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RECOGNITION OF HELPER T CELL EPITOPES IN ENVELOPE (E) GLYCOPROTEIN OF JAPANESE ENCEPHALITIS, WEST NILE AND DENGUE VIRUSES

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Abstract—Helper T (Th) cell antigenic sites were predicted from the primary amino acid sequence (approximately 500 in length) of the envelope (E) glycoprotein (gp) of Japanese encephalitis (JE), West Nile (WN) and Dengue (DEN) I–IV flaviviruses. Prediction of Th epitopes was done by analyzing the occurrence of amphipathic segments, Rothbard–Taylor tetra/pentamer motifs and presence of alpha helix-preferring amino acids. The simultaneous occurrence of all these parameters in segments of E gp were used as criteria for prediction as Th epitopes. Only one cross reactive epitope was predicted in the *C*-terminal region of the E gp predicted segments of all flaviviruses analyzed. This region is one of the longest amphipathic stretch (approximately from 420 to 455) and also has a fairly large amphipathic score. Based on the predicted findings three selected peptides were synthesized and analyzed for their ability to induce *in vitro* T cell proliferative response in different inbred strains of mice (Balb/c, C57BL6, C3H/HeJ). Synthetic peptide I and II prepared from *C*-terminal region gave a cross reactive response to JE, WN and DEN-II in Balb/c and C3H/HeJ mice. Synthetic peptide III prepared from *N*-terminal region gave a proliferative response to DEN-II in Balb/c strain only, indicating differential antigen presentation.

INTRODUCTION

The family Flaviviridae, comprising of over 70 related viruses, has a common evolutionary origin, and has been classified serologically into several subgroups (Westaway *et al.*, 1985). In India and South East Asia major flaviviral epidemics are of Japanese encephalitis (JE), Dengue (DEN) and to some extent West Nile (WN) viruses. The mature extracellular flavivirus has three distinct structural proteins (i) 14 kD core (C) protein; (ii) 7 kD membrane (M) protein; and (iii) 50 kD envelope E gp encoded as polyprotein from the genomic positive strand RNA of approximately 11 kb (Westaway *et al.*, 1985). Among these, E gp is responsible for most biological activities of these viruses and sequences of these proteins for JE, WN and DEN (I–IV) viruses are known (Sumiyoshi *et al.*, 1987; Wengler *et al.*, 1985; Mason *et al.*, 1987; Dubel *et al.*, 1986; Osatomi *et al.*, 1988; Zhao *et al.*, 1986). The comparison of sequences of E gp from JE, WN and DEN (I–IV) has shown over 80% homology between JE and WN, and among DEN (I–IV types), and somewhat less homology occurs between JE/WN and DEN (I–IV).

To develop strategies for synthetic vaccine, it is necessary to delineate both dominant B and T cell epitopes on a protein of these viruses. In the existing killed flaviviral vaccines the E gp constitutes the major component. Immunodominant B cell epitopes/domains have been identified on E gp in some of these viruses (Nowak and Wengler, 1987; Kimura-Kuroda and Vasui, 1983; Cecilia *et al.*, 1988; Heinz, 1986). However, reports are lacking for identification of helper T cell (Th) epitopes on E protein. Thus, delineation of regions of E protein which are recognized by Th cell may be useful for T-B epitopes conjugated synthetic vaccines for these viruses.

The mechanism of antigen recognition by T cell differs significantly from those of B cells. T cells with membrane bound antigen specific T cell receptors (TCRs) recognize processed antigen fragments (epitopes) only in association with major histocompatibility complex (MHC) class I or class II molecules. The helper T cells recognize the processed antigen fragments—oligopeptides of 7–11 amino acid length capable of binding with MHC II molecules to form a peptide–MHC II complex. Such a complex is then recognized by helper TCRs (Benacerraf, 1978; Schwartz, 1985).

To identify Th epitopes, Margalit *et al.* (1987) have developed a method to predict Th epitopes which makes use of the helical amphipathic nature of such regions. Similarly Rothbard and Taylor (1988) have pointed out from their sequence analysis of known T cell epitopes that a specific sequence pattern should be present in these epitopes. They have shown the occurrence of tetra/pentapeptide having charged or glycine residue in the ith position followed by hydrophobic residues in $i+1$ and $i+2$ position (proline or hydrophobic in $i+3$ position if pentapeptides) and finally a polar or glycine residue for the 4th and 5th position respectively for tetra/pentapeptides.

Such motifs are observed in almost all experimentally known T cell epitopes. Besides, Th cell response for oligopeptides is also dependent on specific MHC molecule as the presence of allele specific subpatterns in T cell epitopes are also known to occur (Lamb *et al.*, 1988).

In the present study we tried to combine the approach developed by Margalit *et al.* (1987), that of Rothbard and Taylor (1988) and occurrence of alpha helix preferring amino acids. Most of the oligopeptides which are experimentally known to be Th cell recognition sites contain alpha helix preferring amino acids and form part of alpha helix in the parent molecule as was first shown by Pincus *et al.* (1983). The simultaneous occurrence of all the above three parameters was considered as the criterian for Th epitopes. Synthetic peptides prepared from such predicted segments were used in the experimental analysis of Th epitopes in the E gp of JE, WN and DEN II viruses.

MATERIALS AND METHODS

(*A*) *Prediction of Th epitopes by algorithmic programs*

(i) The AMPHI computer program developed by Margalit *et al.* (1987) was used to pick up amphipathic segments in E gp of JE, WN, DEN (I–IV). These authors have shown that block length of 7 amino acids with hydrophobicity scale developed by Fauchere and Pliska (1983) gives the best results and therefore in our analysis we have used this scale.

(ii) A computer program was developed to pick up the pattern, which was observed by Rothbard and Taylor (1988) at tetra/pentapeptide level, in a given protein sequence. This program was used to pick up such motifs in the E gp of JE, WN and DEN (I–IV).

(iii) Analysis of experimentally known T cell epitopes given by Rothbard and Taylor (1988) shows that except a few, all Th recognition sites (oligopeptides) are likely to be part of alpha helix since they contain alpha helix preferring amino acids. This analysis was carried out using the rules which are similar to those developed by Chou and Fasman (1978). We have developed a programme which picks up at least four consecutive alpha helix preferrers followed by either alpha helix preferrers or indifferent amino acids. Such regions are likely to take alpha helical conformation.

(*B*) *Experimental analysis of Th epitopes*

Synthesis of peptides. Peptides SIGKAVHQVF (Pep I, 436–445, JE), SLGKAVHQVF (Pep II, 430–439 DEN-IV) from the cross reactive *C*-terminal region and RDFVEGVSGGA (Pep III, 9–19 DEN-II) from *N*-terminal region of E gp were synthesized by the conventional method using t-BOC amino acids. The synthesis on solid phase resin was performed by the Merrifield's method for peptide synthesis at Centre for Cellular and Molecular Biology (CCMB), Hyderabad. The peptides were cleaved from the resin by TFMSA method and purified on P-2 column for peptide 1 and 3 and peptide 2 was purified on Sephadex G-10 column. The peptides were further purified by reverse phase HPLC. The purified peptides were analyzed and used for the study.

Preparation of JE, WN and DEN-II antigens. JE (Nakayama) and WN (E101) were grown in PS cell culture maintained in Earle's MEM with 10% goat serum. The infected fluid was harvested from burlars after 3-4+ CPE. Further processing was done at 4°C. The fluid after clarification at 3000 rpm was treated with 1 mg/ml protamine sulphate for 30 min, then centrifuged at 10,000 rpm for 30 min. The virus was pelleted at 34,000 rpm for 5 hr in Type 35 rotor using Beckman L5-75 B ultracentrifuge. The pellet was soaked in NTE buffer (100 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA, pH 8.0) overnight and this was loaded on sucrose gradient (10%–40% w/v) and centrifuged at 25,000 rpm in SW27 rotor for 2 hr. The fractions were collected and those with highest HA activity were pooled and further ultracentrifuged by pelleting at 40,000 rpm for 4 hr in Beckman SW Ti40 rotor. Pellets were soaked in NTE buffer (pH 8.0) overnight and used as a source of E gp. The inactivated antigen was prepared by treating the purified virus with beta propiolactone (BPL) 0.01%. Antigen was stored at −70°C (Cecilia *et al.*, 1988).

DEN-II (TR1751) antigen was prepared from infected infant Swiss albino mouse brains. Briefly, 2 day old Swiss albino mice were inoculated intracerebrally with a 10-fold dilution of mouse brain virus suspension in 0.75% BSA in phosphate buffered saline (PBS). On the 4th PI day when the mice were sick, the infected brains were harvested. A 20% suspension of this was made in 0.01 M Tris buffered saline (TBS) (pH 8.2) in a Waring blender. The suspension was centrifuged at 10,000 ***g*** for 3 min at 4°C. The supernatants were treated with 1 mg/ml protamine sulphate for 30 min at 4°C. Further processing for antigen purification was similar to that of JE and WN antigens.

Immunization of mice. Balb/c (H2d), C3H/HeJ (H2k), C57BL6 (H2b) inbred strains of mice, maintained at NIV, were immunized with 10 μg BPL inactivated JE antigen, or 25 μg peptide, in 50% complete Freund's adjuvant by subcutaneous route at the base of tail. The dose for immunization of antigen/peptide was optimal (data not shown).

In vitro *T cell proliferation assay.* Antigen specific T cell proliferation assay was performed using immune draining lymph node cells of Balb/c, C3H/HeJ and C57BL6 mice. The inguinal lymph nodes were harvested 5–7 days after immunization. The lymph nodes were squeezed in a glass tube with a loose fitting Teflon rod and the lymph node cells were washed thrice in 0.01 M PBS. The viable cell count was taken by trypan blue dye exclusion. The cells were suspended in RPMI-1640 containing 20 mM glutamine,

Table 1. Predicted amphipathic segments in envelope glycoprotein

Mid points of blocks	Angles (°)	Amphipathic scores (AS)	Amino acid segments	Sequence
JE				
52–57	105–135	13.3	49–60	EASQLAEVRSYC
66–74	105–135	23.0	63–77	ASVTDISTVARCPTT
113–117	95–120	12.2	110–120	KGSIDTCAKFS
125–129	80–105	13.8	122–132	TSKAIGRTIQP
182–184	120–135	8.7	179–187	KLGDYGEVT
263–268	85–100	15.2	260–271	GGLHQALAGAIV
312–317	85–120	14.1	309–320	SFAKNPADTGHG
351–354	90–120	9.7	348–357	MTPVGRLVTV
395–399	80–115	8.8	392–402	INHHWHKAGST
401–408	80–110	18.7	398–411	KAGSTLGKAFSTTL
412–420	105–135	19.1	409–423	TTLKGAQRLAALGDT
429–454	80–125	69.2	426–457	DFGSIGGVFNSIGKA-VHQVFGGAFRTLFGGMS
462–465	80–95	10.1	459–468	ITQGLMGALL
WN				
12–16	90–120	13.0	9–19	RDFLEGVSGAT
64–67	105–135	10.4	61–70	YLASVSDLST
99–103	80–90	11.7	96–106	VVDRGWGNGCG
113–117	95–120	12.5	110–120	KGSIDTCAKFA
178–180	110–120	8.6	175–183	KLGEYGEVT
259–264	85–95	15.5	256–267	GALHQALAGAIP
299–306	80–135	17.3	296–309	TTYGVCSKAFKFAR
308–314	90–135	15.5	305–317	FKFARTPADTGHG
338–351	90–135	34.4	335–354	PISSVASLNDLTPVG-RLVTV
356–359	95–120	8.8	353–362	TVNPFVSVAT
400–405	80–100	15.0	397–408	GSSIGKAFTTTL
409–417	105–135	19.1	406–420	TTLRGAQRLAALGDT
426–451	80–125	66.8	423–454	DFGSVGGVFTSVGKA-IHQVFGGAFRSLFGGMS
DEN-1				
12–16	95–120	13.8	9–19	RDFVEGLSGAT
20–24	85–105	10.6	17–27	GATWVDVVLEH
31–33	90–110	8.0	28–36	GSCVTTMAK
99–103	80–90	11.5	96–109	FVDRGWGNGCG
153–157	80–110	10.5	150–160	QVGNETTEHGT
310–313	80–105	8.4	307–316	KLEKEVAETQ
398–404	100–120	17.8	395–407	GSSIGKMFEATAR
406–410	80–120	12.3	403–413	EATARGARRMA
424–440	80–125	46.4	421–443	DFGSIGGVFTSVGK-LIHQIFGTA
DEN-2				
12–15	95–120	10.8	9–18	RDFVEGVSGG
31–33	90–110	8.0	28–36	GSCVTTMAK
54–57	85–120	8.3	51–60	KQPATLRKYC
66–69	90–120	8.4	63–72	AKLTNTTTES
99–103	80–90	11.7	96–106	MVDRGWGNGCG
154–160	85–120	18.3	151–163	VGNDTGKHGKEIK
309–314	95–135	15.5	306–317	FKIVKEIAETQH
348–351	85–105	10.3	345–354	RHVLGRLITV
397–404	80–125	18.5	394–407	KGSSIGQMFETTMR
407–410	100–125	9.5	404–413	TTMRGAKRMA
424–443	85–125	57.6	421–446	DFGSLGGVFTSIGK-ALHQVFGAIYGA
446–449	80–125	8.1	443–452	IYGAAFSGVS
461–465	95–120	13.0	458–468	LIGVIITWIGM
DEN-3				
12–16	95–120	13.8	9–19	RDFVEGLSGAT
20–24	85–105	10.6	17–27	GATWVDVVLEH
31–33	90–110	8.0	28–36	GGCVTTMAK
99–103	80–90	11.8	96–106	YVDRGWGNGCG
396–402	100–120	17.8	393–405	GSSIGKMFEATAR
404–408	80–120	12.3	401–411	EATARGARRMA
422–438	80–125	48.1	419–441	DFGSVGGVLNSLGKMV-HQIFGSA
440–447	85–115	16.9	437–450	IFGSAYTALFSGVS
461–463	100–120	8.1	458–466	GVLLTWIGL
DEN-3				
12–16	80–120	13.0	9–19	RDFVEGVSGGA
31–34	90–110	10.4	28–37	GGCVTTMAQG
47–51	80–120	10.1	44–54	ELTKTTAKEVA
70–75	80–120	9.8	67–78	NITTATRCPTOG
97–103	80–90	16.1	94–106	RDVVDRGWGNGCG
126–129	85–120	9.1	123–132	GKITGNLVRI
261–264	80–90	9.5	258–267	GAMHSALAGA
348–353	80–95	15.3	345–356	EKVVGRIISSTP
370–374	95–115	12.9	367–377	IELERPLDSYI
397–409	85–120	33.2	394–412	GSSIGKMFESTYRGAK-RMA
423–448	85–125	70.1	420–451	DFGSVGGLFTSLGKA-VHQVFGSVYTTMFGGVS

For calculating amino segments 3 amino acids are added at the left and right of the mid point of blocks.

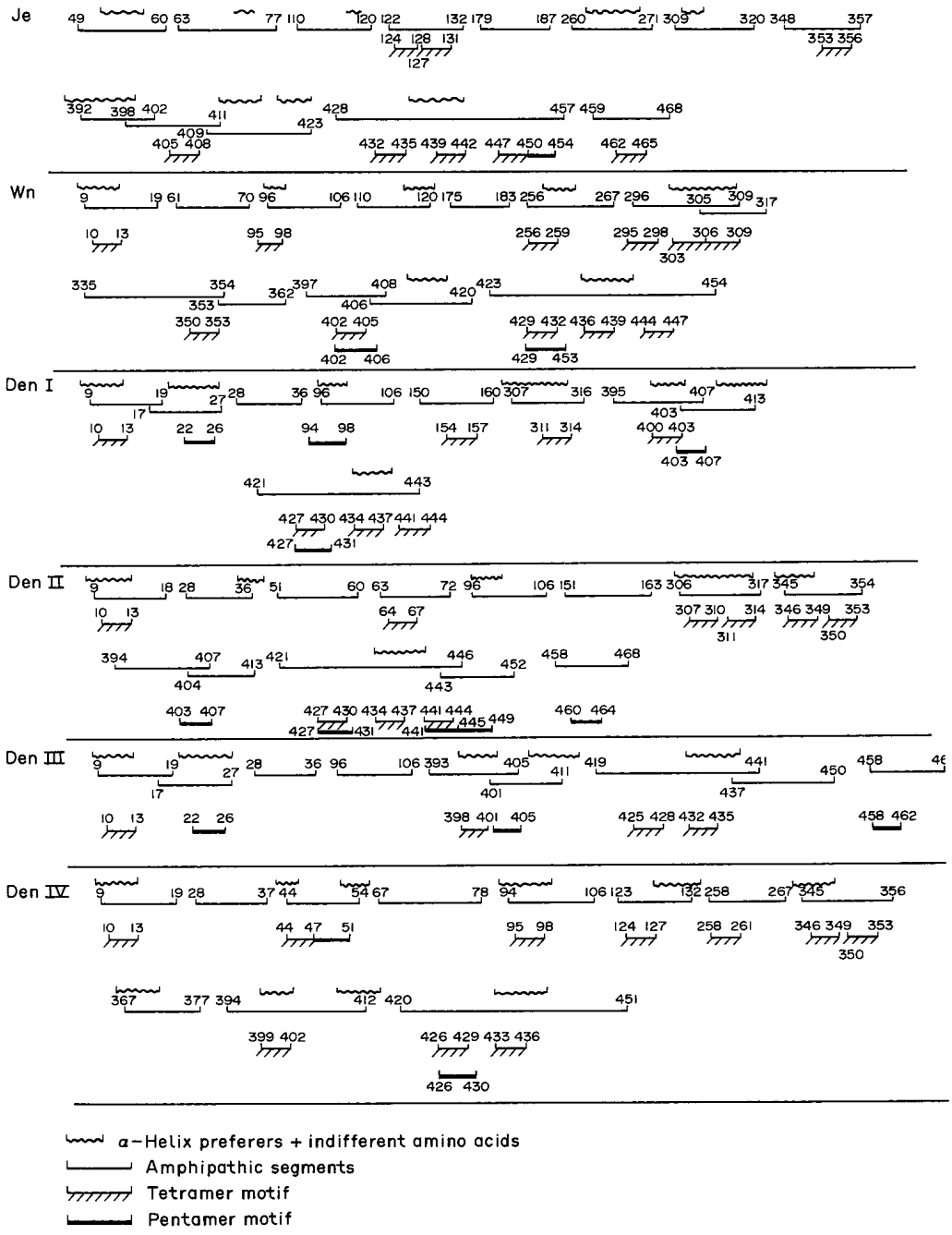

Fig. 1.

penicillin and streptomycin, supplemented with 10% FCS at a cell density of 2×10 cells/ml. Cell suspension (0.2 ml) was added per well to 96 well plates (NUNC, Denmark). Cultures were stimulated with predetermined optimum JE, WN and DEN-II antigens (5 μg/ml) and peptide concns (10 μg/ml). After 80 hr the cultures were pulsed with 1 μCi tritiated-thymidine (specific activity 15000 mCi/mM) and 16 hr later they were terminated. The cells were harvested onto glass fibre filter paper using semiautomated PHD cell harvester. The dried filter discs were counted for radioactivity in PPO and POPOP based scintillation fluid using LKB Rackbeta scintillation counter. The counts are reported as counts per minute (cpm).

RESULTS AND DISCUSSION

The AMPHI program predicts a large number of amphipathic segments (Table 1) which are probable Th recognition sites. Thirteen amphipathic segments were scored for JE, WN and DEN-II envelope protein, 11 amphipathic segments for DEN-IV and 9 for DEN-I and DEN-III, respectively. If the presence of tetra/pentapeptide motif in the amphipathic segments

Table 2. Predicted T-helper cell recognition sites on envelope (E) glycoprotein

WN (9–19)	RDFLEGVSGAT
DEN-1 (9–19)	RDFVEGLSGAT
DEN-2 (9–18)	RDFVEGVSGG
DEN-3 (9–19)	RDFVEGLSGAT
DEN-4 (9–19)	RDFVEGVSGGA
DEN-1 (17–27)	GATWVDVVLEH
DEN-3 (17–27)	GATWVDVVLEH
DEN-4 (94–106)	RDVVDRGWGNGCG
WN (256–267)	GALHQALAGAIP
WN (296–317)	TTYGVCSKAFKFARTPADTGHG
DEN-1 (307–316)	KLEKEVAETQ
DEN-2 (306–317)	FKIVKEIAETAQH
DEN-2 (345–354)	RHVLGRLITV
DEN-4 (345–356)	EKVVGRIISSTP
DEN-1 (395–407)	GSSIGKHFEATAR
DEN-3 (393–405)	GSSIGKMFEATAR
DEN-4 (394–402)	GSSIGKHFESTYRGAKRMA
JE (426–457)	DFGSIGGVFNSIGKAVHQVFGGAFRTLFGGMS
WN (423–454)	DFGSVGGVFTSVGKAIHQVFGGAFRSLFGGMS
DEN-1 (421–443)	DFGSIGGVFTSVGKLIHQIFGTA
DEN-2 (421–446)	DFGSLGGVFTSIGKALHQVEGAIYGA
DEN-3 (419–441)	DFGSVGGVLNSLGKMVHQIFGSA
DEN-4 (420–451)	DFGSVGGLFTSLGKAVHQVFGSVYTTMFGGVS

Table 3. *In vitro* antigen specific T cell proliferation with JE, WN, DEN II antigens and peptide I, II, III

Immunized with	Mouse strain	*In vitro* stimulation with JE Ag	Pep. I	Pep. II	Pep. III	WN	DEN-II
JE Ag.	Balb/c	98988 (4.9)	26871 (2.1)	23005 (2.0)	3655 (1.1)	ND	ND
	C3H/HeJ	63644 (3.8)	24750 (2.1)	25621 (2.1)	5497 (1.2)	ND	ND
	C57BL6	23619 (2.1)	54 (1.03)	47 (1.4)	11 (1.09)	ND	ND
PEP. I (SIGKAVHQVF)	Balb/c	716 (4)	261 (2.1)	317 (2.3)	20 (1.06)	— (0.5)	— (0.6)
	C3H/HeJ	4870 (7.4)	804 (2.06)	860 (2.13)	21 (1.02)	— (0.96)	13840 (19.2)
	C57BL6	28 (1.2)	19 (1.1)	(0.9)	19 (1.1)	— (0.66)	— (1.05)
Pep. II (SLGKAVHQVF)	Balb/c	1619 (5.17)	3842 (10.9)	2083 (6.36)	(0.89)	2393 (7.16)	1696 (7.9)
	C3H/HeJ	2323 (5.67)	544 (2.09)	3300 (7.63)	45 (1.09)	25 (1.05)	3326 (7.69)
	C57BL6	70 (1.56)	69 (1.56)	64 (1.52)	58 (1.49)	73 (1.59)	94 (1.7)
Pep. III (RDFVEGBVSGGA)	Balb/c	148 (1.76)	— (0.77)	— (0.9)	1132 (6.8)	— (0.95)	899 (5.6)
	C3H/HeJ	60 (1.34)	— (0.70)	— (0.8)	174 (1.98)	20 (1.1)	— (0.75)
	C57BL6	30 (1.21)	— (0.97)	— (1.04)	2186 (16.5)	— (1.0)	— (0.7)

Results are expressed as net CPM (Experimental – Control), Stimulation indices S.I. (Experimental/Control) in parentheses.
ND—Not done.

is considered as a necessary condition then 5 segments in JE, 6 segments in DEN-III, 7 segments in DEN-I and 8 segments each in WN, DEN-II and DEN-IV satisfy both these conditions (Fig. 1). The third condition viz. Th cell recognition sites are also part of alpha helical segment, if imposed, only 23 segments given in Table 2 satisfy all the three conditions simultaneously. There is thus reduction in the predicted Th epitopes. Application of any one of the above conditions to other protein sequences points out that sites predicted are with approximately 75% confidence limit (Margalit *et al.*, 1987). It may be mentioned here that simultaneous application of these conditions will help to pick up few potential regions which may more likely be Th epitopes. Such an approach has the limitation that some latent Th epitopes will be missed. The predicted Th epitopes can however, be used as first candidate in experimental analysis. Therefore among the predicted Th epitopes, we have picked up the region 420–455, which is likely to be cross-reactive to JE, WN and DEN viruses for our experimental studies. This region is one of the longest stretches and has fairly large amphipathic score, with angular value in range of 85–125. Similar region was also predicted as potential T cell determinant in Tick borne encephalitis virus (Mandl *et al.*, 1989). Lymph node cells from synthetic peptides I and II prepared from this cross-reactive region when immunized in mice gave a T cell proliferative response to JE, WN and DEN-II antigen in Balb/c and/or C3H/HeJ mice (Table 3) However, there was no proliferative response in C57BL6 strain indicating a defect in these peptides binding to H2B haplotype. The other peptide (Pep III) was chosen from *N*-terminal region. It is also known that in flaviviruses *N*-terminal sequences of E-protein are virus specific (Heinz, 1986; Nowak and Wangler, 1987). Peptide III gave a proliferative response to homologous peptide immune lymph node cells in all the three strains of mice analyzed. The stimulation experiments carried out with JE, WN and DEN-II antigens for peptide III immune mice showed that only DEN-II gave a proliferative response to lymph node cells from Balb/c mice. The absence of response to JE, WN and DEN-II in C57BL6 and C3H/HeJ may be explained in terms of antigen processing where this fragment may be cleaved in these strains,

although peptide binding is positive (Table 3). In conclusion, our studies have pointed out that there is a Th epitope on E gp of JE, WN and DEN viruses which can be used in cross-reactive T cell responses.

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