

Conserved amino acids 193–324 of non-structural protein 3 are a dominant source of peptide determinants for CD4⁺ and CD8⁺ T cells in a healthy Japanese encephalitis virus-endemic cohort

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Our earlier identification of the non-structural protein 3 (NS3) of Japanese encephalitis virus (JEV) as a dominant CD4⁺ as well as CD8⁺ T cell-eliciting antigen in a healthy JEV-endemic cohort with a wide HLA distribution implied the presence of several epitopes dispersed over the length of the protein. Use of various truncated versions of NS3 in lymphocyte stimulation and interferon (IFN)- γ secretion assays revealed that amino acids (aa) 193–324 of NS3 were comparable with, if not superior to, the full-length protein in evoking Th1 responses. The potential of this 14.4 kDa stretch to stimulate IFN- γ production from both subtypes of T cells in a manner qualitatively and quantitatively similar to the 68 kDa parent protein suggested the presence within it of both class I and II epitopes and demonstrated that the entire immunogenicity of NS3 was focused on aa 193–324. Interestingly, this segment contained five of the eight helicase motifs of NS3. Analysis of variability of the NS3 protein sequence across 16 JEV isolates revealed complete identity of aa 219–318, which is contained within the above segment, suggesting that NS3-specific epitopes tend to cluster in relatively conserved regions that harbour functionally critical domains of the protein.

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INTRODUCTION

The genus *Flavivirus* belonging to the family *Flaviviridae* includes many arthropod-transmitted viral pathogens including the dengue viruses, Murray Valley encephalitis virus, Japanese encephalitis virus (JEV), tick-borne encephalitis virus complex, yellow fever virus and West Nile virus. Among the medically important flaviviruses, JEV, which causes acute encephalitis in humans, has the highest mortality rate and is one of the chief threats to public health in several parts of Asia (Tsai *et al.*, 1999). While the disease incidence has been reduced to low levels in developed countries such as Japan, Korea and Taiwan, principally due to routine childhood immunization, JEV remains the most important cause of acute epidemic viral encephalitis worldwide and continues to expand its geographic domain to previously unaffected areas like Indonesia and continental Australia (Mackenzie *et al.*, 2001, 2002). One of the major problems with the above-mentioned internationally licensed mouse brain-derived inactivated JEV vaccine is the lack of long-term immunity necessitating repeated booster doses for effective reduction in disease rates (Ku *et al.*, 1994). The success of the live attenuated yellow fever virus vaccine, which has an excellent record of safety and effectiveness with life-long

immunity (memory) achieved by administration of a single dose (Monath, 1999), is probably attributable to the generation of T cell help required for effective induction of quick and enhanced magnitudes of antiviral B and T cell responses. Identification of T cell epitopes that are immunogenic in viral infections could thus be of immense value in designing vaccine vectors that can supersede the use, and thereby the inherent risks involved, of live attenuated viruses, which are currently the ideal vaccine candidates by virtue of their close resemblance to natural virus infections.

There have been indications from previous reports that human anti-JEV-specific T cells produced during natural infection target predominantly the viral non-structural proteins (produced only during live virus infections) and not the structural proteins (the major constituent of the killed JEV vaccine) (Konishi *et al.*, 1995; Desai *et al.*, 1995; Kumar *et al.*, 2003b). The cell-mediated immune parameters that contribute towards protection against JEV can be investigated in individuals who have experienced subclinical infections of JEV and in whom, therefore, development of immune responses prevented virus invasion into the central nervous system and consequently fulminant encephalitis. To broaden our understanding of the T cell responses in JEV

infections and their potential role in resistance and recovery, we had carried out a screen in healthy individuals from JE-endemic areas of South India for T cell target antigens wherein the non-structural protein 3 (NS3) of JEV scored as a highly immunogenic antigen with nearly 86% of the cohort displaying Th1 responses to this protein. Analysis of the responding T cell frequencies revealed activation of mainly CD4⁺ T cells ascribable to the antigen preparation used, i.e. fixed lysates of recombinant baculovirus-infected Sf21 cells expressing the NS3 protein (Kumar *et al.*, 2003b). A comparison of the antigenicity of these proteins of JEV provided in the form of fusions to the 11 amino acid (aa) human immunodeficiency virus (HIV) TAT protein transduction domain (PTD; YGRKKRRQRRR), to ensure the intracellular delivery of protein upon exogenous addition of antigen via transduction across cell membranes, also revealed

a dominant recognition of NS3 in 96% of a cohort of children with a previous incidence of JEV subclinical infections (Kumar *et al.*, 2004a). In the present study, we used truncated versions of the NS3 protein to show that in most of the JEV-exposed children who formed our cohort, almost the entire NS3-specific T cell response (inclusive of CD4⁺ and CD8⁺) localized to an immunodominant region between aa 193 and 324.

METHODS

Cells and viruses. The JEV P20778 strain (National Centre for Cell Science, Pune, India) was propagated in the *Aedes albopictus* cell line C6/36 at 28 °C and in Vero cells at 37 °C in minimal essential medium supplemented with 10% fetal bovine serum.

Construction of recombinant proteins fused to HIV TAT.

Purified viral genomic RNA extracted from JEV-infected C6/36 cells was reverse-transcribed and the 1857 nt long NS3 gene (4608–6464 nt in the JEV genome) PCR amplified using the Expand RT system (Roche Diagnostics) with the following primer pairs (start and stop codons in bold, restriction sites underlined): NS3 sense, 5'-GGATACTCGAGCATATGGGGGGCGTGTTTTGGGACAC-3' (*XhoI*); NS3 antisense, 5'-CGCGGAATCCTTATCTCTTCCTGCTGCAAGTCTTT-3' (*EcoRI*). The PCR-amplified NS3 gene was digested with *XhoI* and *EcoRI* and cloned into the corresponding sites of the bacterial expression vector pTAT-HA (a kind gift from Steven F. Dowdy, Howard Hughes Medical Institute, St Louis, MO, USA) in frame with the N-terminal six-histidine leader, 11 aa PTD of HIV TAT protein and a haemagglutinin tag provided by the vector (Becker-Hapak *et al.*, 2001) to give pTAT-NS3. Various N- or C-terminally deleted versions and internal fragments of JEV NS3 generated with all forward primers containing a *XhoI* site and a start ATG codon and reverse primers with an *EcoRI* site and a stop codon were identically cloned. Similarly expressed and purified unrelated green fluorescent protein (GFP) was the control antigen. Recombinant TAT fusion proteins expressed in *E. coli* BL21 (DE3) were purified by electroelution from SDS-polyacrylamide gels, precipitated and solubilized in PBS. All proteins were tested for purity by N-terminal sequencing of the electroeluted protein using Edman

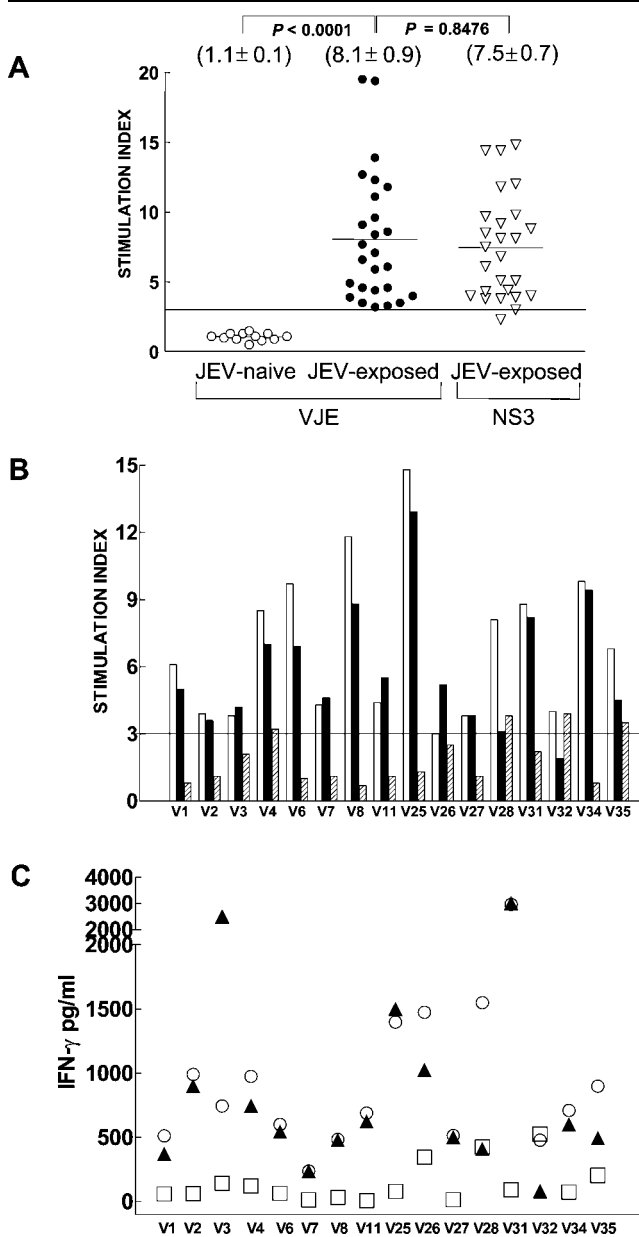


Fig. 1. (A) Virus-specific lymphocyte responses in healthy JEV-immune donors. Proliferation of PBMC was measured in response to fixed lysates of JEV-infected Vero cells or NS3 protein after a 5 day incubation by incorporation of [³H]thymidine during the last 18 h of culture. SI values are ratios of mean c.p.m. in the presence of VJE or NS3 to that of VUI or GFP, respectively, in triplicate wells. The line denotes the cut-off of 3.0 for a positive response. The mean SI value observed in each group (horizontal bars and values in parentheses) is shown. (B) Comparative analysis of lymphoproliferative responses in 16 JEV-exposed donors to the full-length protein (filled bars), and the first 324 (NS3₁₋₃₂₄, open bars) and last 296 (NS3₃₂₄₋₆₁₉, hatched bars) aa of NS3. SI values were ratios of mean counts incorporated by PBMC in the presence of test antigen and GFP in triplicate wells. The line denotes the positive cut-off of 3.0 for SI. (C) Comparative analysis of secreted IFN- γ levels in 3 day cultures of PBMC stimulated with 0.7 mM full-length protein (○), NS3₁₋₃₂₄ (▲) and NS3₃₂₄₋₆₁₉ (□). IFN- γ levels were obtained by subtracting values for GFP-stimulated PBMC from test values as means of triplicate wells.

chemistry, endotoxin absence using the Pyrogen plus Gel-clot LAL test kit (BioWhittaker) and finally their ability to transduce into lymphocytes before use in assays.

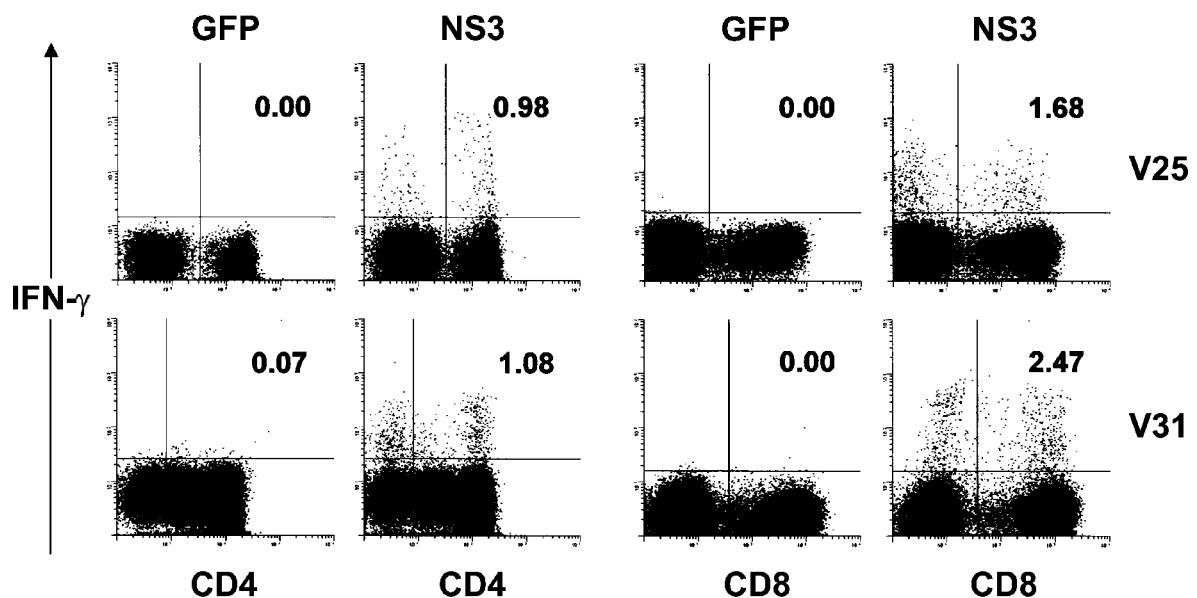
Human peripheral blood mononuclear cells (PBMC). Peripheral blood specimens were obtained from children belonging to the JE-endemic regions of the states of Karnataka and Andhra Pradesh between August 2002 and January 2003 at the district hospital, Vijayanagar Institute of Medical Sciences, Bellary, Karnataka, India. Twenty-six healthy non-vaccinated JEV-seropositive children (15 males, 11 females, 5–16 years old, mean age 9.6 ± 0.6 years) with no history of clinical encephalitis, but with $>1 \log_{10}$ serum plaque reduction neutralization test (PRNT) antibody titres to JEV (Rao-Bhau *et al.*, 1988; Ting *et al.*, 2001), as well as serum antibodies to non-structural proteins by radioimmunoprecipitation (Kumar *et al.*, 2003b), constituted the test group having experienced previous subclinical JEV infections. Serum PRNT-ELISAs to dengue and West Nile viruses were confirmed to be negative ($<0.4 \log_{10}$). Volunteers V1–V8, V10, V11, V15, V16, V18, V21 and V22 were also included in our previous study cohort (Kumar *et al.*, 2004a) and have been designated the same numbers. Volunteers V25–V35 were recruited only for this study. The control group also formed part of our previous study (Kumar *et al.*, 2004a). This group included 12 healthy non-vaccinated children with no history of clinical encephalitis, with a similar age and sex distribution (7 males, 5 females; 6–11 years old, mean age 8.1 ± 0.4 years) and $<0.2 \log_{10}$ serum-PRNT titres to all three flaviviruses. Measles, tuberculosis, hepatitis and HIV/AIDS were all ruled out in the study population. Blood was drawn following informed consent of the guardians of the children in the study

after explaining the purpose and consequences of the investigation. All the procedures and protocols were conducted in conformity with the ethical guidelines of the Indian Council of Medical Research.

Proliferative responses of PBMC. Proliferation assays were performed as previously described (Kumar *et al.*, 2003b). PBMC (1.5×10^5) were cultured with total JEV proteins (VJE) at a concentration equivalent to 16 ng E protein per well (Aihara *et al.*, 1998; Kumar *et al.*, 2003b) or purified recombinant TAT peptide fusion proteins, i.e. NS3 at the optimal concentration of $50 \mu\text{g ml}^{-1}$ (0.7 mM) or truncated proteins at molar concentrations equivalent to that of full-length NS3. Uninfected Vero cell lysates (VUI) and GFP also provided as a TAT peptide fusion were the control antigens. Proliferative response was expressed as stimulation index (SI), the ratio of the mean c.p.m. incorporated by PBMC in triplicate wells in the presence of test and control antigens. We scored a positive response to viral antigens based on the criteria that: (i) the $\text{SI} \geq 3.0$ since the highest SI value in control individuals in response to the test antigen plus 1.96 times the SD of the mean was <2.5 ; and (ii) the mean c.p.m. obtained on stimulation with viral antigen was ≥ 500 . Recovery of and counts incorporated by PBMC from blood of donors of both groups was similar.

Interferon (IFN)- γ ELISA. IFN- γ in 3 day culture supernatants was assayed using commercial capture ELISA kits (Endogen). The lower limit of detection was $15 \text{ pg cytokine ml}^{-1}$.

Flow cytometry. Antigenic stimulation of 0.5 ml whole blood was carried out as previously described (Kumar *et al.*, 2003a) with $50 \mu\text{g}$ of recombinant NS3 (at the optimal concentration of 1.4 mM) or



V25: A*2409, B*5205, B*6215, C3, C4

V31: A1, A*3219, B*5717, B*4921, C6, C7

Fig. 2. Phenotypic analysis of T cell subsets contributing to NS3-specific IFN- γ production in JEV-exposed donors V25 and V31. Whole blood stimulated with 1.4 mM NS3 or GFP provided as TAT fusions for 6 h was processed for flow cytometry. After sequentially gating on lymphocytes and CD3, cells were analysed for CD8/CD4 and IFN- γ . Numbers indicate frequencies as percentages of IFN- γ -positive CD8 $^+$ or CD4 $^+$ T cells after subtracting background frequencies with GFP. No positive staining was observed with matched isotype control antibodies.

equivalent concentrations of GFP and NS3_{193–324}, all provided as fusions to the TAT peptide, for 6 h at 37 °C with 3 μM monensin included during the last 4 h. Intracellular IFN-γ was detected using an antibody cocktail made up of anti-CD3-FITC, anti-CD4/CD8-phycoerythrin and anti-IFN-γ-biotin followed by Streptavidin-Cy-Chrome (BD Biosciences Pharmingen). Data were acquired on a Becton Dickinson FACScan flow cytometer. For each analysis, a total of 50 000 CD3⁺CD4⁺/CD3⁺CD8⁺ T cell subsets were acquired and data were analysed using Winlist software (Verity Software House Inc., Topsham, ME, USA). Positive staining was determined after comparing data with that obtained using isotype-matched controls and by comparing dot plots of test and control antigen-stimulated cultures.

HLA typing. PBMC of JEV-exposed donors were typed for HLA by microlymphocytotoxicity using HLA typing trays purchased from Biotest.

Measurement of protein variability. NS3 protein sequences of 37 independently isolated flaviviruses including 11 dengue, 16 JEV, one Kunjin, one Murray Valley encephalitis, six West Nile and two yellow fever virus isolates (GenBank accession nos P14340, A42551, GNWVD3, GNWVDF, GNWV26, GNWVJA, P27914, AAK29447, AAG30730, AAK67712, AAA42964, U15763, M55506, U47032, M18370, AF080251, L48961, AAL77444, AF014160, AF014161, AF069076, AAD16275, AAD16276, AAD16277, AF045551, AF075723, GNWVKV,

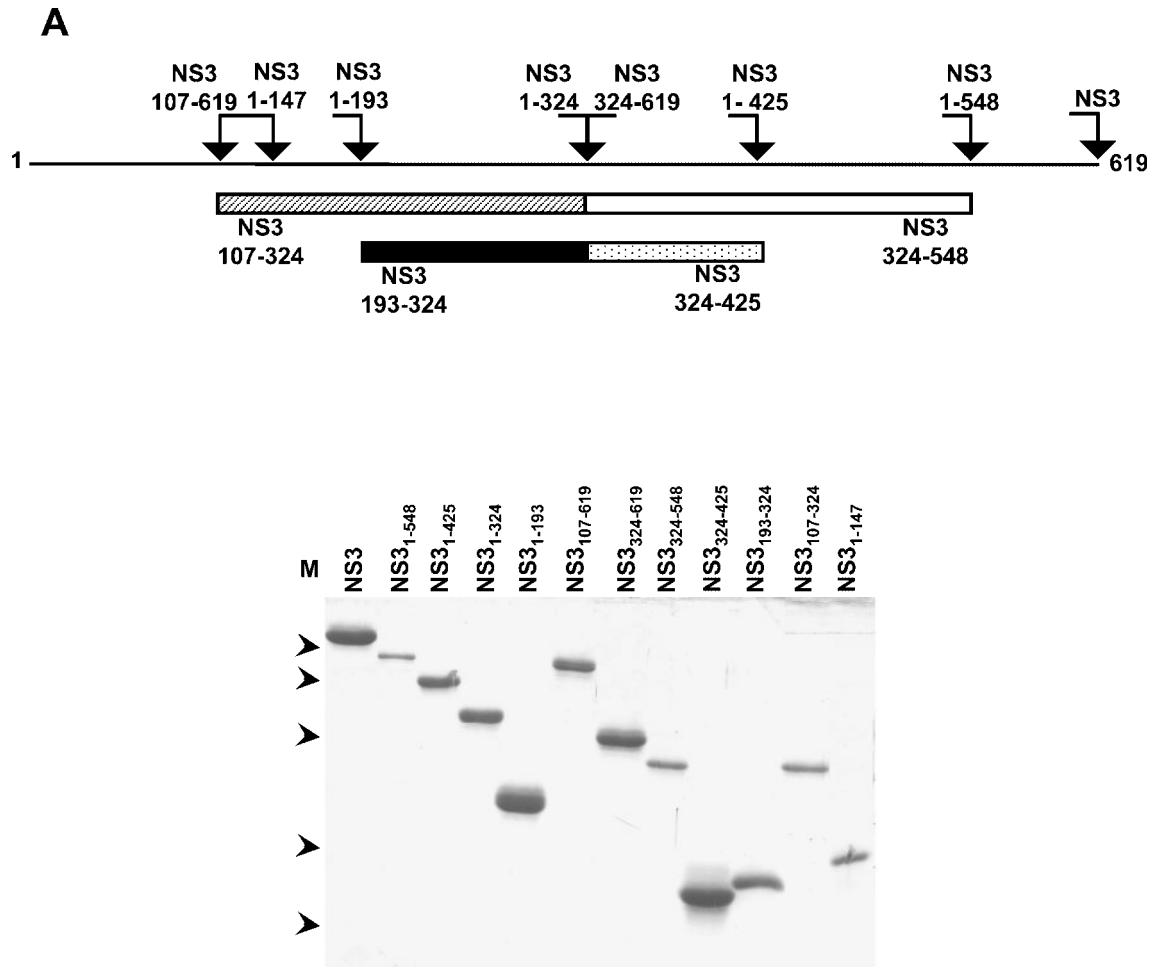


Fig. 3. (A) Schematic representation of the truncated NS3 proteins expressed in the pTAT-HA vector. Bent arrows on the NS3 protein represent the truncation sites. NS3_{107–619} and NS3_{324–619} are C-terminal fragments. All other truncations represented by arrows are N-terminal fragments. The internal fragments of NS3 are shown as separate bars. The Coomassie blue-stained 12.5% SDS-polyacrylamide gel shows purified NS3 (lane 1, ~72 kDa), NS3_{1–548} (lane 2, ~64 kDa), NS3_{1–425} (lane 3, ~51 kDa), NS3_{1–324} (lane 4, ~40 kDa), NS3_{1–193} (lane 5, ~25 kDa), NS3_{107–619} (lane 6, ~60 kDa), NS3_{324–619} (lane 7, ~37 kDa), NS3_{324–548} (lane 8, ~29 kDa), NS3_{324–425} (lane 9, ~15 kDa), NS3_{193–324} (lane 10, ~19 kDa), NS3_{107–324} (lane 11, ~28 kDa) and NS3_{1–147} (lane 12, ~20 kDa), all expressed as fusions to the HIV TAT peptide. Arrows indicate migration of marker proteins of 68, 50, 35, 21 and 14 kDa molecular mass. (B) Localization of the immunogenic region on JEV NS3 protein recognized by PBMC of JEV-exposed donors V25 and V31. Secreted IFN-γ (○, ◆) and proliferation (closed and open bars) in PBMC cultures were measured after 3 and 5 days, respectively, of culture with 0.7 mM full-length or the various truncations of NS3. SI values were ratios of mean counts incorporated by PBMC in the presence of test antigen and control GFP antigen in triplicate wells. IFN-γ levels were obtained by subtracting values for GFP-stimulated PBMC from test values as the means of triplicate wells.

AF217620, NP_722535, AAP20887, P06935, AAK06624, AAG02040, AAG02039, AAF18443, NP_776005 and AAA99713, respectively) were aligned using the Bioedit Sequence Alignment Editor version 5.0.9 (Hall, 1999). A Shannon entropy score was then calculated for each position in the protein alignment using the same program to obtain a measure of the amino acid variability at a given position taking into account both the number of possible amino acids allowed and their frequency (calculated as $\sum P_{aa} \log P_{aa}$, where P_{aa} is the proportion of each amino acid in the respective position) (Yusim *et al.*, 2002). To examine the relative levels of variability over the entire NS3

protein sequence, the entropy scores were smoothed by averaging over a window of 9 aa, the typical size of an epitope binding to HLA class I molecules, and individual as well as smoothed scores for each amino acid position in each overlapping window was determined. Positions where the majority of the sequences had gaps were excluded from consideration (Yusim *et al.*, 2002). When only a minority of sequences (less than eight) had gaps, however, the position was included and the gaps were treated as separate symbols.

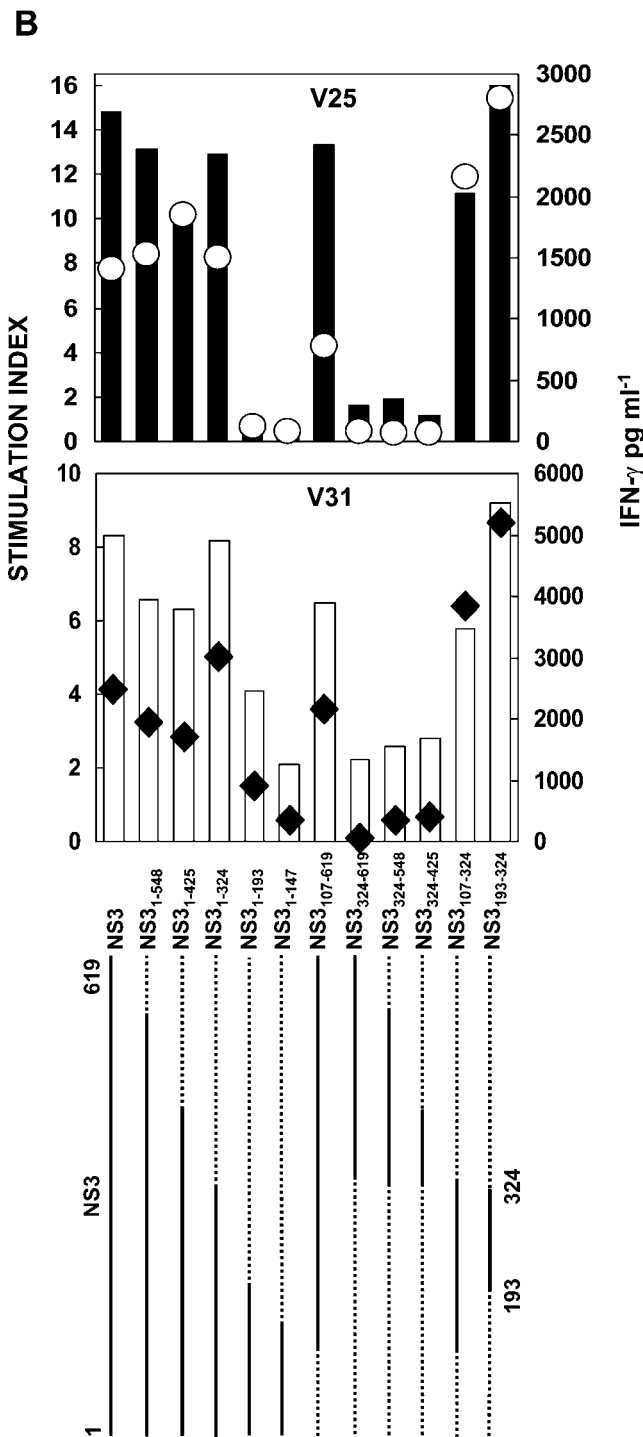
Statistical analysis. Results of lymphoproliferation and ELISA are represented as mean SI values and pg ml^{-1} , respectively, \pm SEM. All analyses were performed with GraphPad Prism version 3.00 for Windows (GraphPad Software) at a significance of $P < 0.05$. The non-parametric Mann-Whitney U test was used to compare the two groups, the Wilcoxon rank sum test for paired measurements for comparison between antigens and the Friedman test for comparison between three or more matched groups, as appropriate. Correlations were quantified using Spearman's rank correlation coefficient. The age and sex of the study individuals were found not to influence the parameters measured as analysed by logistic regression.

RESULTS

Memory T cell responses to stimulation with JEV antigen

PBMC from healthy children with $> 1 \log_{10}$ PRNT titres to JEV (range $1.10\text{--}3.11$) and serum antibodies to NS3 indicative of a prior subclinical infection with the virus were stimulated with total proteins from JEV. All donor PBMC exhibited positive proliferation ($\text{SI} \geq 3.0$, range $3.2\text{--}19.5$) to JEV antigen pointing to the priming of virus-specific T cells during a prior subclinical infection (Fig. 1A), while minimal proliferation was induced in control PBMC. As described by us previously, most of the JEV-exposed donors analysed in two independent, non-overlapping cohorts also displayed dominant T cell reactivity to the NS3 protein of JEV (Kumar *et al.*, 2003b, 2004a). NS3-specific CD4^+ as well as CD8^+ T cells secreted significant levels of the type 1 cytokine IFN- γ when NS3 was provided as a TAT fusion whose ability to translocate into the cytoplasm of PBMC was established by flow cytometry and confocal microscopy (Kumar *et al.*, 2004a). In the cohort under consideration, the dominant antigenicity of NS3 was again reinforced by a 96.2% frequency of recognition, since it induced proliferative responses in all but one donor (mean $\text{SI } 7.5 \pm 0.7$; Fig. 1A).

We initially screened for responses to the first 324 (NS3₁₋₃₂₄) or last 296 (NS3₃₂₄₋₆₁₉) aa of NS3 in 16 donors with NS3-specific responses. Proliferation levels to NS3₁₋₃₂₄ exceeded 70% of that elicited by the full-length protein in 13 of the 15 responders to NS3₁₋₃₂₄ (Fig. 1B). In contrast, PBMC from 12 of these 13 donors did not respond to NS3₃₂₄₋₆₁₉ ($\text{SI} < 3.0$). Measurement of secreted IFN- γ levels revealed a similar picture, with NS3₃₂₄₋₆₁₉ stimulating $< 100 \text{ pg ml}^{-1}$ from PBMC of 10 donors and $< 30\%$ of the total NS3-induced IFN- γ in PBMC from all but V32, whose response alone was directed only towards NS3₃₂₄₋₆₁₉ (Fig. 1C). Both halves of NS3 contributed to total IFN- γ in V26, V28 and V35. Thus, the first 324 aa of NS3 appeared



to contain the most immunogenic epitope(s) targeted by T cells during natural infections with JEV.

Mapping of immunogenic regions of NS3 using truncated versions of the protein

Phenotypic analysis of NS3-specific T cells from V25 and V31, both of whom displayed impressive responses to only the first half of NS3, revealed high proportions of IFN- γ -producing CD4⁺ as well as CD8⁺ T cells (Fig. 2), indicating that NS3 contained epitopes recognized by both subsets of T cells in these donors. Furthermore, both these donors were found to completely differ in their class I HLA alleles (Fig. 2) pointing to the presence of at least two distinct class I epitopes in NS3. To demarcate the epitope-bearing regions of NS3, six N-terminal and two C-terminal NS3 fragments along with four additional internal stretches of the NS3 protein, designated according to the boundary amino acids, were generated and purified as TAT fusion proteins (Fig. 3A) to examine their *in vitro* lymphocyte-stimulating capacity in V25 and V31.

Progressive truncation of the NS3 protein from aa 619 to aa 324 did not significantly reduce the proliferative response in either donor (Fig. 3B). Further reduction to aa 193 totally abrogated responses in V25, while in V31, NS3₁₋₁₉₃ evoked significantly reduced responses, which further declined to minimal levels with NS3₁₋₁₄₇. Thus aa 193–324 houses epitopes recognized in both these individuals. These observations were further corroborated using N-terminal truncations and internal regions of NS3 (Fig. 3B). Predictably, only proteins NS3₁₀₇₋₆₁₉, NS3₁₀₇₋₃₂₄ and NS3₁₉₃₋₃₂₄ successfully stimulated PBMC, the latter containing only the amino acid stretch under investigation, eliciting maximal SI values comparable with those attained with the full-length protein. The levels of IFN- γ produced followed the trend observed in SI values for each of the NS3 truncations (Fig. 3B). NS3₁₉₃₋₃₂₄ induced maximal IFN- γ production (2800 and 5200 pg ml⁻¹ compared with 1400 and 2972 pg ml⁻¹ observed with NS3 in V25 and V32, respectively) at a molar concentration equivalent to that of full-length NS3, probably reflecting an increased efficiency in processing of this smaller protein stretch. In view of the dissimilar HLA alleles in these two individuals, these results point to the presence of more than one T cell epitope between aa 193 and 324 of NS3.

Aa 193–324 of JEV NS3 protein dominate in their ability to stimulate PBMC from the majority of JEV-exposed individuals

We investigated the potential of NS3₁₉₃₋₃₂₄ to stimulate PBMC of the 26 children who formed our JEV-exposed cohort. Twenty-three donors displayed positive lymphoproliferation towards NS3₁₉₃₋₃₂₄ with SI values ranging from 43 (V28) to 219% (V29) of that obtained with the full-length protein (Fig. 4A) and comparable means of 7.5 ± 0.7 for the full-length NS3 and 6.8 ± 0.8 for NS3₁₉₃₋₃₂₄ ($P=0.4868$, Mann-Whitney U test). Similarly, the secreted IFN- γ levels towards

NS3₁₉₃₋₃₂₄ in 18 of the 23 donors were comparable with or greater than those seen in response to NS3 (Fig. 4B), implying that the epitopes targeted by T cells in each of these individuals were all positioned between aa 193 and 324 of NS3. Moreover, in 14 of the 15 children identified with positive responses to NS3₁₋₃₂₄ in the initial screen (Fig. 1B), no significant differences could be noted in mean levels of the measured parameters to the full-length, the first half and aa 193–324 of NS3 (Fig. 4C, $P>0.05$, Friedman test). NS3₁₉₃₋₃₂₄ accounted for the entire response, with the first 193 aa contributing partly only in V27. Thus, in a JEV-exposed healthy population, the Th1 response to the NS3 protein of JEV is focused on aa 193–324 of NS3.

NS3₁₉₃₋₃₂₄ is capable of stimulating IFN- γ secretion from both CD4⁺ and CD8⁺ T cells

We next addressed the question of whether the immunogenic NS3₁₉₃₋₃₂₄ contained epitopes presented by HLA class I and II alleles. The profile of T cells responding to NS3₁₉₃₋₃₂₄ was remarkably similar to that observed with the full-length protein, which stimulated IFN- γ from both CD4⁺ and CD8⁺ T cells in almost all 18 individuals studied (representative dot plots are shown in Fig. 5 and Table 1) establishing without ambiguity the presence of both class I and class II epitopes in this stretch of NS3. The secreted IFN- γ levels closely correlated with the percentage of T cells activated to produce IFN- γ (Table 1, Spearman's rank correlation coefficient $r=0.8209$, $P<0.0001$). While mean fluorescence intensities (data not shown) and frequencies of IFN- γ -producing T cell subsets were not significantly different with either antigen (Wilcoxon rank sum test, $P>0.05$), a perceptible increase in IFN- γ -positive T cell frequencies upon stimulation with NS3₁₉₃₋₃₂₄ was documented in V1, V3, V25, V29 and V31 (Table 1). Moderate reductions in frequencies of either or both T cell subtypes occurred in V5, V27, V28 and V35 suggestive of minor contributions from recognition of epitopes elsewhere in NS3. Haplotyping of the class I alleles of volunteers whose target epitopes lay within NS3₁₉₃₋₃₂₄ revealed 12 HLA A, 12 HLA B and 5 HLA C alleles. The existence of at least three volunteers with no common HLA alleles whose CD4⁺ and CD8⁺ T cells recognized NS3₁₉₃₋₃₂₄ pointed to a minimum of three epitopes restricted by independent class I alleles and at least one class II epitope mapping to this region of NS3. V32, whose PBMC alone did not respond to NS3₁₉₃₋₃₂₄, had a single HLA allele [A26(10)] that was not found in any other donor, suggesting that this donor's weak CD8⁺ T cell response to NS3 (Table 1) was restricted by this allele.

Analysis of the relationship between amino acid variability and occurrence of T cell epitopes

The fact that NS3₁₉₃₋₃₂₄ was an antigenic target of T cells from individuals with diverse HLA haplotypes was considered to be a feature worth investigating, especially since any variation in sequence of the infecting strain would influence the assessment of these immune responses. To evaluate links, if any, between sequence variability of NS3

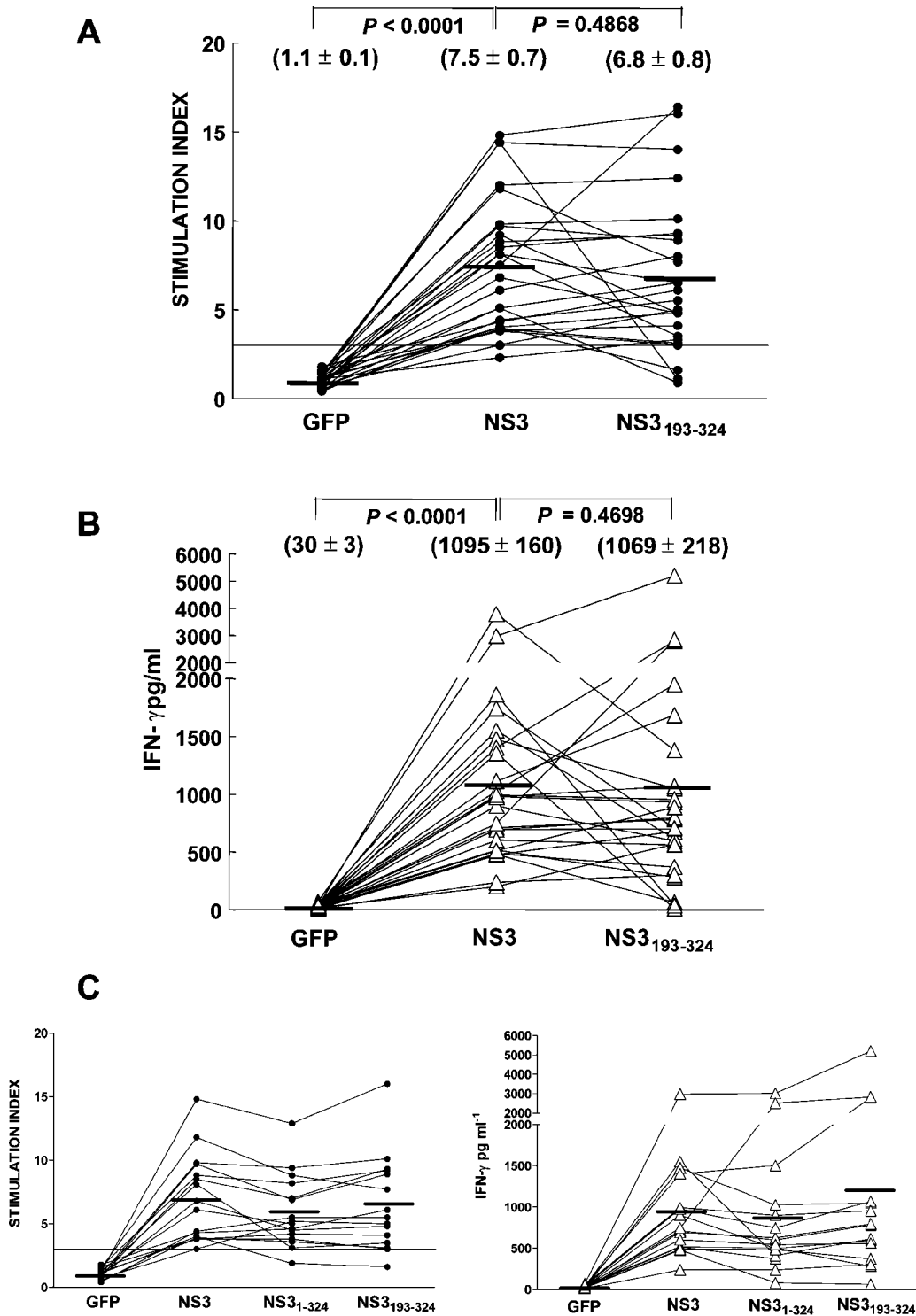


Fig. 4. Aa 193–324 are dominantly recognized in donors with NS3-specific lymphocyte responses. Proliferation (A) and secreted IFN- γ (B) in PBMC cultures were measured after 5 and 3 days of culture, respectively, with 0.7 mM full-length NS3, NS3_{193–324} or GFP. The mean SI and IFN- γ values observed in each group (horizontal bars and values in parentheses) are shown. SI values were ratios of mean counts incorporated by PBMC in the presence of viral antigen and GFP in triplicate wells. IFN- γ levels were obtained by subtracting values for GFP-stimulated PBMC from test values as the means of triplicate wells. The SI values and IFN- γ levels for GFP-stimulated PBMC were obtained in comparison with cultures of unstimulated PBMC. (C) Comparison of proliferation (left panel) and secreted IFN- γ levels (right panel) of PBMC stimulated with 0.7 mM GFP, full-length NS3, NS3_{1–324} and NS3_{193–324} in the 16 individuals who were compared in Fig. 1(B) and (C). The mean positive SI value (cut-off > 3) and IFN- γ level observed in each group (horizontal bars) are shown.

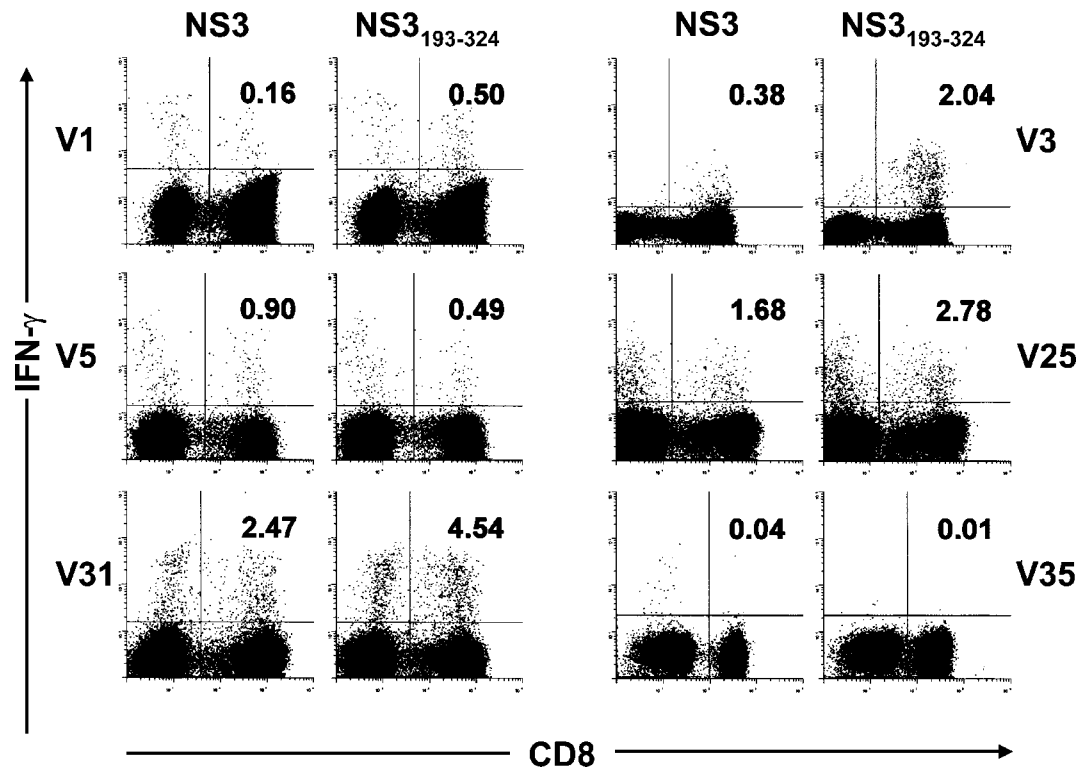


Fig. 5. Intracellular cytokine analysis in response to the full-length NS3 and NS3_{193–324} in JEV-exposed healthy donors. Whole blood was stimulated with 1.4 mM purified TAT peptide fusion proteins for 6 h, fixed and stained for CD3, CD8 and IFN- γ . Cells were gated on lymphocytes, followed by CD3, and analysed for CD8 and IFN- γ . The frequencies in the upper quadrants are IFN- γ -producing cells as percentages of the total number of CD8⁺ cells after subtracting the background frequencies observed with GFP-stimulated cultures. GFP did not stimulate significant frequencies of IFN- γ -producing T cells in any donor. No positive staining was observed with matched isotype control antibodies.

and virus-specific T cell responses, we assessed the relative variability of NS3 by aligning the amino acid sequences of this protein from 16 different isolates of JEV. The mean entropy of all positions of the protein was estimated by taking into account both the frequency and spectrum of amino acids at each position. Site-by-site analysis revealed only 50 positions of variability in the 619 aa long NS3, reflecting the highly conserved nature of this protein (Fig. 6A). Remarkably, the largest stretch of the protein that recorded absolutely no variation in any of the 16 sequences compared was found to be aa 219–318, which interestingly was encompassed within aa 193–324 of NS3. The documented existence of flavivirus cross-reactive epitopes prompted us to align and compare the variability of NS3 sequences across 37 different flaviviruses including dengue, JEV, Murray Valley encephalitis virus, West Nile virus and yellow fever virus. Since variability was highly non-uniform within different flavivirus NS3 protein sequences, we calculated regional entropy scores averaged over a 9 aa window, the size of a typical class I epitope. Again, the smoothed entropy scores were least in the region corresponding to aa 193–324 (Fig. 6B), with the mean entropy of this region scoring lower than that for an equivalent length

of any other region in NS3 or the entire NS3 itself. Calculation of entropy scores smoothed over a window of 20 aa, so as to include class I as well as class II epitopes, yielded typically similar results with aa 193–324 again ranked the lowest (data not shown).

DISCUSSION

Following our earlier observation that NS3-specific T cells producing IFN- γ were present in a vast majority of healthy exposed individuals of varied HLA haplotypes from a JE-endemic region, it was of particular interest to investigate the distribution of epitopes responsible for CD4⁺ and CD8⁺ T cell responses on this protein. Our approach of using NS3 segments fused to the TAT peptide of HIV, thus enabling detection of both subsets of T cells, identified a single, highly immunodominant region comprising aa 193–324 of NS3 as primarily responsible for stimulating T cells in two healthy JEV-exposed children with different class I HLA haplotypes. NS3_{193–324} elicited a Th1 response in more than 88% of the volunteers, often more efficiently than the parent protein, presumably due to improved processing and consequent presentation of this smaller protein.

Table 1. HLA and phenotype of IFN- γ -producing lymphocytes stimulated by NS3 and NS3_{193–324}

Donor	HLA	NS3			NS3 _{193–324}		
		IFN- γ *	CD4 ⁺ †‡	CD8 ⁺ †	IFN- γ *	CD4 ⁺ †‡	CD8 ⁺ †
V1	A1, A29, B7, B57(17), Cw6, Cw17	512	0·31	0·16	887	0·30	0·50
V2	A1, A24(9), B13, B35, Cw4, Cw6	991	0·20	0·30	954	0·17	0·30
V3	A2, A11, B7, B60(40), Cw6, Cw7	745	0·00	0·38	2833	0·10	2·04
V4	A2, A24(9), B51(5)	975	0·13	0·31	1068	0·14	0·33
V5	A2, A32(19), B51(5), B49(21), Cw7	1740	0·59	0·90	705	0·59	0·49
V6	A32(19), A68(28), B51(5), B61(40)	602	0·18	0·28	561	ND	ND
V7	A2, A28, B35, B61(40)	238	0·08	0·13	300	0·10	0·18
V11	A31(19), B51(5), B8, Cw6, Cw7	691	0·31	0·40	795	0·29	0·49
V15	A11, B35, Cw4	691	0·27	0·34	701	0·29	0·38
V18	A23(9), A24(9), B7, B44(12), Cw4	980	0·94	0·80	928	0·96	0·74
V25	A24(9), B52(5), B62(15), Cw3, Cw4	1400	0·98	1·68	2800	1·58	2·78
V26	A24(9), A33(19), B44(12), B57(17), Cw6, Cw7	1475	0·65	1·60	1050	0·72	1·54
V27	A2, A3, B51(5), B49(21), Cw7	515	0·24	0·30	280	0·15	0·19
V28	A28, B35, B51(5), Cw4, Cw6	1550	0·55	0·49	615	0·12	0·35
V29	A24(9), B61(40)	1025	0·46	0·30	1950	0·86	0·60
V31	A1, A32(19), B57(17), B49(21), Cw6, Cw7	2972	1·08	2·47	5200	1·60	4·54
V32	A3, A26(10), B8, B62(15), Cw6, Cw7	478	0·26	0·04	64	0·03	0·01
V35	A11, A32(19), B52(5), B60(40), Cw3, Cw4	900	0·31	0·29	595	0·16	0·12
Mean \pm SEM...		1027 \pm 149	0·42 \pm 0·07	0·62 \pm 0·15	1238 \pm 296	0·48 \pm 0·12	0·91 \pm 0·30

*Data shown are in pg ml⁻¹ and were obtained by subtracting mean IFN- γ levels secreted by PBMC in triplicate wells in the presence of GFP from that measured in the presence of specific antigen.

†The frequencies of IFN- γ -producing cells are shown as percentages of each T cell subtype after subtracting the background frequencies observed with control GFP antigen. GFP did not elicit responses in PBMC (mean CD4⁺ = 0·02 \pm 0·00 %, mean CD8⁺ = 0·02 \pm 0·00 %).

‡The percentages of responding CD4⁺ T cells were obtained independently by staining with antibodies to CD4.

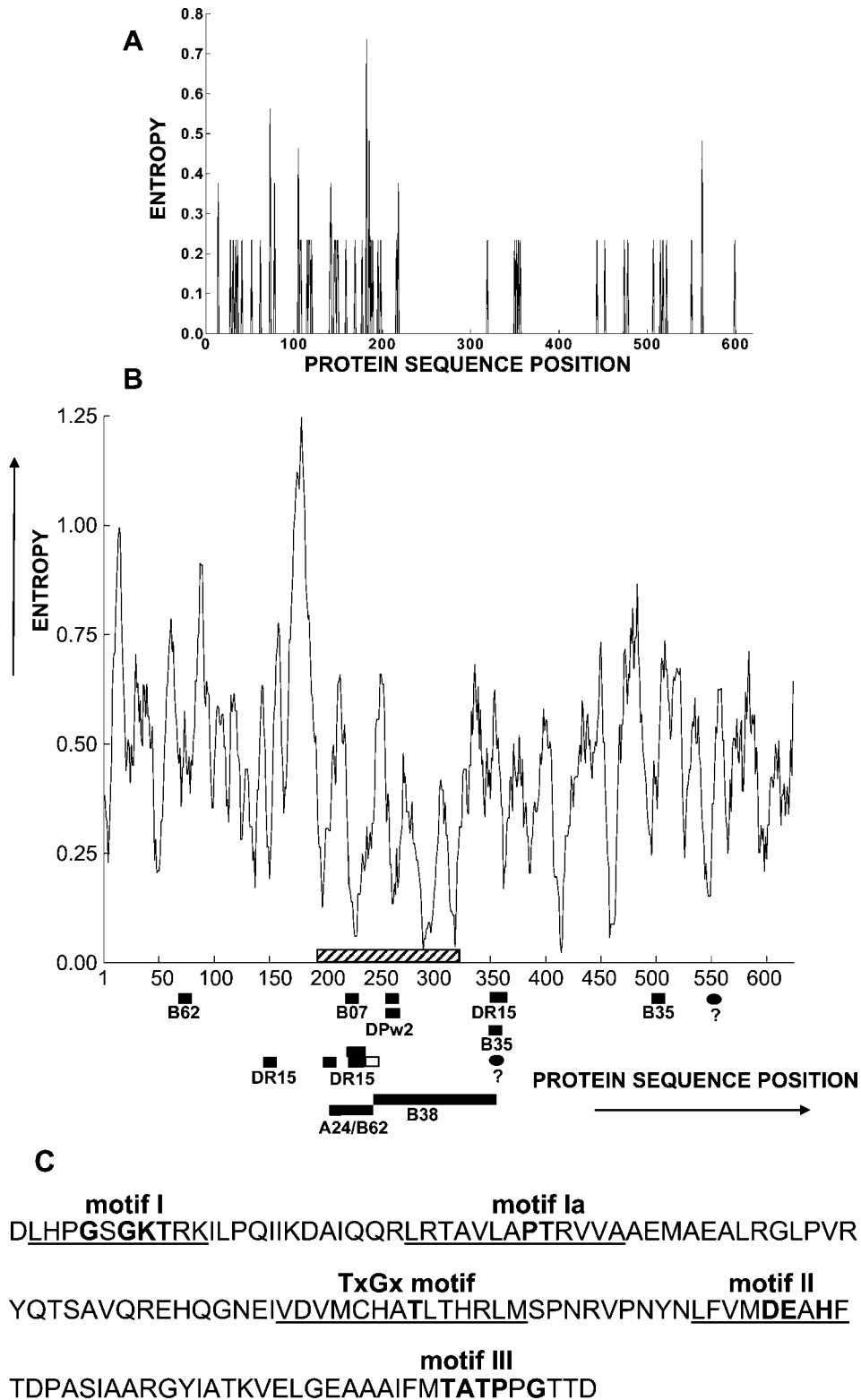
ND, Not determined.

It was indeed noteworthy that the phenotype of T cells responding to the full-length NS3 and the 132 aa segment were almost identical, both qualitatively and quantitatively. Th1 responses to NS3_{193–324} were mediated by CD4⁺ as well as CD8⁺ T cells, revealing the presence in this segment of both class I and II epitopes that are processed and presented during the natural course of JEV infection. Moreover, this region was highly conserved, with absolutely no change in aa 210–318 among 16 different strains of JEV. The high α -helicity of this stretch (53·44 %, data not shown) is in consonance with the known propensity of T cell epitopes to lie within α -helical regions of proteins (Yusim *et al.*, 2002).

NS3, one of the most conserved proteins among flaviviruses (Billoir *et al.*, 2000), is known to perform the functions of a protease, nucleoside triphosphatase and helicase (Rice & Lindenbach, 2001). The first 180 aa residues of NS3 contain the catalytic triad required for its trypsin-like protease activity, while the region C-terminal to this contains conserved motifs found in the nucleoside triphosphate-binding proteins and DexH family of RNA helicases, the last function being implicated in an unwinding step during genomic RNA replication. Of the seven motifs that are characteristic of RNA helicases of the DexH subfamily, four map to aa 193–324 of NS3 (Fig. 6C) (Lin & Kim, 1999). In addition, the

conserved TxGx/CHAT and SIAARG motifs (Billoir *et al.*, 2000) also map to this region. Mutations within as well as just outside any of these conserved motifs have been known to have deleterious effects ranging from non-viability/ altered phenotype of flaviviruses to slow replication rates/ attenuation (Lin & Kim, 1999; Matusan *et al.*, 2001), justifying the high conservation of aa 193–324 of NS3.

The flavivirus NS3 protein has been a dominant source of antigenic peptides in the mouse model (Lobigs *et al.*, 1994). Studies on the human cell-mediated immune response to dengue and yellow fever viral proteins have also identified NS3 as a predominant target for both CD4⁺ and CD8⁺ T cells in vaccine recipients as well as in natural infections. It was interesting to note that several epitopes bound by HLA A24, B07, B35, B38, B62, DPw2 and DR15 have all been mapped to the region corresponding to aa 193–324 of NS3 (Kurane *et al.*, 1993, 1995, 1998; Mathew *et al.*, 1996, 1998; Zeng *et al.*, 1996; Okamoto *et al.*, 1998; Zivny *et al.*, 1999; Loke *et al.*, 2001; Co *et al.*, 2002; Zivna *et al.*, 2002). When we aligned the positions of these experimentally identified epitopes in the NS3 protein with the smoothed entropy scores, the region corresponding to aa 193–324 of NS3 harboured nine of the 16 identified epitopes, both class I and II, restricted by at least five different HLA alleles



(Fig. 6B). Notably, each of the experimentally defined epitopes mapped with striking regularity to regions with a dip in entropy, revealing the concentration of epitopes of varied HLA specificities in conserved regions within the aa 193–324 segment of NS3.

Some of the dengue virus NS3 epitopes mentioned above are cross-reactive and were recognized by human CD8⁺ or CD4⁺ T cell clones specific for not just the four different serotypes of dengue virus, but also other flaviviruses such as West Nile virus and yellow fever virus. According to a recent

Fig. 6. Analysis of variability of the NS3 protein sequence. (A) Shannon entropy scores for all positions in alignments of NS3 protein sequences from 16 JEV isolates. The scores varied from 0.2333 at 41 positions (only one of the 16 isolates compared had a variant amino acid) to 0.7356 at residue 182 (three different amino acids were found in this position among the 16 isolates compared). (B) Shannon entropy scores for all positions in alignments of NS3 protein sequences from 37 independently isolated flavivirus strains. The entropy data were smoothed by using a window of 9 aa (the size of a typical class I epitope). The hatched bar corresponds to aa 193–324 of JEV NS3. The positions of experimentally identified flavivirus NS3 epitopes and their restricting HLA alleles are also shown. The oval symbols refer to epitopes whose restricting alleles have not been identified. (C) Amino acid sequence between aa 193 and 324 of JEV P20778 NS3 protein. The underlined sequences constitute the residues that characterize the conserved motifs characteristic of DexH RNA helicases.

report, cytotoxic T cells against two immunodominant H-2K^k-restricted determinants on the Murray Valley encephalitis virus NS3 lysed target cells pulsed with peptides from the corresponding amino acid stretches on NS3 of six other flaviviruses including JEV (Regner *et al.*, 2001). Extremely immunogenic T helper cell epitopes have been identified in the core, NS3 and NS4 proteins of the related hepatitis C virus, which, besides being highly conserved among the known isolates, were also found to be very promiscuous because they could be presented to T cells by several different class II alleles (Diepolder *et al.*, 1997; Lamonaca *et al.*, 1999). Similarly, naturally processed viral peptides have also been known to be presented in the context of two or more class I alleles (Tomiyama *et al.*, 2000; Thimme *et al.*, 2001). Fine mapping of the T cell epitopes in this segment currently underway should help to resolve the question of whether the high recognition frequency of NS3_{193–324} might also be ascribed, among other reasons, to similar promiscuity.

A likely contributing factor to the identification of immunoreactive peptides in this region could be a selection effect, as T cells that target variable regions may go undetected due to sequence substitutions in reagents used to define the T cell response. Thus, the decrease in immunogenic potential of the variable regions in NS3 may reflect past immune escape concentrated in regions of the virus that readily tolerate change or might ensue from actual clustering of epitopes in the relatively conserved regions. The former possibility, however, appears unlikely in view of the reasonably high degree of conservation observed in other regions, especially the second half of NS3, despite which this region was targeted in only a few of the donors with NS3 reactivity. In addition, the mean rate of non-synonymous (amino acid-changing) substitutions for mosquito-borne flaviviruses (7.5×10^{-5} year⁻¹; Zanotto *et al.*, 1996) is nearly 200 times less than that observed in a highly mutating virus such as HIV (3.9×10^{-3} year⁻¹; Li *et al.*, 1988), where clustering of epitopes in relatively conserved regions of various proteins is a recognized phenomenon (Yusim *et al.*, 2002). Hence, despite possible reference strain experimental bias, there could be additional as-yet-unidentified immunological reasons that account for the paucity of epitopes in variable regions of NS3. We are tempted to speculate that the low incidence of symptomatic JEV infections (1 in every 250 infected individuals) could be due to the restricted spread of the virus in the periphery on account of selective targeting by

the immune system of regions in viral proteins that cannot tolerate change in sequence owing to their crucial function.

Our earlier studies have implicated a role for IFN- γ in recovery from encephalitis in JEV patients (Kumar *et al.*, 2004b). That NS3_{193–324} also elicits this antiviral Th1 cytokine in healthy individuals residing in endemic areas who represent a ‘naturally immunized’ population makes it an important correlate of protective immunity to JEV. NS3 is one of the most conserved proteins among different strains of JEV and among flaviviruses, which enhances the probability that the highly antigenic aa 193–324 stretch may be recognized by T cells against different strains of not only JEV but also other flaviviruses. Therefore, incorporation of this highly immunodominant region from one of the most conserved proteins of flaviviruses in immunizing preparations would help in the development of a highly cross-reactive flavivirus vaccine, capable not only of eliciting CD8⁺ T cells required for ultimate virus clearance, but also in providing T cell help for long-lasting neutralizing antibody generation, a mechanism that is believed to be crucial for protective immunity against JEV (Pan *et al.*, 2001). In conclusion, immunodominance, sequence conservation and probable cross-reactivity make the stretch of amino acids from aa 193 to 324 of NS3 an ideal additive to a humoral response-eliciting vaccine against JEV.

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