.1 (0.2)	0.3 (0.04)
	10	4.1 (1.9)	6.2 (1.4)[d]	6.9 (1.0)	1.1 (0.2)	0.3 (0.06)
VE2pep07	10	0.4 (0.06)	0.4 (0.1)	0.2 (0.02)	0.2 (0.04)	0.3 (0.05)

[a] Antigen used in in vitro stimulation for the analyses.

[b] Representative results of four independent tests with DEN 17. Data represent mean [^{3}H]TdR counts per minute (10^3) incorporated and standard deviation (in parentheses). The lymphoblastogenesis test (LBT) and IL-2 assay were performed as described in Materials and Methods. ND, not determined.

[c] Isotope incorporation by normal HT-2 cells.

[d] Differences in the lymphoblastogenesis test between peptide-primed, peptide-stimulated or peptide-primed, virus-stimulated cells were significantly different ($P < 0.05$) from results derived with peptide-primed, unstimulated cells.

immunization (data not shown). DEN 79, 5-7, 142-1, 208, and 67 were poorly immunogenic regardless of the mouse strain or the presence or absence of DEN 17. We interpreted this result to mean that no B-cell epitopes were present on these seronegative peptides.

Structural requirements for T-cell help. We were interested in defining the structural requirements for the observed T-B-cell interactions (Table 5). We chose DEN 04 as a BALB/c-restricted candidate B-cell epitope donor which was unable to elicit antibody or in vitro T_h-cell activity. DEN 04 has an encoded amino-terminal cysteine residue. As previously demonstrated, the BALB/c antibody response to DEN 04 was enhanced when DEN 04 was mixed with DEN 17 (Table 4). Two experiments were performed to determine whether free cysteine residues in both T- and B-cell epitope-containing peptide donors were required for the enhanced antibody response. Initially, the amino-terminal cysteine residue in DEN 04 was alkylated with iodoacetamide to produce DEN 04alk. DEN 04alk was 87% alkylated. DEN 04 and DEN 04alk were equally immunogenic in outbred animals ($n = 10$). Alkylation of DEN 04 to make DEN 04alk had no effect on peptide antigenicity, since both anti-DEN 04 and anti-DEN 04alk antibody were fully reactive with either DEN 04 or DEN 04alk. DEN 17 could not enhance the BALB/c antibody response to DEN 04alk (Table 5). Second, we resynthesized DEN 17 without the nonencoded carboxy-terminal cysteine [Table 1, DEN 17 (-cys)]. DEN 17(-cys) was fully antigenic by itself. When DEN 17(-cys) was mixed with DEN 04, a minimal antibody response to DEN 04 was observed. A final experiment was performed in which DEN 04 and DEN 17 were cosynthesized. No glycine spacer bridge between peptides was included, and the carboxy-terminal cysteine residue of DEN 17 and the amino-terminal cysteine of DEN 04 were deleted to more closely mimic the probable conformation of a dimerized DEN 04- DEN 17 mixture. BALB/c animals immunized with the DEN 04-17 copolymer again raised high-level antibody to both DEN 04 and DEN 17. The antibody elicited by the colinear peptide was more reactive with itself than was the antibody elicited by mixing DEN 04 and DEN 17. As previously observed, all animals with demonstrable anti-DEN 17 or anti-DEN 04 antibody also had comparable antiviral ELISA titers.

Analysis of T_h-cell activity of a heterologous flavivirus T-cell epitope. To study the ability of these peptides to furnish heterologous T_h-cell stimulating activity, we analyzed the ability of MVE 17 and DEN 17 to help the antibody response to the B-cell epitopes on MVE 04 or DEN 04 (Table 6). As previously mentioned, the MVE 04 and DEN 04 peptides were unable to induce antibody only in NIH-Swiss mice. MVE 17 did not appear to be as efficient as DEN 17 at providing T-cell help for homologous B-cell-containing peptides following two inoculations. The MVE 17 mixture required three inoculations to elicit an anti-DEN 04 response similar to the antipeptide response observed following two inoculations with the DEN 17 mixture ($P < 0.05$). DEN 17 was also more efficient than MVE 17 at eliciting an antibody response to MVE 04 following two inoculations.

DISCUSSION

This is the first report of a biologically active flavivirus T_h-cell epitope capable of providing help for a homologous flavivirus B-cell antibody response to another peptide that was genetically restricted in an inbred strain of mice (BALB/c, H-2^d). At least three other peptides from the E glycoprotein of MVE virus have also been implicated as possible T_h-cell epitope donors (21). The observation that the T- and B-cell epitopes must be linked for full activity is not new (5, 6, 8, 9, 27). It is surprising to us that this covalent linkage can apparently occur by simply mixing two peptides which contain free sulfhydryl groups. The timing and characteristics of this linkage are unclear. We do not know whether the peptides cross-linked during refrigeration of the adjuvant immunogen mixture or following inoculation. It is also possible that peptides cross-link to the immune effector cells. Our results suggest that the most effective association of the T- and B-cell epitopes occurs when the peptides are colinearly synthesized. These results are similar to those observed with other viral peptides (5, 6, 8, 9, 28). In this study, an interpeptide glycine spacer bridge was not necessary for full antigenicity of the copolymer. While the copolymer was synthesized with the B-cell epitope-containing peptide on the amino terminus of the dipeptide, cross-linking of peptide mixtures would result in a carboxy-terminal

TABLE 5. Structural requirements for the T_h-cell epitope activity of DEN 17 with DEN 04 in BALB/c mice

Antiserum	ELISA reactivity to[a]:					
	17	17(-cys)	04	04alk	04-17	Virus
17	4.4 (0.4) (20)	3.5 (0.6) (10)	<1.6 (0.2) (*)	ND	3.5 (0.5) (10)	3.0 (0.2) (20)
17(-cys)	3.1 (0.5) (10)	3.2 (0.6) (10)	1.6 (0) (*)	ND	3.5 (0.6) (10)	2.3 (0.2) (10)
04	<1.6 (0) (*)	<1.6 (0) (*)	1.6 (0.4) (20)	1.7 (0.4) (10)	<1.6 (0) (10)	1.6 (0) (20)
04alk	ND	ND	1.5 (0.2) (10)	1.5 (0.2) (10)	ND	<1.6 (0) (10)
17 + 04	4.2 (0.6) (26)	2.6 (0.4) (16)	3.4 (0.7) (26)	3.9 (0.9) (10)	2.9 (0.5) (16)	3.3 (0.2) (26)
17 + 04alk	4.8 (0.4) (10)	ND	1.8 (0.5) (10)	1.8 (0.5) (10)	ND	3.0 (0.3) (10)
17(-cys) + 04	3.0 (0.5) (6)	3.4 (0.3) (6)	2.2 (0.4) (6)	ND	3.5 (0.3) (6)	2.7 (0.2) (6)
04-17	4.1 (0.2) (10)	4.5 (0.4) (10)	4.2 (0.6) (10)	ND	4.6 (0.5) (10)	4.0 (0) (10)

[a] Geometric mean ELISA titer on the homologous peptide (log_{10}) and standard deviation (in parentheses). The number of animals (n) tested is shown in parentheses beneath the mean value. Starting dilution was 1:40 for all samples. n = * is the mean of three tests of a pool of 10 animals. ND, not determined.

TABLE 6. Comparison T-cell activity of MVE 17 and DEN 17 with heterologous and homologous B-cell epitopes in BALB/c mice

Antiserum	No. of immunizations	Titer[a]			
		MVE 17	DEN 17	MVE 04	DEN 04
MVE 17	2	3.1 (0)[b]	ND	ND	ND
DEN 17	2	ND	4.3 (0)	ND	ND
MVE 04	2	ND	ND	1.5 (0.2)[c]	ND
DEN 04	2	ND	ND	ND	1.7 (0.4)[d]
MVE 17 + MVE 04	2	2.8 (0)[b]	ND	1.9 (0)[b]	ND
DEN 17 + MVE 04	2	ND	4.0 (0)	3.0 (0.2)	ND
DEN 17 + DEN 04	2	ND	4.2 (0.6)[d]	ND	3.4 (0.7)[d]
MVE 17 + DEN 04	2	2.9 (0)	ND	ND	1.6 (0)
MVE 17 + DEN 04	3	3.8 (0.1)	ND	ND	2.5 (0)

[a] Geometric mean ELISA titers on the homologous peptide ($\log_{10}$) and standard deviations (in parentheses) of antisera raised against MVE or DEN peptides or mixtures. Serum pools of six animals were tested in triplicate unless otherwise noted. Starting serum dilution was 1:40 for all samples unless otherwise noted in previous tables. ND, not determined.

[b] Data from Table 2.

[c] Data from reference 29 for comparison. Geometric mean ELISA titers for four animals.

[d] Data from Table 5.

position of the B-cell epitope-containing peptide, as judged from the location of the free cysteine residues. In either case, the dipeptide is quite immunogenic, which indicates that there is no directional characteristic to the immunogenicity of this dipeptide.

The observation that the DEN 17 provides help for the heterologous MVE virus B-cell epitope-containing peptide 04 and vice versa is significant. Identification of flavivirus group reactive T_h-cell epitopes might mean that a single T_h-cell epitope could provide T_h-cell stimulatory activity for many flaviviruses. This approach has advantages over inclusion of heterologous T_h-cell epitopes from unrelated antigens such as those found on inert carrier proteins, because flavivirus-specified T_h-cell epitopes would prime for epitopes encountered in subsequent flavivirus infections. Although we did no cross-priming experiments, the low sequence homologies between MVE 17 and DEN 17 predict that this region is not optimal for heterologous T_h-cell priming. Other T_h-cell active peptides with more sequence homologies (e.g., MVE 06) may be more likely heterologous T_h-cell epitope candidates.

We are currently producing a complete T_h-cell epitope map of the E glycoprotein of DEN 2 Jamaica in multiple haplotypes of mice, using synthetic peptides that represent the entire E-glycoprotein gene sequence. In this way, we hope to identify the most promising flavivirus T_h-cell epitopes for inclusion in subunit flavivirus vaccines. We hope that by delineating regions important in the T_h-cell response, new vaccines derived from site-directed mutagenized infectious cloned virus can be attenuated but still elicit a fully functional T_h-cell response.

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REFERENCES

1. **Allan, J. E., and P. C. Doherty.** 1986. Stimulation of helper/delayed-type hypersensitivity T cells by flavivirus infection: determination by macrophage procoagulant assay. J. Gen. Virol. **67:**39–46.
2. **Barnett, B. C., C. M. Graham, D. S. Burt, J. S. Skehel, and D. B. Thomas.** 1989. The immune response of BALB/c mice to influenza hemagglutinin: commonality of the B cell and T cell repertoires and their relevance to antigenic drift. Eur. J. Immunol. **19:**515–521.
3. **Borras-Cuesta, F., J. Petit-Camurdan, and Y. Fedon.** 1987. Engineering of immunogenic peptides by co-linear synthesis of determinants recognized by B and T cells. Eur. J. Immunol. **17:**1213–1215.
4. **Brandt, W. E.** 1990. Development of dengue and Japanese encephalitis vaccines. J. Infect. Dis. **162:**577–583.
5. **Cox, J. H., J. Ivanyi, D. B. Young, J. R. Lamb, A. D. Syred, and M. J. Francis.** 1988. Orientation of epitopes influences the immunogenicity of synthetic peptide dimers. Eur. J. Immunol. **18:**2015–2019.
6. **Ertl, H. C. J., B. Dietzschold, M. Gore, L. Otvos, Jr., J. K. Larson, W. H. Wunner, and H. Koprowski.** 1989. Induction of rabies virus-specific T-helper cells by synthetic peptides that carry dominant T-helper cell epitopes of the viral ribonucleoprotein. J. Virol. **63:**2885–2892.
7. **Francis, M. J., G. Z. Hastings, B. E. Clarke, A. L. Brown, C. R. Beddell, D. J. Rowlands, and F. Brown.** 1990. Neutralizing antibodies to all seven serotypes of foot-and-mouth disease virus elicited by synthetic peptides. Immunology **69:**171–176.
8. **Francis, M. J., G. Z. Hastings, A. D. Syred, B. McGinn, F. Brown, and D. J. Rowlands.** 1987. Non-responsiveness to a foot-and-mouth disease virus peptide overcome by addition of foreign helper T-cell determinants. Nature (London) **330:**168–170.
9. **Francis, M. J., G. Z. Hastings, A. D. Syred, B. McGinn, F. Brown, and D. J. Rowlands.** 1988. Peptides with added T-cell epitopes can overcome genetic restriction of the immune response, p. 1–7. *In* H. Ginsberg, F. Brown, R. A. Lerner, and R. M. Chanock (ed.), Vaccines 88. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **Gao, X.-M., F. Y. Liew, and J. P. Tite.** 1989. Identification and characterization of T helper epitopes in the nucleoprotein of influenza A virus. J. Immunol. **143:**3007–3014.
11. **Gao, X-M., F. Y. Liew, and J. P. Tite.** 1990. A dominant Th cell epitope in influenza nucleoprotein: analysis of the fine specificity and functional repertoire of T cells recognizing a single determinant. J. Immunol. **144:**2730–2737.
12. **Gillis, S., and K. A. Smith.** 1977. Long-term culture of cytotoxic T lymphocytes. Nature (London) **268:**154–156.
13. **Heber-Katz, E., S. Valentine, B. Dietzschold, and C. Burns-Purzycki.** 1988. Overlapping T cell antigenic sites on a synthetic peptide fragment from herpes simplex virus glycoprotein D, the degenerate MHC restriction elicited, and functional evidence for antigen-Ia interaction. J. Exp. Med. **167:**275–287.
14. **Heinz, F. X., and J. T. Roehrig.** 1990. Flaviviruses, p. 289–305. *In* M. H. V. van Regenmortel and A. R. Neurath (ed.), Immunochemistry of viruses, vol. II. The basis for serodiagno-

sis and vaccines. Elsevier Science Publishers B.V. (Biomedical Division), Amsterdam.

15. **Johnson, B. J. B., J. R. Brubaker, J. T. Roehrig, and D. W. Trent.** 1990. Variants of Venezuelan equine encephalitis virus that resist neutralization define a domain of the E2 glycoprotein. Virology **177**:676–683.
16. **Kurane, I., B. L. Innis, A. Nisalak, C. Hoke, S. Nimmannitya, A. Meager, and F. A. Ennis.** 1989. Human T-cell responses to dengue virus antigens. Proliferative responses and interferon gamma production. J. Clin. Invest. **83**:506–513.
17. **Kurane, I., A. Meager, and F. A. Innis.** 1989. Dengue virus-specific human T cell clones. Serotype crossreactive proliferation, interferon γ production, and cytotoxic activity. J. Exp. Med. **170**:763–775.
18. **Kurata, A., T. J. Palker, R. D. Streilein, R. M. Scearce, B. F. Haynes, and J. A. Berzofsky.** 1989. Immunodominant sites on human T cell lymphotropic virus type 1 envelope protein for murine helper T cells. J. Immunol. **143**:2024–2030.
19. **Kutubuddin, M., A. S. Kolaskar, S. Galande, M. M. Gore, S. N. Ghosh, and K. Banerjee.** 1991. Recognition of helper T cell epitopes in envelope (E) glycoprotein of Japanese encephalitis, West Nile and dengue viruses. Mol. Immunol. **28**:149–154.
20. **Margalit, H., J. L. Spouge, J. L. Cornette, K. B. Cease, C. DeLisi, and J. A. Berzofsky.** 1987. Prediction of immunodominant helper T cell antigenic sites from the primary sequence. J. Immunol. **138**:2213–2229.
21. **Mathews, J. H., J. E. Allan, J. T. Roehrig, J. R. Brubaker, and A. R. Hunt.** 1991. T-helper cell and associated antibody response to synthetic peptides of the E glycoprotein of Murray Valley encephalitis virus. J. Virol. **65**:5141–5148.
22. **Mathews, J. H., and J. T. Roehrig.** 1989. Specificity of the murine T helper cell immune response to various alphaviruses. J. Gen. Virol. **70**:2877–2886.
23. **Milich, D. R.** 1989. Synthetic T and B cell recognition sites: implications for vaccine development. Adv. Virus Res. **45**:195–282.
24. **Milich, D. R., A. McLachlan, A. Moriarty, and G. B. Thorton.** 1987. A single 10-residue pre-S(1) peptide can prime T cell help for antibody production to multiple epitopes within the pre-S(1), pre-S(2), and S regions of Hbs Ag. J. Immunol. **138**:4457–4465.
25. **Milich, D. R., A. McLachlan, G. B. Thorton, and J. L. Hughes.** 1987a. Antibody production to the nucleocapsid and envelope of the hepatitis B virus primed by a single synthetic T cell site. Nature (London) **329**:547–549.
26. **Nicholas, J. A., M. E. Levely, M. A. Mitchell, and C. W. Smith.** 1989. A 16-amino acid peptide of respiratory syncytial virus 1A protein contains two overlapping T-cell-stimulating sites distinguishable by class II MHC restriction elements. J. Immunol. **143**:2790–2796.
27. **Partidos, C. D., C. M. Stanley, and M. W. Steward.** 1991. Immune responses in mice following immunization with chimeric synthetic peptides representing B and T cell epitopes of measles virus proteins. J. Gen. Virol. **72**:1293–1299.
28. **Partidos, C. D., and M. W. Steward.** 1990. Prediction and identification of a T cell epitope in the fusion protein of measles virus immunodominant in mice and humans. J. Gen. Virol. **71**:2099–2105.
29. **Roehrig, J. T., A. R. Hunt, A. J. Johnson, and R. A. Hawkes.** 1989. Synthetic peptides derived from the deduced amino acid sequence of the E-glycoprotein of Murray Valley encephalitis virus elicit antiviral antibody. Virology **171**:49–60.
30. **Roehrig, J. T., A. J. Johnson, A. R. Hunt, R. A. Bolin, and M. C. Chu.** 1990. Antibodies to dengue 2 Jamaica E-glycoprotein synthetic peptides identify antigenic conformation. Virology **177**:668–675.
31. **Rothbard, J. B.** 1986. Peptides and the cellular immune response. Ann. Inst. Pasteur **137E**:518–526.
32. **Rothbard, J. B., R. I. Lechler, K. Howland, V. Bal, D. D. Eckels, R. Sekaly, E. O. Long, W. R. Taylor, and J. R. Lamb.** 1988. Structural model of HLA-DR1 restricted T cell antigen recognition. Cell **52**:515–523.
33. **Rothman, A. L., I. Kurane, Y.-M. Zhang, C.-J. Lai, and F. A. Ennis.** 1989. Dengue virus-specific murine T-lymphocyte proliferation: serotype specificity and response to recombinant viral proteins. J. Virol. **63**:2486–2491.
34. **Sarin, V. K., S. B. H. Kent, J. P. Tam, and R. B. Merrifield.** 1981. Quantitative monitoring of solid-phase peptide synthesis by the ninhydrin reaction. Anal. Biochem. **117**:147–157.
35. **Scherle, P. A., and W. Gerhard.** 1986. Functional analysis of influenza-specific helper T-cell clones in vivo. T cells specific for internal viral proteins provide cognate help for B cell responses to hemagglutinin. J. Exp. Med. **164**:1114–1128.
36. **Uren, M. F., P. C. Doherty, and J. E. Allan.** 1987. Flavivirus-specific murine L3T4$^+$ T cell clones: induction, characterization and cross-reactivity. J. Gen. Virol. **68**:2655–2663.