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TITLE

Chimeric T helper–B cell peptide vaccine for Japanese encephalitis virus.

FIELD OF INVENTION

This invention relates to chimeric T helper–B cell peptide as a vaccine for Japanese encephalitis virus.

BACKGROUND OF INVENTION

Amongst insect borne viral diseases, Japanese encephalitis and dengue have a notoriety of encompassing entire region of South East Asia. The envelope glycoprotein contains at least five determinants that seem to be correlated with the important biological properties of hemagglutination and neutralization. Envelope glycoprotein is responsible for the attachment of the virus and thus is associated with infectivity. The existing vaccine for Japanese encephalitis is purified, killed virus vaccine prepared from infant mouse brain that consists of mainly envelope glycoprotein of the virus. Three injections of mouse brain derived killed purified vaccine followed by a booster after 12 to 18 months, can give effective immunization as judged by induction of neutralizing antibodies. Mouse brain vaccine shows neutralizing antibodies against Indian strain also. The efficacy of the vaccine has been noted only if three doses are administered.

U.S. Patent 5,824,506 Chan, et al. October 20, 1998 discloses Peptide antigens derived from the dengue virus type-2 glycoprotein NS1 are provided. The peptide antigens are specifically immunoreactive with sera from individuals infected with the dengue virus. The antigens are useful as diagnostic tools in determining whether an individual has been or is infected with dengue virus, and for discriminating between infection with dengue virus and infection with related flaviviruses. The antigens are also useful in vaccine compositions for immunizing individuals against infection with the dengue virus.

U.S. Patent no.5,494,671 Lai, et al. Discloses C-terminally truncated flavivirus envelope proteins 80-81% in size which are more immunogenic than their counterpart full-length proteins. The aforesaid patent further discloses recombinant viruses that encode the truncated protein and to host cells infected therewith. Host cells express the truncated protein on their outer membrane and secrete it into the medium. The patent discloses vaccines for use against flavivirus infection. The vaccines include either a recombinant vaccinia virus expressing the truncated envelope protein, and the truncated envelope protein produced by a recombinant baculovirus.

In China, attenuated Japanese encephalitis virus vaccine

consisting of Japanese encephalitis virus strain SA 14-14-2 is being used. The attenuation has been carried out in hamster kidney cells and have unknown passage histories. Mice inoculated intracerebrally with the SA 14-14-2 vaccine strains survived without showing any signs of CNS involvement. The virus titers in brains persisted at low levels for several days and could not be detected after 10 days (Hase T, Dubois, Dr. Summers, PL, Downs, MB and Ussery, MA. (1993) Comparison of replication rates and pathogenicities between the SA14 parent and SA14-14-2 vaccine strains of Japanese encephalitis virus in mouse brain neurons. Arch Virol 130 131-43. Dubois T., Dr. Summers, PL. Downs, MB and Ussery, MA (1993) Comparison of replication rates and pathogenicities between the SA14 parent and SA 14-14-2 vaccine strains of Japanese encephalitis virus in mouse brain neurons. Arch Virol 130 131-43). The safety and immunogenicity of this vaccine has been tested. 1,026 children between the ages of 5 and 12 years, were vaccinated with live-attenuated Japanese encephalitis virus vaccine. None of the group of 47 of the vaccinated children, has temperature >37.4 °C. Seroconversion rates in seronegative children were 100% (GMT 35.3) (Xin YY, Ming, ZG, Peng, GY, Jain, A and Min, LH (1988) Safety of a live-attenuated Japanese encephalitis virus vaccine (SA14-

14-2) for children. Am J Trop Med Hyg 39 214-7).

Many attempts are being carried out to develop recombinant vaccine for Japanese encephalitis virus. These include expression of various proteins and then immunizing animals with the products. It was realized very early that expression and immunization with envelope glycoprotein alone was not very useful (Mason PW, McAda, PC, Dalrymple, JM, Fournier, MJ and Mason, TL (1987) Expression of Japanese encephalitis virus antigens in Escherichia coli. Virology 158 361-72). Mice immunized with recombinant baculovirus infected cells containing envelope glycoprotein and NS-1 genes were challenged with Japanese encephalitis virus. Survival was increased from about 30% in unimmunized mice to 70% in envelope glycoprotein and polyprotein recipients but not in NS-1 receipients (McCown J, Cochran, M, Putnak, R, Feighny, R, Burrous, J, Henchal, E and Hoke, C (1990). Protection of mice against lethal Japanese encephalitis with a recombinant baculovirus vaccine. Am J Trop Med Hyg 42 491-9). Immunization of mice with purified extracellular subviral particles composed of prM and E proteins in recombinant vaccinia viruses could protect mice from 4.9 x 10^5 LD50 of Japanese encephalitis virus. (Konishi E, Pincus, S, Paoletti, E, Shope, RE, Burrage, T and Mason, PW. (1992) Mice immunized with a subviral particle containing the

Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection. Virology 188 714-20). These particulate antigens were also shown to induce Japanese encephalitis virus specific CTL response in mice. (Konishi E, Win, KS, Kurane, I, Mason, PW, Shope, RE and Ennis, FA (1997) Particulate vaccine candidate for Japanese encephalitis induces long-lasting virus-specific memory T lymphocytes in mice. Vaccine 15 281-6).

Vaccinia recombinants that co-expressed the genes for premembrane and envelope glycoprotein elicited high levels of neutralizing and HI antibodies in mice and protected mice from a lethal challange by Japanese encephalitis virus. Recombinants expressing only the gene for NS1 induced antibodies to NS1 but provided low levels of protection from a similar challange dose of Japanese encephalitis virus. Immunization of mice with vaccinia recombinant viruses containing PrM gene along with NS-1 and envelope glycoprotein protected them from challenge with Japanese encephalitis virus. Pox virus (Canary pox and viccinia) based Japanese encephalitis recombinant vaccines have been constructed and shown to produce Japanese encephalitis virus specific CTLs in mice. (Konishi, E, Kurane, I, Mason, PW, Shope, RE and Ennis, FA (1997) Poxvirus-based Japanese encephalitis vaccine candidates induce JE virus specific

CD8+ cytotoxic T lymphocytes in mice. Virology 227 353). Poxvirus-based recombinant JE vaccine candidates, NYVAC-JEV and ALVAC-JEV, encoding the Japanese encephalitis virus prM, E and NS1 proteins were examined for their ability to induce Japanese encephalitis virus-specific CTLs. The volunteers received subcutaneous inoculations with each of these candidates on days 0 and 28. Anti-E and anti-NS1 antibodies were elicited in most vaccinees inoculated with NYVAC-JE virus and in some vaccinees inoculated with ALVAC-JEV, PBMCs obtained from approximately one half of vaccinees showed positive proliferation in response to stimulation with live Japanese encephalitis virus. Presence of the Japanese encephalitis virus-specific CD8+CD4- cytotoxic T cells in vitro-stimulated peripheral blood mononuclear cells obtained from two NYVAC-JEV and two ALVAC-JEV vaccinees was demonstrated. (Konishi E, Kurane, I, Mason, PM, Shope, RE, Kanesa-Thasan, N, Smucny, JJ, Hoke, CH Jr and Ennis, FA (1998). Induction of Japanese encephalitis virus-specific cytotoxic T lymphocytes in humans by poxvirus-based JE vaccine candidates. Vaccine 16 842-9). A chimeric Yellow fever (YF)/JE virus (ChimeriVax-JE virus) was constructed by insertion of the prM and envelope glycoprotein genes of an attenuated human vaccine strain (SA14-14-2) of Japanese encephalitis virus between C and NS genes of a YF 17D

infectious clone. Mice inoculated subcutaneously with one dose of >/=10^3 pfu of ChimeriVax-JE virus were protected against IP challenge with a virulent Japanese encephalitis virus.

In recent years, it has been shown that fragments of proteins in the form of synthetic peptides can be used to induce T helper and antibody responses. Attempts to delineate B cell epitopes from Japanese encephalitis virus have resulted in delineation of Met 303 to Trp 396 as the shortest sequence capable of reacting with 10 MAbs. Disulfide bond between cys 304 and 335 was required for presentation of the binding site(s) for these MAbs. However, it was not an effective immunogen for inducing neutralizing or protective antibodies in mice (Mason PW, Dalrymple, JM, Gentry, MK, McCown, JM, Hoke, CH, Burke, DS, Fournier, MJ and Mason, TL (1989) Molecular characterization of a neutralizing domain of the Japanese encephalitis virus structural glycoprotein. J Gen Virol 70 2037-49). The fragment carrying the coding sequence of amino acid 373-399 of envelope glycoprotein elicited the highest neutralizing antibody titer (1:75). HI antibodies were not induced by this fusion protein (Seif SA, Morita, K and Igarashi, A (1996) A 27 amino acid coding region of JE virus E protein expressed in E. coli as fusion protein with glutathione-S-

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transferase elicit neutralizing antibody in mice. Virus Res 43 91-6). Neutralizing antibody inducing epitopes have been detected on C terminal regions of envelope glycoprotein (Seif SA, Morita, K, Matsuo, S, Hasebe, F and Igarashi, A (1995) Finer mapping of neutralizing epitope(s) on the C-terminal of Japanese encephalitis virus E-protein expressed in recombinant Escherichia coli system. Vaccine 13 1515-21 and Jan LR, Yang, CS, Henchal, LS, Sumiyoshi, H, Summers, PL, Dubois DR and Lai, CJ (1993) Increased immunogenicity and protective efficacy in outbred and inbred mice by strategic carboxyl-terminal truncation of Japanese encephalitis virus envelope glycoprotein. Am J Trop Med Hyg 48 412-23). Peptides from C protein have also been delineated for reactivity with sera from Japanese encephalitis and dengue patients. Pep91-105 and 8-22 belonged to group-specific epitopes that reacted with both Japanese encephalitis and dengue-1 patient sera. Pep 1-15 and 34-48 belonged to subcomplex-specific epitopes that reacted only with Japanese encephalitis but not with dengue-1 patient sera. (Huang JH, Wey, JJ, Lee, HF, Tsou, TL, Wu, CS, Wu, JR, Chen, HM, Chin, C, Chien, LJ, Chen, LK, Wu YC, Pan, MJ and Wang, TM (1996) Identification of immunodominant, group-specific and subcomplex-specific, continuous epitopes in the core regions of Japanese

encephalitis virus using synthetic peptides. Virus Res 41 43-53).

DISADVANTAGES OF THE PRIOR ART

In case of mouse brain derived killed purified vaccine, three doses of the injectable vaccine are to be administered. In a trial with vaccine made in Japan was carried out in South Arcot district of Tamil Nadu, India. Of a total of 113 school children, 72% showed antibody response while the responders increased to 87.8% after booster dose of Biken Japanese encephalitis vaccine after one year. Only about 20 per cent of the children had persisting antibodies one year after the primary vaccination. (Mohan Rao CVR, Risbud, AR, Dandawate, CN, Umarani, UB, Ayachit, VM, Rodrigues, FM and Pavri, KM (1993) Serological response to Japanese encephalitis vaccine in a group of school children in South Arcot district of Tamil Nadu Indian J Med Res 97 53-59). The problems of strain variation and the protection offered by the inactivated vaccine based on Nakayama have always been noted.

In case of attenuated Japanese encephalitis vaccine used in China, although efficacy of the vaccine has been proven in many studies by now, there are some problems associated with the licensing of this vaccine all over the world. The passage history and the laboratory practices, which were

used in generation of this vaccine, have not been known completely. Thus, attempts to re-invent the attenuated strain from the same SA14-14-2 strain have been carried out. In a Japanese study antibodies against both envelope glycoprotein and NS 1 were observed in mice infected with the attenuated Japanese encephalitis virus strain SA(A) derived from the live Japanese encephalitis vaccine strain SA14-14-2. (Lee T, Komiya, T, Watanabe, K, Aizawa, C and Hashimoto, H (1995) Immune response in mice infected with the attenuated Japanese encephalitis vaccine strain SA14-14-2. Acta Virol 39 161-4).

The problems associated with vaccine for Japanese encephalitis are, e.g. discrepancy in the age at which Japanese encephalitis vaccine should be administered. The cost of currently available vaccine is very high. The additional cost of administering three doses will also have to be taken into consideration. It is not known whether the effect of Japanese encephalitis vaccine will be long lasting, in absence of exposure to Japanese encephalitis after the third dose. Whether yearly boosters are required or not, until natural immunity due to natural Japanese encephalitis infection is not known. Thirdly, immune response to Japanese encephalitis virus is very low (Pavri KM (1984)) Problems of JE immunization in India. Proc. Of

National Conference on Japanese encephalitis. Ind.J. Med. Res Suppl pp 81-84). In addition, the question of immunity to the local strains by Nakayama, or Beijing strains of virus used in vaccine will have to be taken into consideration. As the vaccine is mouse brain derived there are allergic reactions to the vaccine and the frequencies of allergic mucocutaneous reactions varied from 1-17 per 10,000 vaccinees during 1983-1995 (Plesner AM and Ronne, T (1997) Allergic mucocutaneous' reactions to Japanese encephalitis vaccine. Vaccine 15 1239-43).

13. How there drawbacks have been overcome by your invention. In recent years, it has been shown that fragments of proteins in the form of synthetic peptides can be used to induce T helper and antibody responses. (Ref) Thus, neutralizing antibody-inducing epitopes from envelope glycoprotein have been delineated. As these peptide sequences are not sufficient for inducing protective immunity, a chimeric peptide has been prepared incorporating T helper epitope along with the virus neutralizing antibody inducing B cell epitope. This will ensure that both T helper and B cell immunity is generated for the protection from Japanese encephalitis virus challenge. The said chimeric vaccine is designed and thus more than one chimeric peptide can be added to formulate the effective vaccine as per

requirement in the future.

OBJECTS OF THE INVENTION

It is an object of the present invention to propose safe and effective vaccines against flaviviruses for humans and animals. Various other objects and advantages of the present will become apparent from the ensuing description.

DESCRIPTION OF THE INVENTION

According to this invention there is provided a vaccine composition for humans and animals against or Japanese encephalitis virus infection, comprising a chimeric synthetic peptide, said chimeric peptide selected from envelope glycoprotein, consisting of amino acids Egp 149-SENHGHYSAQVGASQ-163 and Egp 427-GSIGGVEFNSIGKAVHQVFG-445 of Japanese encephalitis virus envelope glycoprotein wherein chimeric peptide in an amount sufficient to induce protective immunity against Japanese encephalitis virus infection.

In one embodiment, the present invention relates to vaccine composition for humans and animals against or Japanese encephalitis virus infection, comprising a chimeric synthetic peptide. Chimeric peptide was selected from envelope protein, consists of amino accids 149-SENGHYSAQVGASQAAKF-167 AND 427-GSIGGVFNSIGKAVHQVFG-445.

In another embodiment, the present invention also relates to

the vaccine composition wherein said peptide 149-SENHGHYSAQVGASQAAKF-167 ... induces neutralizing antibodies against Japanese encephalitis virus.

In a further embodiment, it also relates to a vaccine composition for humans and animals against Japanese Encephalitis Virus infection comprising a neutralizing antibody inducing peptide sequences from envelope glycoprotein of Japanese encephalitis virus. The said sequences are amino acids 46-PTLDVRMINIEA-55, 266-EYSSSVKLTSG-272.

In another embodiment, the present invention also relates to peptide sequences from Japanese encephalitis virus envelope glycoprotein, Capsid protein, Membrane protein, Non Structural protein-1 and Non Structural protein-3 capable of stimulating T helper cells from immunized animals.

In another embodiment, the present invention also relates to a combination of peptides mentioned above resulting in chimeric T helper B cell peptides capable of inducing protective immunity against Japanese encephalitis virus infection.

Either B or T lymphocytes through their receptors may define epitopes as the regions of part of proteins that are recognized. Based on the cell involved epitopes may be classified as B cell, T helper cell or CTL epitopes that

stimulate B cells, T helper (CD4+) and CTLs respectively. MHC molecules present T cell epitopes to the TCR present on the T cells. B cell epitopes in contrast to the T cell epitopes have dependence on the three dimensional structure. B cell epitopes can be predicted using several different methods B cell determinants of envelope glycoprotein of Bakura (India) strain (733913) were identified. Briefly, antigenic propensity values are assigned to each of the twenty amino acid residues based on their frequency of occurrence in experimentally confirmed B cell antigenic determinants. These parameters along with appropriate cutoff values were used in a computer program developed. Table 1 shows the predicted B cell determinants of envelope glycoprotein of Japanese encephalitis virus.

Table 1. Predicted B cell epitopes from envelope glycoprotein of Japanese encephalitis virus.

Kolaskar & Tongaokar's method	Kutubuddin et al 1993
18-TWVDLVLEGDSCLTIM-33	
40-TLDVRMI-46	
48-IEAVQLAEVRSYCYHASVTDISTVARCP-75	75-CPTTGEAHNEKRADSSYV-92
88-SSYVCKQG-95	
113-IDTCAKFSCTSK-124	
155-YSAQVGASQAAKFTVTPNAPSITLKL-180	147-TTTSENHGNYS-157
155-YSAQVGASQAAKFTVTPNAPSITLKLGD-182	
185-EVTLDCE-191	
199-EAFYVHTV-206	
208-SKSFLVHRE-216	226-WTPPSSTAWRNR-237
262-LHQALAG-268	243-FEEAHATKQ-251
273-EYSSSVKLTSGHLKCRLK-290	
292-DKLALKG-298	309-FAFAKNPADTG-319
320-GTVVIELSYS-329	328-SYSGSDGP-335
351-VGRLVTVN-358	
367-NSKVLVEME-375	363-ATSSANS-369
379-GDSYIVVGR-387	

The Table 1 depicts amino acid sequences predicted from

envelope glycoprotein of Japanese encephalitis virus. Amino acid sequence derived is from Japanese encephalitis virus strain Bankura (733913).

In order to arrive at the unique sequences that might be useful in vaccine development amino acid sequences from other related flaviviruses viz., WNV, MVEV, DENV and YFV were downloaded from protein data banks and multiple alignments were carried out using CLUSTAL program. Similarly in order to understand amino acid sequences from a few Japanese encephalitis virus strains were also subjected to multiple alignment.

The multiple alignments were carried out using CLUSTAL program and the outputs are shown in Figure-1 and Figure-2.

As seen in the figure 1, the regions in the envelope glycoprotein which were non-homologous with other flaviviruses were:

1. 149-SENHGNYSAQVGASQ-163
2. 40-TLDVRMINIEA-50
3. 270-IVVEYSSSVKLTS-282

These sequences were conserved within different Japanese encephalitis virus strains (Figure 2). This means that regions 40-50, 155-163 and 270-290 are unique to Japanese encephalitis virus. Antibodies induced against these peptides from envelope glycoprotein would thus have better

chances of being neutralizing antibody. It should be noted here that there are no major differences between the amino acid sequence of Japanese encephalitis virus Nakayama strain and Bankura strain.

SYNTHESIS OF PEPTIDES

These peptides were synthesized by the standard solid phase synthesis protocols. The synthesis was carried out using FMOC(9-Fluorenylmethoxycarbonyl) amino acid pentafluoro-phenyl (O PFP) esters on Novasyn PA 500 resin (Novabiochem Ltd. UK) that yielded peptide acids on cleavage. Completion of the coupling of amino acid was strictly monitored to avoid short substituted peptides by qualitative monitoring of the coupling reaction color test for detection of free terminal amino groups in the solid-phase for detection of free amino groups. Coupring also was checked at 290 nm by monitoring the FMOC group released during the deprotection step by 20% piperidine solution in dimethyl formamide. Peptides were cleaved from the resin by trifluoroacetic acid (TFA) with appropriate scavengers dictated by the sequence. The peptides were precipitated in super dry diethyl ether. The ether precipitated peptides were purified on HPLC and peaks collected. The purity was checked by assessing the single peak elution of the peptide under a linear gradient elution of 80% acetonitrile in water containing 0.1% TFA on

reverse phase 18 column. Purified peptides were lyophilized and stored at -20.

Any peptide with a purity >85% was used for conjugation purposes. Alternatively, peptide in the region of 270-290 were synthesized on Pin head peptide synthesis modules using Fmoc amino acids using manufacture's protocols. Side chains of these peptides were also cleaved by Trifluroacetic acid cleavage keeping peptides attached to the pins.

Reactivity of peptides with monoclonal antibodies against JE virus

A panel of anti Japanese encephalitis virus monoclonal antibodies that have different immunological properties Epitope mapping of Japanese encephalitis virus envelope protein using monoclonal antibodies against an Indian strain developed at NIV. These MAbs were used to probe the anti-gemcity of the peptides. The purpose of this study was to determine the epitope recognized by the MAbs and also find the optimum peptide length and sequence as an epitope. This would also elucidate the structural requirement if any, for antibody binding. The MAbs included Hs-1, Hs-2, Hs-3 that are Japanese encephalitis virus specific, Hemagglutination inhibition (HI) positive Epitope mapping of Japanese encephalitis virus envelope protein using monoconal antibodies against an Indian strain, J. Gen Virol 69 2714-7).

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Peptides (1ug/well) were passively coated on ELISA wells (Immulon II, Nunc) suing Na CO_3 /HCO_3 byffer pH 9.6. Blocking was carried out by 1% BSA in PBS. Reaction of with peptide and washing were carried out in 1% BSA in PBS (0.01 M phosphate 0.15 M NaCL, pH 7.2). Monoclonal antibody was diluted either 1:50 or 1:100 for reaction. Hundred micro liters of antibody diluted in 1% BSA in PBS, was added and incubated for 60 min at 37 °C. After washing with PBS containing 0.1% Tween 20, bound antibodies (Sigma, USA). In case of ELISA on Pin heads after blocking pins with peptides were dipped into wells containing antibody. The color development was carried out using H_2O_2 and O-phenyl diamine (OPD) as the chromogen. The OD values were monitored at 492nm Epitope mapping of Japanese encephalitis virus envelope protein using monoclonal antibodies against an Indian strain. SP2/0 AF was used as negative control. Data in Table 2 clearly indicates that peptides 39-PTLDVRMINI-48 and 269-AIVVEYSSSVKLT-281 react with MAb Hs-1 and peptide 151-NHGNYSAQVGASQAAKF-167 reacts with MAb Hs-2 and MAb Hs-3.

ELISA with JE virus

ELISA wells (Nunc Immulon II) were coated with purified JE antigen (10g/well) overnight, in sodium carbonate buffer (0.05 M, pH 9.8). Subsequently, wells were blocked with 1% bovine serum albumin (BSA) in PBS (0.01 M phosphate 0.15 M

NaCl, pH 7.2). Hundred micro liters of antibody diluted in 1% ovalbumin in PBS, was added and incubated for 60 min at 37 °C. After washing with PBS containing 0.1% Tween 20, bound antibody was probed with goat-anti mouse Ig-horseradish peroxidase conjugated antibodies (Sigma, USA). The color development was carried out using H_2O_2 and O-phenyl diamine (OPD) as the chromogen. The OD values were monitored at 492 nm (Cecilia et. al., 1988). Antibody response against JE virus was tested for anti-peptide antibody sera. In all these experiments, sera from PBS control mice and ovalbumin immunized mice were used as negative controls. Polyclonal immune AF was used as the positive control and peritoneal AF from the nonimmune mice was used as negative control. In experiments with MAbs, AF obtained from SP2/0 inoculated mice were used as negative control.

Table 2

Reactivity of Peptides with monoclonal Antibodies against JE virus

PEPTIDE	Hs-1	Hs-2	Hs-3
33-IMANDKPT-40	0.110	0.016	0.017
35-ANDKPTLD-42	0.022	0.016	0.052
37-DKPTLDVR-44	0.029	0.016	0.035
39-PTLDVRMI-46	0.313	0.006	0.025
40-TLDVRMIN-47	0.329	0.008	0.064
41-LDVRMINI-48	0.277	0.007	0.082
42-DVRMINIE-49	0.155	0.008	0.043
43-VRMINIEA-50	0.338	0.012	0.133
45-MINIEASQ-52	0.177	0.001	0.000
47-NIEASQLA-54	0.07	0.003	0.081
155-YSAQVGASQ-163	0.118	0.492	0.334
151-NHGNYSAQVGASQ-163	0.074	0.294	0.258
149-SENHGNYSAQVGASQ-163	0.093	0.415	0.377
155-YSAQVGASQAAKF-167	0.106	0.501	0.377
151-NHGNYSAQVGASQAAKF-167	0.084	0.561	0.259
149-SENHGNYSAQVGASQAAKF-167	0.095	0.412	0.400
269-AIVVEYSS-276	0.696	0.041	0.115
270-IVVEYSSS-277	0.637	0.030	0.112
271-VVEYSSSV-278	0.659	0.029	0.090
272-VEYSSSVK-279	0.290	0.040	0.106
273-EYSSSVKL-280	0.538	0.045	0.110
274-YSSSVKLT-281	0.616	0.054	0.153
275-SSSVKLTS-276	0.765	0.053	0.136
276-SSVKLTSG-277	0.447	0.061	0.123

Conjugation of peptide with carier immunization of mice

Peptides 40-TLDVRMINIEASQ-52 AND 149-SENHGNYSAQVGASQA-163 were synthesized, purified, and conjugated to the carrier molecule. The carrier molecule used in these experiments was Ovalbumin (OA). 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 solution (in water 10 mg ml^{-1}) was slowly added. During the reaction pH was maintained between 8-9. 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-dimethyl-aminopropyl - 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 ^{o}C.

For immunization, 40 µg of peptide-protein conjugate emulsified in Freund's complete adjuvant (1:1) was injected

by sub cutaneous route in mice. A group of four mice was used per peptide-protein carrier conjugate. Two boosters containing the similar amount of peptide-carrier conjugate were administered at weekly intervals in Freund's incomplete adjuvant. Control groups received carrier protein without peptide and PBS, emulsified with adjuvant. The mice were bled from the retro-orbital plexus, on 7th, 14th and 21st days post immunization for checking the antibody titer. Sera were separated and stored at -20° C until used. Anti Japanese encephalitis virus immune response was checked by ELISA where the anti-peptide sera was allowed to react with the native Japanese encephalitis virus virion by the method mentioned above. Figure 3 shows the reactivity at different dilutions. In ELISA

In order to fine tune this region in terms of antibody eliciting and antibody binding capacity, set of overlapping peptides were synthesized that were used to probe the same. Following overlapping peptides were synthesized

155-163	YSAQVGASQ
151-163	NHGNYSAQVGASQ
149-163	SENHGNYSAQVGASQ
155-167	YSAQVGASQAAKF
151-167	NHGNYSAQVGASQAAKF

All these peptides were conjugated to ovalbumin as essential

According to protocol mentioned above. Four BALB/c mice each were immunized with 40 ug per mouse per dose of these peptide OA conjugates along with Freund's adjuvant. The mice were administered booster doses at weekly intervals and bled through the retro-orbital route at weekly intervals. The anti Japanese encephalitis virus response was monitored by ELISA by the method mentioned above. Values mentioned are mean optical density readings obtained.

Table 3:

Reactivity of Anti peptide sera with Japanese encephalitis virus by ELISA

Mice immunized with adjuvant + conjugated with peptide	Sera collected on post inoculation days		
	7	14	21
155YSAQVGASQ163	0.212+0.076	0.236+0.028	0.195+0.047
151NHGNYSAQVGASQ163	0.286+0.153	0.270+0.62	0.246+0.090
149SENHGNYSAQVGASQ163	0.242+0.157	0.268+0.096	0.268+0.057
155YSAQVGASQAAKF167	0.223+0.037	0.262+0.067	0.198+0.0178
151NHGNYSAQVGASQAAKF167	0.226+0.060	0.220+0.033	0.144+0.021
149SENHGNYSAQVGASQAAKF167	0.191+0.058	0.255+0.050	0.217+0.029
Ovalbumin	0.127+0.022	0.116+0.072	0.153+0.003
PBS	0.113+0.034	0.124+0.029	0.183+0.029

The Table 3 shows the reactivity of the antipeptide antibodies against Japanese encephalitis virus by ELISA. Anti peptide antibody response reactive to Japanese encephalitis virus could be observed in all peptide immunized mice by 7th day P1, i.e after the first dose. Antisera against all peptides showed reactivity with native Japanese encephalitis virus in ELISA confirming the correctness of the prediction method.

Delineation of T helper epitopes from structural and nonstructural proteins of Japanese encephalitis virus

The importance of T helper cells in the immune response is well known. In course of development of B cells and maturation of immune response. B cells require certain localized cognate signals and cell-cell contact originating from T cells. Using combination of methods to predict amphipathic, tetramer and pentamer motifs, along with prediction of helix preferers and sequences which would have I-A and I-E binding motifs were predicted for all proteins of JE virus. Prediction of Th epitopes was carried out using EPIPLOT which included, amphipathic helix segments predicted by AMPHI program developed by Margalit et al (Margalit H, Sponge J.L., Cornette J.L., Cease K.B., DeLisi C. and Berzofsky J.A. (1987) Prediction of immunodominant helper T cell antigenic sites from the primary sequence. J.

Immunology 138, 2213-2229). For prediction of amphipathic segments Fauchere and Pliska hydrophobicity scales of amino acids were used, block length of seven was selected. Fauchere J.L. and Pliska V., (1983) Hydrophobic parameters II of amino acid side chains from the partitioning of N-acetyl amino acid Eur. J. Med. Chem. 18, 369-375. Tetramer and pentamer motif [charged residues or glycine followed by 2-3 hydrophobic residues and then a polar residue] by Rothbard and Taylor (Rothbard J. and Taylor W.R. (1988) A common sequence pattern in T cell epitopes. EMBO J.7, 93-100). Prediction of sequence motifs of immunodominant secondary structures capable of binding to MHC [Ia/Ie] with high affinity was carried out by method of[Sette et al. (Sette A., Buus S., colon S., Miles C. and Grey H.M. (1989) Structural analysis of peptides capable of binding to more than one Ia antigen. J. Immunol 142, 35-40). Multiple alignment of flaviviuses by using ALIGN program to choose flavivirus cross-reactive Th epitopes. T helper peptides with high scores in each of these prediction programs were chosen. Cross-reactive epitopes were chosen on the basis of homology amongst flavviviruses. After sequence alignments and homology analysis between various flaviviruses 14 peptide sequences were chosen.

These peptides were synthesized on Chiron Pin-head modules

using cleavable option as per the manufacturer's methodology. Peptides were cleaved by Trifluroacetic acid method as mentioned above. BALB/c mice were primed with 10^{-2} 0.1ml, dilution of mouse brain live Japanese encephalitis virus, West Nile virus intra-peritonealy, Primed mice were inoculated sub-cutaneously with 0.1 ml of Japanese encephalitis virus, West Nile virus inactivated mouse brain antigen [10 ug] and Normal antigen in equal volume of Complete Freund's adjuvant. Booster dose of above antigens were given similarly in Incomplete Freund's Adjuvant four days before harvesting. T helper cell proliferation assay was performed using spleenocytes from immunized and unimmunized mice. Spleenocytes were added to 96-well flat bottom plates at a concentration of $2 * 10^{5}$ cells/well/0.1ml. Cultures were stimulated with 0.1 ml of JEV antigen [5,2.5 & 1 ug/ml] and peptide [10,5 & 2.5 ug/ml] After 6 days, cultures were pulsed with 1uCi [^{3}H] Thymidine for 18h. Cells were harvested and counted for radioactivity. Values are depicted as Net cpm Net cpm=cpm in virus immune lymphocytes-cpm in non-immune lymphocytes. As shown in the Figure and Table peptides especially from the nonstructural proteins of JE virus have shown better stimulation.

Fig 4 shows the stimulations of Japanese encephalitis virus immune spleenocytes with T helper peptides.

Table 4 delineation of T helper peptides from JE virus

	Protein	Sequence	Response to	
			JE	WN
Peptide 1	WN Egp	135-IKYEVAIFVHG-145	++	+
Peptide 2	JE Egp	260-GALHQALAGAI-270	-	-
Peptide 3	JE Egp	140-GIFVHGTTTSE-150	+	+
Peptide 4	JE Egp	346-HVLGRLTTVN-355	+++	-
Peptide 5	JE M	17-EAWLDSTKAT-26	++++	-
Peptide 6	JE PrM	36-WVRAIDVG-43	++	-
Peptide 7	JE NS1	37-ETPRSLAKIVHKAH-50	+	-
Peptide 8	JE NS1	297-SVRTTTDSGKLITD-310	++++	+
Peptide 9	JE NS1	156-EDFGFGITSTRV-167	++++	-
Peptide 10	JE NS1	404-TDLARYVVL-412	+++	++
Peptide 11	JE NS3	202-KILPQIIKDAIQ-212	+	-
Peptide 12	JE NS3	17-DTTTGVYRIMARG-29	++	++
Peptide 13	JE NS3	534-FLELLRTAD-543	+	-
Peptide 14	JE Egp	439-SIGGVFNSIGKAVHQ-455	+++	+

Based on the results shown in Figure and Table following peptides have been chosen as candidate T helper cell peptides to be incorporated in Chimeric T helper B cell vaccine.

JE Egp 346-HVLGRLTTVN-355

JE M 17-EAWLDSTKAT-26

JE NS1 297-SVRTTTDSGKLITD-310

JE NS1 156-EDFGFGITSTRV-167

JE NS1 404-TDLARYVVL-412

JE Egp 439-SIGGVFNSIGKAVHQ-455

Preparation of Chimeric T helper and B cell peptides immunization and protection studies

T cell epitopes were derived from envelope glycoprotein as their potential to stimulate T cells was earlier checked in our laboratory (Kutubuddin, M, Kolaskar, AS, Galande, S, Gore MM, Ghosh. SN, Banerjee, K. (1991) Recognition of helper T cell epitopes in envelope (E) glycoprotein of Japanese encephalitis West Nile and Dengue virus. Mol. Immunol 28 149-54). It was thus decided to make a synthetic peptide which has both T helper and B cell epitopes in tandem. The peptide that was co-linearly synthesized was:

NH2-SENHGNYSAQVGASQGSIGGVFNSIGKAVHQVFG-COOH

This peptide had two components viz.,

B cell epitope 149-SENHGNYSAQVGASQ-163

T cell epitope 429-SIGGVFNSIGKAVHQVFG-445

The peptide was synthesised as a single peptide co-linearly using solid phase synthesis using FMOC amino acids as mentioned above.

The purified peptide collected as a single peak was lyophilized and used for immunizations. The immunization was

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done in different strains of adult mice that were both inbred and outbred (n=8). The inbred strains were BALB/C ($H\text{-}2^d$) and C3H ($H\text{-}2^k$) while the outbred strain was SWISS/Albino. The antigens used for immunization were, the chimeric peptide, B cell peptide, commercial Japanese encephalitis virus vaccine (Obtained from Central Research Institute, Kasauli, India) and PBS (sham). The immunizations were done with a variation in schedule. The immunization schedule was a dose of 50 ug of peptide per mouse per dose through sc route, administered alongwith Freund's adjuvant every 14 days. For the first dose Freund's complete adjuvant while for the subsequent doses incomplete adjuvant was used. A total of two booster doses were given. All the mice were bled through the retro-orbital route on days -3 (preimmune sera), 10, 22, 34 days post first dose. The mice were challenged with a lethal dose of live virus on day 35, which was a week after the last dose. The mice were scored for 21 days post challenge for sickness and death. The immune response was also characterized using usual methods like ELISA, Immunofluorosence assays and virological assays like neutralization and challenge experiments.

Anti Japanese encephalitis virus response was checked by ELISA. As depicted in the Figure 5 a,b,c, it can be seen that the peptides are capable of eliciting immune response

against JE virus especially if the chimeric peptide is the immunogen. In terms of immune response, best response was seen in mice immunized with Japanese encephalitis virus vaccine followed by Chimeric peptide and B cell peptide in that order.

In vitro virus neutralization by anti chimeric peptide sera. Neutralization experiments were performed by plaque reduction assay. Antichimeric peptide sera were diluted 1:50 in culture medium. Fifty microliters of the diluted serum and equal volume of virus pool containing 48 plaque forming units were mixed and incubated at 37 C for one hour. Virus antibody mixture was added on to the preformed Porcine stable kidney cells in 24 well plates. After one hour adsorption caboxymethyl cellulose overlay was added and plates were incubted for 3 days. Percentage plaque inhibition was calculated using virus plaque numbers without antibody. The Figure 6 shows the efficiency of the anti-peptide sera to neutralize the virus. As seen from the figures only antibodies raised against the chimeric peptides in C3H mice are capable of neutralizing Japanese encephalitis virus.

Protection of mice immunized with chimeric peptide from lethal challenge of Japanese encephalitis virus

Since the peptides are potential vaccine candidate, the mice

immunized were assayed for their ability to resist the virus challange. Lethal dose of Japanese encephalitis virus was administered according to the protocol by (Yeolekar & Banerjee 1996 Yeolekar, LR and Banerjee, K. (1996) Immunogenicity of immunostimulating complexes of Japanese encephalitis virus in experimental animals. Acta Virological 40 245-250). Chimeirc peptide immune mice were inoculated intraperitoneally with 0.1 ml Japanese encephalitis virus suspension, immediately followed by inoculation of 0.03ml of 1% starch solution by intracerebral route. The mice were observed for 21 days post challenge. According to the survival curves in Figure 7 it can be seen that the C3H mice that were immunized with chimeric peptides were able to resist the virus challenge. The other strains of mice (BALB/c or SWISS) were not able to neutralize the virus nor were they able to resist the virus challange

Fig 7 shows servival of chimeric peptide KK immune mice from Japanese encephalitis virus challange with a challenge virus dose of 2.3 log LD 50.

The data thus supplied in this document here clearly indicates that it is possible to protect mice from lethal challange of Japanese encephalitis virus. The data also has been supplied to indicate that in addition to the peptide epitope 149-SENHGNYSAQVGASQAAKF-167 B cell epitopes 39-

PTLDVRMINI-48 and 269-AIVVEYSSSVKLT-281 can also induce neutralizing antibodies to Japanese encephalitis virus and thus are unique. Data has also been presented to indicate that peptides JE Egp 346-HVLGRLTTVN-355, JE M 17-EAWLDSTKAT-26, JE NS1 297-SVRTTTDSGKLITD-310, JE NS1 156-EDFGFGITSTRV-167 JE NS1 404-TDLARYVVL-412, JE Egp 439-SIGGVFNSIGKAVHQ-455 can be used as T helper cell peptides. As chimeric T helper B cell peptide has conclusively been shown to protect mice from lethal challenge it is claimed that any any all the combinations of B cell and T helper peptides would be useful in developing vaccine against Japanese encephalitis virus.

WE CLAIM:

1. A vaccine composition for humans and animals against Japanese encephalitis virus infection, comprising a chimeric synthetic peptide, said chimeric peptide consisting of: amino acids Egp 149-SENHGNYSAQVGASQ-163 and Egp 429-SIGGVFNSIGKAVHQVF-445 from Japanese encephalitis virus envelope glycoprotein, wherein the chimeric peptide is in an amount sufficient to induce protective immunity against Japanese encephalitis virus infection.

2. The vaccine composition according to claim 1, further comprising peptide Egp 149-SENHGNYSAQVGASQAAKF-167, wherein the peptides Egp 149-SENHGNYSAQVGASQ-163 and Egp 149-SENHGNYSAQVG QAAKF-167 induce neutralizing antibodies against Japanese encephalitis virus.

3. A vaccine composition as claimed in claim 1, further comprising neutralizing antibody-inducing peptide sequences from envelope glycoprotein of Japanese encephalitis virus, said sequences being amino acids 39-PTLDVRMINIEA-50, and/or 273-EYSSSVKLTSG-283.

4. A vaccine composition as claimed in claim 1 further comprising peptide sequences from Japanese encephalitis virus that are capable of stimulating T helper cells from immunized animals, said sequences being envelope glycoprotein sequence, Egp 346-HVLGRLTTVN-355, membrane protein sequence, M 17-EAWLDSTKAT-26, and/or nonstructural protein-1 sequences, NS-1 297-SVRTTTDSGKLITD-310, NS-1 156-EDFGFGITSTRV-167, or NS-1 404-TDLARYVVL-412.

5. A vaccine composition as claimed in claim 1, further comprising one or more of the peptides Egp 149-SENHGNYSAQVGASQAAKF-167, Egp 39PTLDVRMINIEA-50, Egp 273-EYSSSVKLTSG-283, Egp 346-HVLGRLTTVN-355, M 17FAWLDSTKAT-26 (from membrane protein), NS-1 297-SVRTTTDSGKLITD-310, NS-1 156-EDFGFGITSTRV-l67, or NS-1 404-TDLARYVVL-412 (from nonstructural protein-1), wherein a combination of said peptides results in T helper-B cell chimeric peptides capable of inducing protective immunity against Japanese encephalitis virus infection.