Appendix A

Appendix A

Acronyms

aa	Amino acid
APC	Antigen presenting cell
Boc	Tertiary butyloxycarbonyl
CDC	Center for disease control
CNS	Central Nervous system
CTL	Cytotoxic T lymphocyte
DENV-1	Dengue Virus-1
DENV-2	Dengue Virus-2
DENV-3	Dengue Virus-3
DENV-4	Dengue Virus-4
EBV	Ebstein barr virus
EDC	(1-Ethyl-3-[3-dimethylamino propyl] carbodimiide) hydrochloride
Fmoc	9 Fluorenyl-methyloxycarbonyl
HAT	Hypoxanthine aminopterin thymidine
HIV	Human Immunodeficiency virus
HT	Hypoxanthine thymidine
ic	Intra-cranium
IFN	Interferon
ip	Intra-peritoneum
JE	Japanese encephalitis
JEV	Japanese encephalitis virus
LCMV	Leukocyte choriomeningitis virus
LD	Lethal dose
MAb	Monoclonal Antibody
MHC	Major histocompatibility complex
MVEV	Murray Valley Encephalitis virus
NIV	National Institute of Virology
NK	Natural killer (cell)
PEG	polyethylene glycol
PrM	pre-membrane protein
sc	Sub-cutaneous
TBEV	Tick Borne Encephalitis virus
TFA	Trifloroacetic acid
T_h	T helper (cell)
TNF	Tumor Necrosis Factor
VSV	Vesicular Somatitis virus
WHO	World health organization
WNV	West Nile virus
YFV	Yellow fever virus

Appendix B

Appendix B

PROTOCOLS

Give us the tools and we will finish the job.--- Winston Churchill.

Cell & Tissue Culture Methods

Maintenance Of Porcine Stable (PS) Kidney Cells

Porcine stable kidney cells were maintained using standard protocols used in this Institute. From a fully grown monolayer of cells, medium *i.e.* Earles MEM (*Hi-media*) was aspirated, the monolayer washed with the medium. Pre-warmed TPVG (trypsin) was added to the monolayer at room temperature. The TPVG was then decanted immediately followed by addition of 0.5 ml TPVG and incubation at 37°C for 1-2 minutes. The detached cells were suspended in 1 ml of seeding medium which contained 10 % goat serum (GS) in Earles MEM. The cell inoculum was distributed according to the requirement. Usually for maintenance, split ratio of 1:4 or 1:5 was used. The milk dilution bottles (MD) were seeded with appropriate inoculum and 10 ml of the medium containing goat serum.

Preparation Of JEV Antigen

The procedure followed was as per **Yeolekar & Banerjee (1996)**. Briefly, PS cell culture were infected with the JEV at a multiplicity of infection (m.o.i.) of 0.1. The tissue culture fluid was harvested 48 h post infection after observing the cytopathic effect. Cells and cell debris were removed by centrifugation at 500xg for 20 min. The supernatant was treated with 1 mg/ml protamine sulphate (*Sigma*) with continuous stirring for 20 min, followed by centrifugation at 500 g for 20 min. The virus was concentrated, by pelleting at 150000xg for 3 h in a Sorvall TFT 50.38 rotor. All procedures were carried out at 4 °C. The pellet was resuspended in NTE buffer (0.1 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 8.0) and further purified on a continuous sucrose gradient (10-40%, w/v) in a AH-629 rotor at 100000 x g for 3 h. Two peaks of haemagglutination activity (HA) were observed. Fractions from both the peaks were pooled separately and pelleted by ultracentrifugation (TH641 rotor, *Sorvall Du Pont*, 200000 x g, 4°C, 3 h). Pellets were resuspended in the NTE buffer and soaked in minimum quantity of NTE buffer and kept overnight. The pellet was dispersed and the

protein estimated using standard protocols followed by checker board titration of the minimum quantity of antigen required. This was taken as JEV preparation and used as an antigen in ELISA.

HYBRIDOMA PROCEDURE AND MONOCLONAL ANTIBODY PRODUCTION

The medium (DMEM) and fetal bovine serum required for growing of hybrids were procured from *Gibco BRL* and plasticware from *Nunc, Denmark.*

Mice were immunized with appropriate antigen and sacrificed 3 days after the last dose. Spleenectomy was performed aseptically and the spleen taken in medium (DMEM) with antibiotics in a petridish. Throughout the procedure the medium was without serum except while plating the hybrids. Further procedures were carried out under sterile conditions in the laminar flow hood.

The spleenic tissue was minced finely, teased and single cell suspension prepared. The larger tissue pieces were allowed to settle and the single cell suspension was layered on Histopaque gradient, to get rid of the red blood cells. The cells were centrifuged at 400 g (~ 1500 rpm) for 20 min at room temperature. The mononuclear cells at the interface were washed three times with medium, suspended in medium without serum and viable cells counted. Generally, one immunized spleen yielded around 7-10 x 10^7 cells.

Simultaneously, plasmacytoma cells (SP2/0) in growth phase (between 24-48 hours after transfer), were harvested by jetting the medium on the adherent cells with the pasteur pipette. The floating and adherent cells were collected, washed four times in serum free medium to remove all the traces of serum. The viability of the cells were checked and also the number of cells counted. Usually the viability should be over 80% at the time of fusion.

Before mixing spleen cells and SP2/0 cells, it was made sure that the cells were without serum containing medium. The proportion of spleen cells to plasmacytoma cells was 4:1, though it varies depending on the fusion. The cells were mixed and were centrifuged at low speed followed by the discarding of the medium gently. 0.5 ml of 50% PEG (pre-warmed) was added dropwise to the pellet with constant light shaking (to break up the pellet), over the period of one minute. The pipette used for adding was pre-warmed as the PEG might get solidified in the cold pipette. After 90 seconds, 5.0 ml of medium was added over the period of 5 minutes to dilute out PEG. The tube was left in the upright position for

some time to allow the clumps to settle. To the pellet 40 ml of medium is added slowly and the solution gently pipetted in and out with five ml pipette without force, so that the small clumps and pairs of cells are not broken. The cells were kept at 37°C for 60 min for stabilization. The mixture was distributed in 96-well plate with 0.1 ml per well in which PEG feeders and HAT was already been added 24 hours before the fusion experiment. Generally, the cultures were fed with two changes of HAT for a week followed by two changes of HT. After that the cells were maintained in DMEM containing 10 % serum. Once the wells were 50-70 % full, the supernatants were assayed for the secretors by ELISA. The double positive hybrids were cloned by limited dilution method, positives confirmed and then the clones were cryopreserved. The clones were revived and characterized.

Peptide Synthesis

Peptide synthesis requires chemicals of high purity not only the amino acids but also the solvents. The organic solvents procured were peptide synthesis grade or were distilled.

PURIFICATION OF DIMETHYLFORMAMIDE (DMF)

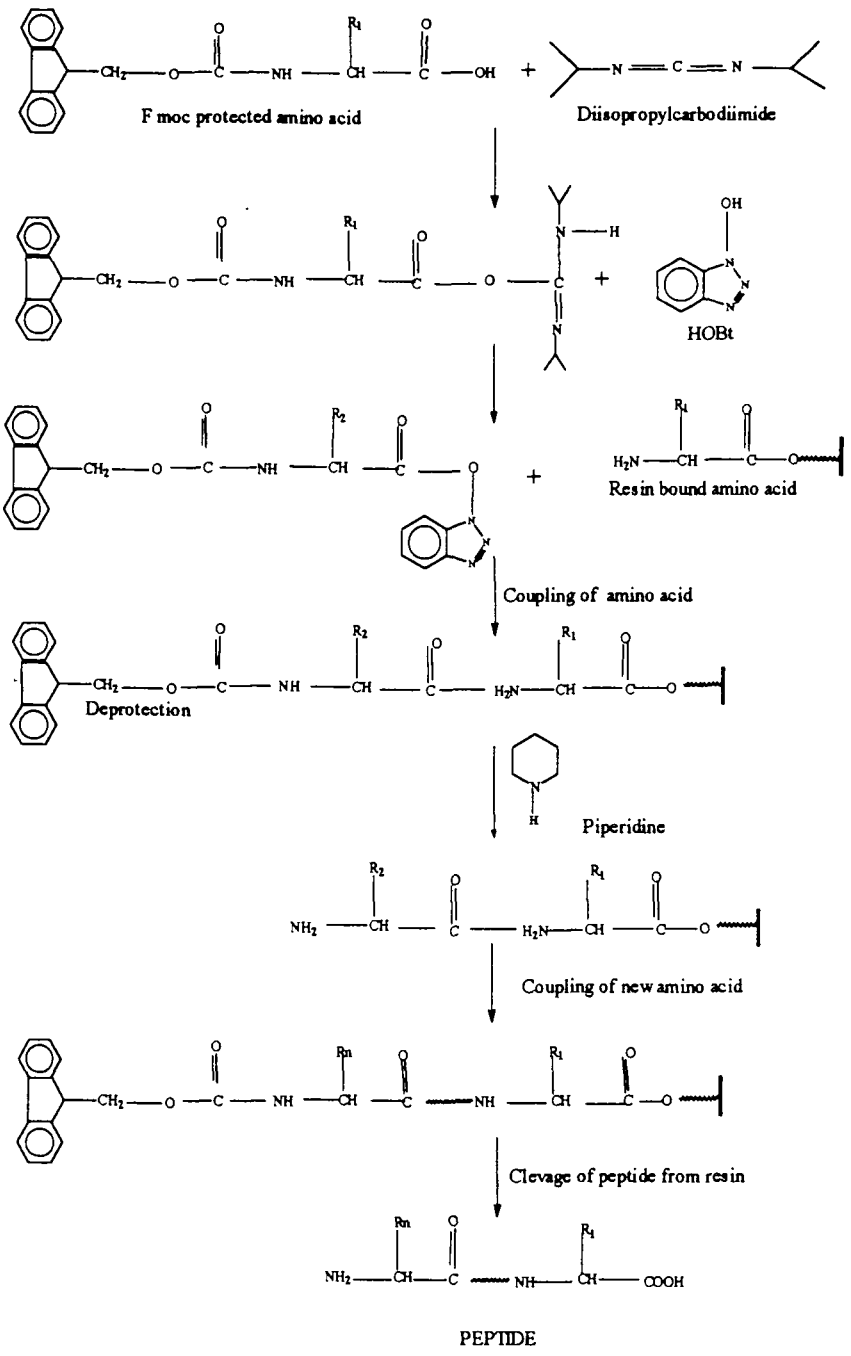
It is necessary to have solvents like DMF free of impurities like formic acid and diethyl amine in order to make it peptide synthesis grade. Hence it was distilled and always used fresh. The commercially available DMF taken as crude, was kept over Potassium hydroxide overnight and distilled over Phosphrous pentoxide in vacuum. Purity of DMF was checked by estimating the Diethyl amine content by using Sanger's reagent, Fluoro dinitro benzene (FDNB). An equal amount of FDNB (1mg ml^{-1} in 95 % ethanol) and distilled DMF was mixed and left for 30 min. The Optical Density (OD) at 381 nm of good DMF should be 0.15 after the blank substraction.

PEPTIDE SYNTHESIS

Fmoc (9-Fluorenylmethoxycarbonyl) Amino acid OPFP esters and other reagents for peptide synthesis were procured from *Sigma, Novabiochem & Bachem, Switzerland.* Except for the amino acids, Trifluoroacetic acid (TFA), carbodiimide, and the catalyst for coupling HOBT (Hydroxybenzotriazole), all other chemicals like DMF, and Diethyl ether were distillated in the laboratory. The general scheme of peptide synthesis is mentioned in

Figure B.1.

Figure B.1 : General Scheme of Peptide synthesis



Attachment of the first amino acid residue using pentafluorophenyl active esters

The resin (*Novasyn PA500* or *p-alkoxybenzylalcohol* resin) 1 g was soaked in 20 ml DMF with intermittent shaking for one to two hrs. The DMF was washed twice with same

volume of DMF. The amino acid (FMOC amino acid opfp ester) and HOBt was dissolved in minimum amount of DMF. The amino acid was four molar equivalents of resin capacity (0.5mmol.). Excess of DMF was removed from the resin and the amino acid solution was added to the wet resin. Dimethyl amino pyridine (DMAP) (0.1mmol.) in DMF 0.5 ml was added to the resin/active ester mixture. The reaction was allowed to occur for one hour with shaking occasionally. The amount of amino acid incorporated was estimated by removing 10 mg of dry resin. 10 mg of resin was filtered after sequential wash with DMF, iso-amyl alcohol, acetic acid, dichloromethane. The degree of attachment was measured spectrophotometrically. In a cuvette 10 mg of dry FMOC-aa-resin was taken. Piperidine 20 %, in DMF (3 ml) was added and mixed properly for 5-10 minutes. After the resin was allowed to settle the solution was read at 290 nm blank being 20% piperdine solution. Using correlation graph an OD of more than 1.485 means 90% attachment. The procedure was repeated till a minimum of 90% attachment was attained.

Chain elongation

After the satisfactory attachment of the first residue the protecting group (FMOC) was removed by five ml of 20% piperdine solution for 20 min with intermittent shaking. The decanted piperidine was monitored for FMOC group at 290 nm for every step. This is deprotection step that exposes the amino group for reaction. Hence, though the peptide is written from N to C termini, the synthesis occurs in the opposite direction.

The resin was washed 5-7 times with DMF so that all the traces of piperidine was removed. After washing, to the soaked resin without excess DMF, Amino acid was added with the catalyst. The amino acid taken was 3 times excess of the molar resin capacity . The coupling solution consisted of amino acid, diisiopropyl carbodiimide ad HOBt all in 4 molar equivalents in minimum DMF. With activated amino acid esters the carbodiimide was not used. The reaction mixture was added to the resin and kept with intermittent shaking for 60 minutes. The coupling was monitored by Kaiser's test mentioned in the next section. If the coupling efficiency was more than 95% only then the reaction was stopped. Otherwise the resin was washed and this step repeated. If the coupling was satisfactory the resin was washed with plenty of DMF and deprotected as per mentioned in earlier protocols and next amino acid added.

All these steps were repeated till the last amino acid was added.

Kaiser's test for detecting amino groups

Few beads of the resin were washed as per the procedure earlier mentioned. To this few drops of phenol-ethanol (7:3) solution, 0.2mM KCN in pyridine and 0.28 M of ninhydrin in ethanol was added. It was then heated in a water bath for few minutes. Blue colored resin or the solution indicated the presence of free amino groups. The blank was the solution without the beads. This technique may be quantified by the procedure by **Sarin *et al*, (1981)**.

Cleavage and isolation of peptide from the resin

The final amino acid was deprotected and resin dried as per earlier mentioned. 50 mg of dried resin was taken in a clean glass flask. To it 5 ml of 95 % TFA (*Sigma*) and scavagers if required were added. The flask was stopped and occasionally stirred. The reaction time depended on the sequence, like Arg (mtr) group containing peptide required more than 6 hrs of cleavage time. Otherwise 1-1.5 hrs was the standard cleavage time. The resin TFA mixture was filtered by sintered funnel under reduced pressure. The resin was washed with 2 ml to 3 ml of TFA. Nitrogen was bubbled through the TFA till the volume became minimum. Another alternative was to evaporate it to minimum value in rotary evaporator. To the concentrated peptide, super dry ether (stored over sodium) was added 5-10 ml. It immediately formed a precipitate, this was ether extraction. The ether was carefully centrifuged at low speed and precipitate was again ether extracted. This was done 2-3 times. The excess ether was removed and precipitation was air-dried.

This is the peptide in crude form. The crude peptide was stored in minimum or appropriate amount of distilled water or 1% acetic acid and lyophilized in glass ampoules.

The ampoules were sealed under nitrogen and stored at subzero temperature.

Estimation of the peptides using the Tyr residue modification by tetranitromethane.

Since the peptides synthesized had only single Tyr residue, it was used to indirectly estimate the amount of peptide. The principle was to modify the Tyr residue with Tetranitro methane (TNM). Number of Tyr residues modified was estimated spectrophotometrically. The molar absorption coefficient for nitrotyrosine at 428 nm is 4200 M^{-1} cm^{-1}. The calculated number of tyrosine modified was used to estimate the amount of peptide concentration.

Fixed volume (0.4 ml) of Tris buffer (0.05 M, pH 8.5) was taken in the cuvette and a fixed volume of peptide dissolved in water was added. After mixing, TNM solution (1:10 diluted in 95 % Ethanol) was added. After 45 min the O.D. of the resultant solution was checked at 428 nm and noted. TNM was again added and procedure repeated. Saturation point was calculated by subtracting the blank reading in each case and plotted on a graph. Only buffer with TNM was taken as blank. The saturation O.D was taken for calculation. The concentration of peptide was indirectly calculated by estimating the nitration of Tyrosine residue according to Beer - Lambert's law. The values were plotted to obtain a saturation graph.

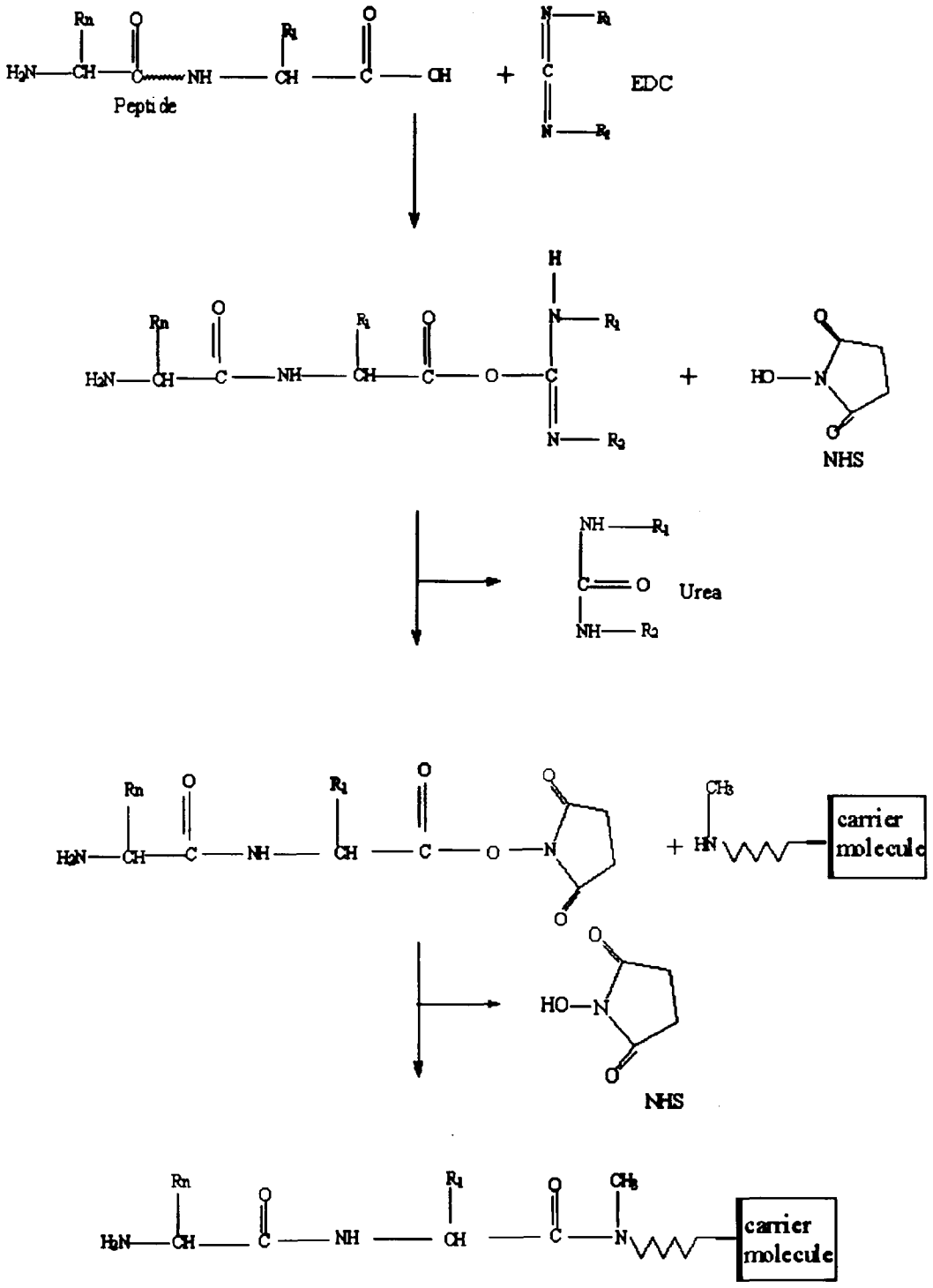
PURIFICATION OF PEPTIDES

Peptides were initially synthesized on a small scale. Once the synthesis was satisfactory the procedure was scaled up to the experimental requirement. The ether precipitated peptides were purified on HPLC. The peptide was first loaded on a semi-preparative C-18 column (*Deltapack, Waters*). This peak was concentrated and analyzed using a C-18 Reverse Phase HPLC analytical column (*Bondapack, Waters*). The mobile phase was a linear gradient of Acetonitrile and 0.1 % Tri-Fluoro Acetic acid (TFA) in water. The purity was checked by assessing the single peak elution of the peptide under a gradient elution of Acetonitrile in Water containing 0.1%TFA. The gradient conditions are shown with the chromatogram.

CONJUGATION OF PEPTIDE TO CARRIER

For immunization purposes the peptide was covalently conjugated to Ovalbumin as a carrier molecule. The covalent linkage of the peptide to the carrier was done using EDC. The general scheme for conjugation is mentioned in *Figure B.2*. For conjugation it is always preferable to have a site directed approach wherein only one of the residues is involved in conjugation. In case of B cell epitopes, it would be advantageous to have the conjugation either through C terminal of N terminal end of the peptide. For this purpose, it is imperative to block all other possible reactive sites in the peptide. Citraconylation is one of the ways by which all the primary amino groups (NH_2) are blocked. The only reactive groups left for the coupling are COOH groups that is present in the COOH terminal of the peptide. The advantage with this (citraconylation) procedure is that blocking is (pH) reversible.

Figure B.2 : Linkage of peptide to carrier molecule



Peptide coupled to carrier molecule

The peptide was dissolved in HEPES buffer (50 mM pH 8.5) at a concentration of 10 mg ml^{-1} and was kept for stirring. While stirring, equal volume of Citraconic anhydride (*Sigma*) solution (in water 10 mg ml^{-1}) was slowly added. During the reaction pH was maintained between 8-9 (monitored, if necessary). The reaction mixture was incubated for one hour at room temperature. The peptide was diluted to a concentration of 1 mg ml^{-1}. EDC (3-dimethylaminopropyl - 3 - ethyl carbodiimide) was added to it at a final concentration of 10 mg ml^{-1}, the pH was kept at 8.0. After a incubation at room temperature for 5 min, equal volume of Ovalbumin was added at a molar ratio of 1:20 (Carrier:Peptide). The reaction was incubated at room temperature for four hrs and was terminated by Sodium acetate pH 4.2 to a final concentration of 100 mM. After incubation for one hour, solution was dialyzed against Sodium acetate pH 4.2 and then against 12 liters of PBS with four changes over 12 hours. The peptide protein conjugate was lyophilized and stored below 0 °C.

IMMUNOLOGICAL METHODS

Peptide Immunization

For immunization purposes, 40 μg of peptide-protein conjugate was injected by sub cutaneous route per mouse (set of four adult SWISS/Albino) per dose for all doses. The first dose was emulsified with complete Freund's adjuvant (1:1). Subsequent doses (weekly schedule for 3 weeks) were given through the same route and similar amount in Incomplete Freund's adjuvant. Controls were carrier protein without peptide and PBS emulsified with adjuvant. The mice were bled through retro-orbital vein for checking the antibody titer on 7, 14 and 21 days post immunization. The sera were tested for their reactivity against purified JEV antigen using ELISA.

ELISA

Wells in 96 well plates were coated with 1 μg per well of purified JEV antigen. Free binding sites were blocked with 1% w/v BSA in PBS for 60 min at 37° C. Diluted serum samples (usually 1:100 diluted unless specified) in 1% BSA in PBS were added to the wells and incubated at 37°C for 60 min. Goat anti-mouse HRP conjugate (*Sigma*) was added as per the manufacturer's direction diluted in PBS Tween-20 (0.1%, v/v). The conjugate was incubated for 60 min. Normal mouse serum or Sp2/0 peritoneal fluid and JEV immune peritoneal fluid were used as negative and positive controls respectively. Wells were washed

three times after each step with PBS containing 0.1% v/v Tween 20. Substrate (OPD) was added and the reaction was stopped after 15 min with 4 N H_2SO_4. Absorbance was read at 492 nm.

Peptide ELISA

Covalink procedure followed was a modified protocol by **Sondergard-Andersen *et al*, (1990)** and the users manual of *Nunc Immuno modules, Covalink, NUNC.*

In 10 µl of distilled water the peptide was taken (1 µg per well). To it 10 µl of 0.1 M N- hydroxysuccinimide (NHS) was added. They were kept at room temperature for 30 min. The solution was diluted in carbonate buffer in appropriate volume so that 50 µl could be coated per well on the Cova-link immuno-modules. The reaction was initiated by addition of 50 µl of EDC. The plate was incubated for two hrs at room temperature. The plate was washed by plenty of PBS. The wells were blocked with 1 % BSA in PBS and consequently the procedure for the usual ELISA followed.

Immunofluorescence Assay

PS cells were cultured on coverslips in Leighton tubes to nearly 90 % confluency. After 24 hours the cells were infected with 0.5-1 m.o.i (multiplicity of infection) of JEV. After 30 hrs post infection (P.I.) the cells were fixed in chilled acetone and kept at -20°C for 20 min. After briefly washing in PBS (Phosphate Buffered Saline) pH 7.2, the antibodies were added. The anti peptide sera and appropriate control sera were diluted 1:100 in PBS, and 50-60 µl were added to the fixed cells and incubated for 60 min at 37°C. After incubation the coverslips were washed 3-4 times in PBS and 50 µl of anti mouse IgG-FITC (Fluorocein isothiocynate) conjugate was added at a dilution as per recommendation of the manufacturer (*Sigma*). Coverslips with conjugate were incubated for 60 min and then washed 7-8 times thoroughly to remove any non specifically bound conjugate. They were further counterstained with Evan's blue. The coverslips were mounted in a Glycerol-PBS mountant and viewed under Fluorescence microscope (Olympus) which had the following filters, 'Excitation filter O455, Barrier Filter Y515'. Apple green fluorescence indicated a positive reaction while red color indicated a negative reaction.

Plaque reduction test

Plaque reduction test or *in vitro* neutralization tests were carried out in PS cells. These tests were performed in 24 well plate (*Nunc*) in which 2×10^6 cells/well of PS cells were first allowed to form a uniform monolayer in the 1 ml of MEM containing 10 % GS. The sera were treated at 56°C for 30 min to inactivate the complement. To the100 µl virus suspension containing appropriate virus particles, 100 µl of serum sample (appropriately diluted) was added and incubated at 37°C for 60 min. To the preformed monolayer, 100 µl of the incubated virus antibody mixture was added. The remaining 100 µl was added to 96 well plate containing preformed monolayer of the same cells for neutralization test by CPE method. The virus suspension contained about 50-100 plaque forming units (PFU). The virus was allowed to adsorb on the monolayer for 60 min at 37°C and the unadsorbed virus was removed. The cells were overlayed with 0.5 % carboxymethyl cellulose in Agar overlay medium (2x MEM) containing 2 % GS. After 72-96 hrs, the overlay medium was removed, washed with PBS and stained with 0.1 % Amido black in fixative. The staining was done by keeping the plates at room temperature for 120 min and washed with water. Plaques were then counted.

In vivo Neutralization Studies

Neutralization studies were carried out *in vivo* using infant mice. The sera were inactivated at 56°C for 30 min and diluted appropriately in BAPS (0.75 %). Virus used for the test was approximately 2 Log LD_{50}. The virus and sera in equal amounts were incubated at 37°C for 60 min. Antibody-virus mixture was inoculated in 2-3 day old mice *i.p* route, 30 µl per mouse. One litter was used as a group that contained eight infants (2-3 day old). The mice were observed for illness and death till day 21 post inoculation.

Immunization with Chimeric peptides and Challenge with JE virus (Bankura strain 733913)

All mice received 50 µg of peptide per mouse per dose, inoculated through sub-cutaneous (sc) route. Appropriate controls like PBS and commercial JE vaccine procurred from CRI Kasauli were also inoculated. The vaccine inoculated was diluted 1:5 in Earles MEM and inoculated 0.1 ml i.p. The mice were boosted on day 14 and 28 post first dose. Pre-immunization sera was collected 3 days prior to the first dose. Sera were collected on

day 10, 21 and 35 post immunization. Mice were challenged with live virus on day 38. The procedure used was as per **Yeolekar & Banerjee 1996**. Briefly, 0.1 ml of live virus isolated from brain tissue of infant mice was inoculated ip followed by 1% starch intra-cranially (ic) after 3 hrs of virus inoculation. The animals were monitored for 21 days post-challenge.

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