

Cell-mediated immune responses in healthy children with a history of subclinical infection with *Japanese encephalitis virus*: analysis of CD4⁺ and CD8⁺ T cell target specificities by intracellular delivery of viral proteins using the human immunodeficiency virus Tat protein transduction domain

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Japanese encephalitis virus (JEV), a single-stranded positive-sense RNA virus of the family *Flaviviridae*, is the major cause of paediatric encephalitis in Asia. The high incidence of subclinical infections in Japanese encephalitis-endemic areas and subsequent evasion of encephalitis points to the development of immune responses against JEV. Humoral responses play a central role in protection against JEV; however, cell-mediated immune responses contributing to this end are not fully understood. The structural envelope (E) protein, the major inducer of neutralizing antibodies, is a poor target for T cells in natural JEV infections. The extent to which JEV non-structural proteins are targeted by T cells in subclinically infected healthy children would help to elucidate the role of cell-mediated immunity in protection against JEV as well as other flaviviral infections. The property of the Tat peptide of *Human immunodeficiency virus* to transduce proteins across cell membranes, facilitating intracellular protein delivery following exogenous addition to cultured cells, prompted us to express the four largest proteins of JEV, comprising 71 % of the JEV genome coding sequence, as Tat fusions for enumerating the frequencies of virus-specific CD4⁺ and CD8⁺ T cells in JEV-immune donors. At least two epitopes recognized by distinct HLA alleles were found on each of the non-structural proteins, with dominant antiviral Th1 T cell responses to the NS3 protein in nearly 96 % of the cohort. The data presented here show that non-structural proteins are frequently targeted by T cells in natural JEV infections and may be efficacious supplements for the predominantly antibody-eliciting E-based JEV vaccines.

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INTRODUCTION

The family *Flaviviridae* includes at least 68 medically important viruses, such as *Dengue virus* (DENV), *Japanese encephalitis virus* (JEV), *Yellow fever virus* (YFV), *West Nile virus* (WNV) and *Hepatitis C virus* (HCV), many of which are emerging as important infectious agents in several hitherto unaffected areas of the world today. Japanese encephalitis (JE), endemic to much of south and south-east Asia, is the most common arthropod-borne human encephalitis in the world. Despite a small proportion of the large number of JEV-infected individuals developing

overt manifestations of encephalitis, this incidence nevertheless accounts for more than 50 000 cases and 10 000 deaths each year (Tsai *et al.*, 1999). In endemic areas, the highest age-specific attack rates occur in children 5–15 years of age with a case fatality rate of approximately 30 %, and nearly half the survivors suffer long-term neuropsychiatric sequelae. In the absence of specific anti-flaviviral drugs, vaccination is an effective approach for reducing infection and hence disease incidence. An inactivated JEV formulation prepared from infected mouse brains is the only internationally approved vaccine used with measurable success in many parts of Asia. This vaccine is nonetheless

very costly to prepare, carries with it the risk of inducing allergic reactions and, most importantly, is limited in its capacity to induce long-term immunity (Ku *et al.*, 1994). While a live-attenuated SA14-14-2 vaccine strain of JEV has been found to be extremely efficacious, production and regulatory standards for this vaccine are not established as yet (Tsai *et al.*, 1999).

Successful development of effectual vaccines will be expedited if the immune responses that contribute to disease control are understood. Effective antiviral immunity expressed by most infected individuals appears to prevent JEV infections from progressing to disease. The single-stranded positive-sense genomic RNA of all flaviviruses encodes 10 proteins: three structural [capsid (C), pre-membrane/membrane (prM/M) and envelope (E)] and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The structural protein E, the major component of the killed JEV vaccine, is the chief mediator of its protective nature owing to its ability to induce strong neutralizing antibodies. The short duration of 4–5 years for neutralization titres induced by the killed JEV vaccine (Tsai *et al.*, 1999) in contrast to the 35-year efficacy window of the live-attenuated YFV vaccine (Poland *et al.*, 1981) possibly implicates the efficient recruitment of T cell help by the latter, which expresses not only the structural but also the non-structural proteins. While the beneficial role of humoral immunity to JEV has been well characterized both in humans (Hoke *et al.*, 1988) and in animal models (Kimura-Kuroda & Yasui, 1988; Konishi *et al.*, 1992, 1999, 2000; Lin *et al.*, 1998) of JEV infections, the role of T cell immunity in JEV protection is less well defined. The ability of JEV-specific T cells to protect against lethal challenge in mice (Mathur *et al.*, 1983; Miura *et al.*, 1990; Murali-Krishna *et al.*, 1996), the induction of CD4⁺ and CD8⁺ T cells to E only in vaccinees receiving the formalin-inactivated vaccine (Aihara *et al.*, 1998) or the pox-virus-based vaccine expressing exclusively the structural proteins of JEV (Konishi *et al.*, 1998) but not during natural infections (Desai *et al.*, 1995; Konishi *et al.*, 1995) and, finally, the expendable nature of E-specific T cells for immunity against JEV in mice (Konishi *et al.*, 1999; Pan *et al.*, 2001) all point to the non-structural proteins being the primary targets of protective T cells. Moreover, identification of non-structural protein-specific cytolytic CD4⁺ and CD8⁺ T cells in virus-immune donors immunized with live-attenuated strains of related flaviviruses (Kurane *et al.*, 1991; Mathew *et al.*, 1996, 1998; Zeng *et al.*, 1996; Co *et al.*, 2002) strengthens the conclusion that both subsets of T cells contribute to flaviviral immunity.

To gain insight into the antigen specificity and immunological characteristics of T cells that contribute to resistance against JE disease, we studied healthy children from JE-endemic areas who had experienced subclinical infections with no encephalitis symptoms and therefore evidently mounted an effective immune response that curbed virus entry into the central nervous system. Our earlier studies

in a similar cohort revealed that NS3 was the dominant target of T cells when provided as lysates of recombinant baculovirus-infected Sf21 cells (Kumar *et al.*, 2003b), a preparation that we found predominantly activated memory CD4⁺ T cells. We have now analysed the competence of the four largest JEV proteins to function as targets of both CD4⁺ and CD8⁺ human T cells when provided as purified recombinants fused to the 11 aa Tat protein transduction domain (PTD; YGRKKRRQRRR) of *Human immunodeficiency virus* (HIV), a form which when provided as exogenous antigen also ensures the intracellular delivery of protein by transduction across cell membranes. The results revealed quantitative differences in the vigour and phenotype of the immune responses to E, NS1, NS3 and NS5 proteins prevalent in healthy subclinically infected children and pointed towards a clear dominant role for NS3 in the induction of cell-mediated immunity, and thus a possible protective role, against JEV.

METHODS

Cells and viruses. JEV strain P20778, WNV strain E101 and strain TR 1751 (National Centre for Cell Science, Pune, India) of *Dengue virus* type 2 (DENV-2) were 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.

Generation of recombinant protein antigens of JEV. The E, NS1, NS3 and NS5 genes were obtained by RT-PCR of the genomic viral RNA extracted from JEV-infected C6/36 cells using the Expand RT system (Roche Diagnostics) with the appropriate primer pairs listed below (start and stop codons in bold, restriction sites underlined). E-sense, 5'-GCGCGCGAATTCTCGAGATGTTCAACTGTCTGGGAATGGGC-3' (*XhoI*); E-antisense, 5'-GCGCGCTTATGCATGCACATTGGTCGTAA-3'; NS1-sense, 5'-GGCCGGAATTCTCGAGATGGACACTGGATGCGCCATTGAC-3' (*XhoI*); NS1-antisense, 5'-CGCGCGTCGACAGGCCTTTATAGCACACATACCTCGCC-3'; NS3-sense, 5'-GGATAGAATTCATATGGGGGGCGTGTTTGGGACAC-3' (*EcoRI*); NS3-antisense, 5'-GATCCTTATCTCTTCCCTGCTGCAAAGTCTTT-3'; NS5-sense, 5'-CCGGCGTCGACAGCCATGGGAAGGCCCGGGGCAGGACG-3' (*SalI*); NS5-antisense, 5'-CCGGCCGGATCCCTAGATGACCCTGTCTTCTGGGTCAAGAC-3' (*BamHI*).

Each of the genes was cloned into the pTAT-HA bacterial expression vector (a kind gift from Dr Steven F. Dowdy, Howard Hughes Medical Institute, St Louis, MO, USA) in-frame with the N-terminal hexahistidine leader, 11 aa PTD of HIV Tat protein and a haemagglutinin tag provided by the vector (Becker-Hapak *et al.*, 2001) as follows. The E (nt 978–2477 of the JEV P20778 genome) and NS1 (nt 2478–3713) genes from the respective PCR-amplified cDNAs were inserted as *XhoI*-blunt PCR fragments between the *XhoI* and Klenow-filled *EcoRI* sites and the NS3 gene (nt 4608–6464) as a blunt fragment after *EcoRI* digestion into the Klenow-filled *XhoI* site of pTAT-HA. The NS5 gene (nt 7677–10394) was cloned as two separate halves to facilitate expression in *Escherichia coli* (*E. coli*). The first 1692 nt of the 2717 nt long NS5 gene (nt 7677–9369, NS5N) were cloned as a Klenow-filled *SalI*-*SphI* (nt 9369 of the JEV genome) fragment between the Klenow-filled *NcoI* and *SphI* sites of pTAT-HA. The last 1209 nt of NS5 were cloned between the Klenow-filled *NcoI* and *EcoRI* sites of pTAT-HA as a blunt fragment after digestion with *EcoRI* (nt 9185) and *BamHI*.

Recombinant Tat-JEV fusion proteins expressed in *E. coli* BL21(DE3)

were purified by electroelution from SDS-polyacrylamide gels, precipitated and solubilized in PBS. Protein purity was confirmed by N-terminal sequencing of the electroeluted protein using Edman chemistry and endotoxin absence by the Pyrogen plus Gel-clot LAL test kit (BioWhittaker). Similarly expressed and purified unrelated green fluorescent protein (GFP) was used as control antigen (Tat-GFP).

Study population. Volunteers drawn from the JE-endemic regions of the states of Karnataka and Andhra Pradesh were recruited between August 2002 and January 2003 at the district hospital, Vijayanagar Institute of Medical Sciences, Bellary, Karnataka, India. All 24 healthy non-vaccinated JEV-seropositive children (5–11 years old) included had no history of clinical encephalitis. Prior exposure to the virus was further confirmed by $>1 \log_{10}$ serum plaque reduction neutralization test (PRNT) antibody titres to JEV. Flaviviral infections due to DENV and WNV were ruled out at the time of sampling based on serum PRNT-ELISAs. Twelve healthy non-vaccinated children with no history of clinical encephalitis, who were matched for age and sex and chosen on the criterion of $<0.2 \log_{10}$ serum-PRNT titres to the flaviviruses JEV, DENV and WNV, were the control donors. 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 under 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.

JEV-PRNT titres. Serum JEV-specific PRNT titres were estimated by a modified PRNT-ELISA as described previously (Ting *et al.*, 2001). PRNT titre was calculated by the standard formula of the National Institutes of Health, Japan (Rao-Bhau *et al.*, 1988). DENV- and WNV-specific PRNT titre determinations were performed similarly.

Radioimmunoprecipitation (RIP) analysis. Serum antibodies to NS3 were detected by RIP of lysates of ^{35}S -methionine-labelled JEV-infected Vero cells (Aihara *et al.*, 1998) and the identity of the NS3 protein was confirmed by Western blotting the immunoprecipitates using NS3-specific rabbit antiserum.

Analysis of protein transduction. Peripheral blood mononuclear cells (PBMCs) were treated with 333 nM recombinant Tat fusion or wild-type GFP proteins, harvested 1 h later, washed and fixed/permeabilized with a -20°C acetone/methanol mixture; the intracellular recombinant proteins were stained using specific antisera and corresponding FITC-labelled secondary antibodies. Samples were then analysed using a Becton Dickinson FACScan flow cytometer. For confocal microscopy, PBMCs harvested 3 h after incubation were plated onto poly-L-lysine (M_r 150 000 to 300 000; Sigma) coated cover slips, treated as above and examined using a Leica DMIRB inverted confocal microscope with the pinhole set at 1.5.

Lymphoproliferation assay (LPA). PBMC isolation, antigen stimulation and lymphoproliferation were carried out as described previously (Kumar *et al.*, 2003b). Viral antigens included glutaraldehyde-fixed, JEV-infected Vero cell lysates (VJE) at 16 ng per well equivalent of E protein (Aihara *et al.*, 1998; Kumar *et al.*, 2003b) and the purified Tat fusion proteins used at the experimentally deduced optimal concentration of $50 \mu\text{g ml}^{-1}$. Control antigens were uninfected Vero cell lysates (VUI) and Tat-GFP. Incubations of PBMCs with phytohaemagglutinin A (PHA; Sigma) at $10 \mu\text{g ml}^{-1}$ and sonicate of *Mycobacterium bovis* BCG ($5 \mu\text{g ml}^{-1}$) were for 3 days. Proliferative response was expressed as the stimulation index (SI), the ratio of the mean c.p.m. incorporated by PBMCs in triplicate wells in the presence of test and control antigens. The highest value of the SI in control individuals in response to the test antigen plus 1.96 times

the standard deviation from the mean was 2.19 and 1.90 for VJE and Tat-NS3, respectively. We therefore scored a positive response on the criteria that (i) the SI was ≥ 3.0 for VJE and 2.0 for Tat-NS3 and (ii) the mean c.p.m. obtained on stimulation with viral antigen was ≥ 500 . Recovery of and counts incorporated by PBMCs from blood of donors of all three groups were similar.

Inducible cytokines. Culture supernatants were collected after 48 and 72 h of stimulation and assayed for interleukin-4 (IL-4) and gamma interferon (IFN- γ), using commercial capture ELISA kits (Endogen). The lower limit of detection in these assays was $15 \text{ pg cytokine ml}^{-1}$.

Flow cytometry for intracellular molecules. Whole-blood cultures were stimulated with Tat-JEV proteins or Tat-GFP at $50 \mu\text{g ml}^{-1}$ for 6 h at 37°C with $3 \mu\text{M}$ monensin included during the last 4 h. Staining for intracellular IFN- γ was carried out as described previously (Kumar *et al.*, 2003a). Lymphocytes were gated based on forward versus side scatter with fluorescence triggering in the FL1 channel (CD3-FITC) to gate on CD3^+ T lymphocytes. For each analysis, 20 000–50 000 gated CD4/8hi (FL3) cells were acquired using a FACScan flow cytometer. Data were analysed using WINLIST software (Verity Software House). Positive staining was affirmed using isotype-matched controls and by comparing the dot-plots of cultures stimulated with test and control antigens.

HLA typing. HLA typing of PBMCs from JEV-exposed donors was performed by microlymphocytotoxicity using HLA typing trays purchased from Biotest (Germany).

Statistical analyses. Results of LPAs (mean SI value \pm SEM) and ELISA ($\text{pg ml}^{-1} \pm$ SEM) are given based on triplicate wells. GRAPHPAD PRISM version 3.00 for WINDOWS (GRAPHPAD Software, San Diego, CA, USA) with a significance threshold of $P < 0.05$ was used. The two study groups were compared using the non-parametric Mann-Whitney U-test. Comparisons between responses to different antigens were made using the Wilcoxon rank sum test for paired measurements as well as Friedman's test followed by Dunn's post test for grouped data as appropriate. Age and sex trends for SI and IFN- γ responses were analysed using logistical regression and were found not to significantly influence the outcome.

RESULTS

Specifications of the study cohort

Table 1 lists the particulars of individuals comprising the study cohort. Only those non-vaccinated volunteers with $>1 \log_{10}$ PRNT titres to JEV, the minimal level considered effective for prevention of mosquito-transmitted JEV infection in humans (Tsai *et al.*, 1999), and serum antibodies to the NS3 by RIP were enrolled for the study, the latter criterion indicative of active virus replication. The control group had $<0.2 \log_{10}$ serum PRNT titres and no detectable antibodies to JEV proteins by RIP. PRNT titres of $<0.4 \log_{10}$ and $<0.2 \log_{10}$ for DENV and WNV prevailed in the test and control groups, respectively. The LPA responses and IFN- γ secretion of the two groups to the mitogen PHA and the BCG sonicate, the latter used as a recall antigen since all individuals also hailed from regions of tuberculosis endemicity, were similar to each other (Table 1) and reminiscent of those observed in healthy immune-proficient donors (Katial *et al.*, 1998; Kumar & Satchidanandam, 2000).

Table 1. Characteristics of the study cohort

All data are shown as the mean \pm SEM for three replicates.

Characteristic	JEV-exposed donors	JEV-naïve donors
No. of subjects	24	12
Male-to-female ratio	13:11	7:5
Age (years)	8.5 \pm 0.3 (range 5–11)	8.1 \pm 0.4 (range 6–11)
Serum JEV-PRNT titre	2.03 \pm 0.10* (range 1.10–3.11)	0.05 \pm 0.02* (range 0.00–0.13)
Serum antibodies to NS3	+†	-†
SI‡ to BCGs	8.5 \pm 1.5§ (range 3.4–33.0)	8.4 \pm 1.8§ (range 3.1–25.6)
IFN- γ (pg ml ⁻¹) to BCGs	2398 \pm 481 (range 451–9148)	2772 \pm 537 (range 1101–6881)
SI‡ to PHA	60.5 \pm 7.4¶ (range 11.1–153.0)	57.4 \pm 7.4¶ (range 19.6–109.1)
IFN- γ (pg ml ⁻¹) to PHA	28 110 \pm 4130# (range 9201–83 970)	33 510 \pm 3741# (range 14 560–61 470)

* $P < 0.0001$.

†The presence of serum antibodies to NS3 was determined by RIP followed by Western blotting of the immunoprecipitates.

‡SI values were obtained as ratios of c.p.m. incorporated into PBMCs in the presence of stimulant to that in the absence. The cut-off SI for a positive proliferative response to BCG sonicate (BCGs) was decided at 3.0.

§ $P = 0.7626$.

|| $P = 0.6357$.

¶ $P = 0.9465$.

$P = 0.1110$, all comparisons between healthy donors with a previous subclinical infection with JEV and healthy JEV-naïve donors made using the Mann-Whitney U-test.

To appraise induction of proliferative responses to JEV proteins due to subclinical infection, we measured [³H]thymidine incorporation in response to VJE. All subjects seropositive for JEV-specific antibodies also displayed positive LPA responses to VJE antigen (SI > 3.0) ranging from 3.1 to 19.5 (mean 8.6 \pm 1.1; Fig. 1). All JEV-naïve donors had SI values < 3.0 (mean 0.5–1.5).

Transduction of Tat PTD-containing JEV proteins

To estimate the contribution of CD4⁺ as well as CD8⁺ T cell subsets towards cell-mediated immune (CMI) responses measured, we expressed the E, NS1, NS3 and NS5 proteins of JEV in fusion to the 11 aa Tat peptide of HIV (Fig. 2a), as Tat PTD-containing proteins have been known to indiscriminately translocate across cell membranes both *in vitro* and *in vivo* (Schwarze *et al.*, 2000). Characterization of the *E. coli*-expressed and purified proteins by SDS-PAGE revealed single species with appropriate sizes (Fig. 2b). Initial flow cytometric experiments suggested that all Tat fusion proteins were efficiently imported into cells within 1 h of addition to PBMCs (Fig. 2c). Subsequent confocal analysis confirmed the intracytoplasmic location, with a majority of the cells staining positive at 3 h. Fig. 2(d) is a representative photograph of PBMCs stained for the presence of intracellular Tat-NS3 and Tat-GFP 3 h after incubation. None of the PBMCs were labelled upon incubation with the purified GFP lacking the Tat peptide, demonstrating the ability of Tat fusion proteins to transduce into cells.

Analysis of target specificities of antiviral proliferative responses in JEV-exposed donors

We next characterized the overall magnitude of JEV-specific lymphoproliferation in the cohort in response to

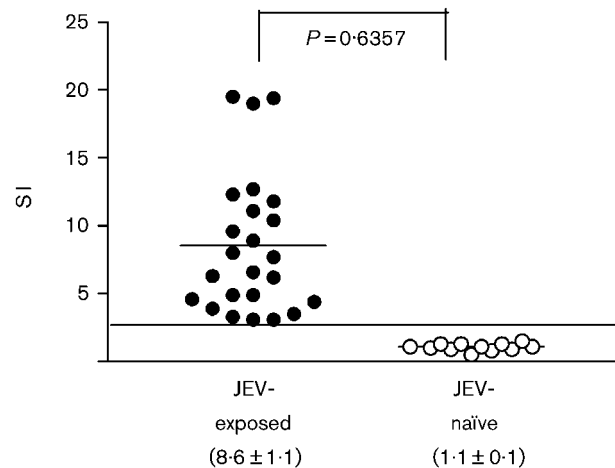


Fig. 1. JEV-infected donors display positive lymphoproliferative responses to total JEV proteins. [³H]Thymidine incorporated by PBMCs during the last 18 h of culture with VJE or VUI antigen for 5 days was measured. SI values denote ratio of mean counts incorporated by PBMCs in the presence of VJE to that of VUI in triplicate wells. The mean positive SI value (cut-off > 3) observed in each group (horizontal bars and values in parentheses) was significantly different from each other.

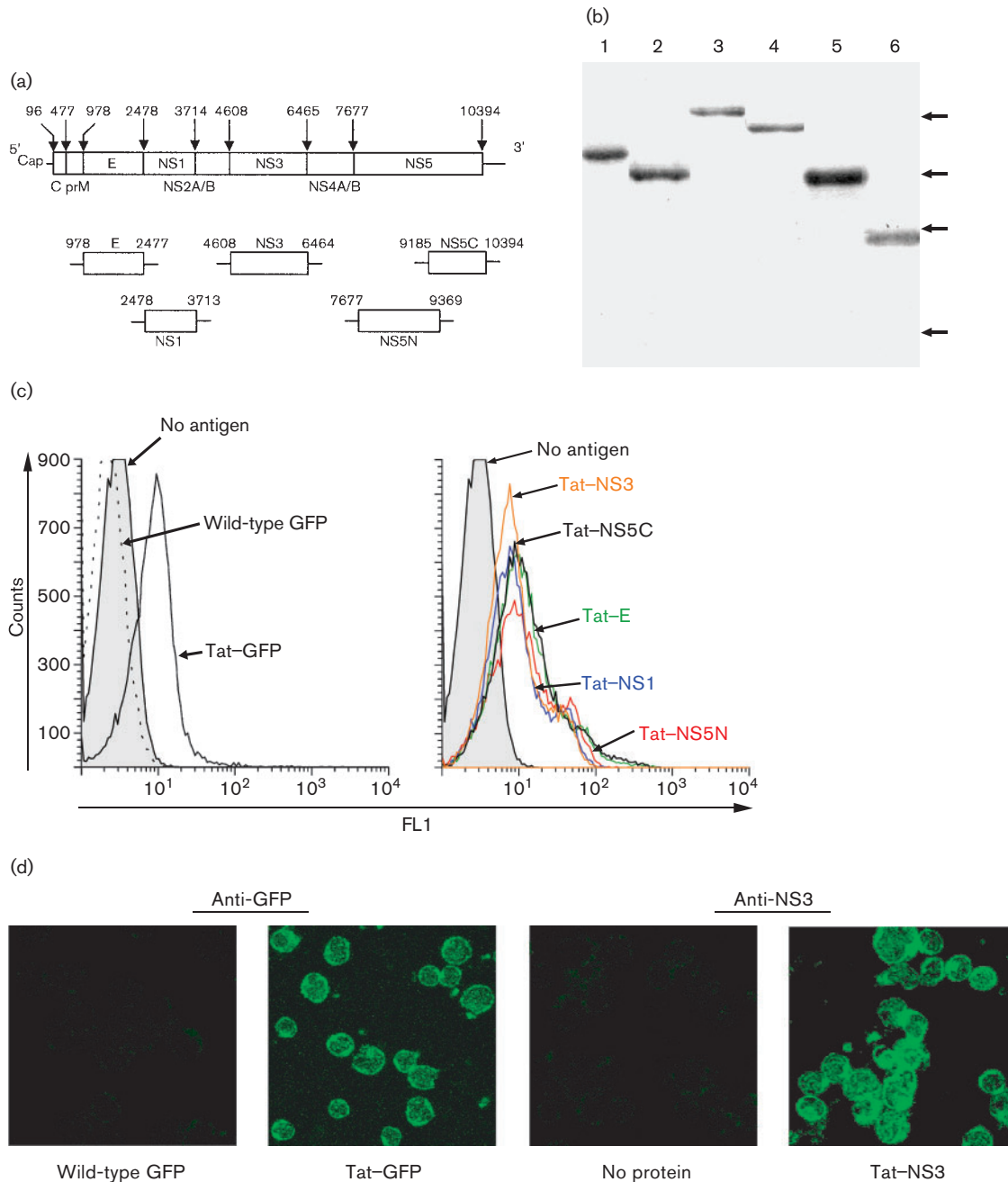


Fig. 2. (a) Schematic diagram of the JEV genome and the pTAT-HA constructs encoding various JEV proteins (Tat-E, Tat-NS1, Tat-NS3 and Tat-NS5 in two halves, Tat-NS5N and Tat-NS5C). The first nucleotide positions of each gene and the cloned fragments are shown. (b) Coomassie blue-stained 10% SDS-PAGE gels in which purified Tat-E (lane 1, ~59 kDa), Tat-NS1 (lane 2, ~49 kDa), Tat-NS3 (lane 3, ~72 kDa), Tat-NS5N (lane 4, ~66 kDa), Tat-NS5C (lane 5, ~48 kDa) and Tat-GFP (lane 6, ~30 kDa) were resolved. Arrows indicate migration of marker proteins of 68, 50, 35 and 21 kDa. (c) Rapid and efficient import of Tat fusion proteins into PBMCs. PBMCs were cultured with purified authentic GFP, Tat-GFP and individual Tat-JEV fusion proteins (333 nM) for 1 h, washed, fixed, permeabilized and stained with a mouse serum to GFP or rabbit sera to each of the individual proteins of JEV followed by corresponding secondary antibodies conjugated to FITC and analysed by flow cytometry. Control cells without added antigen were probed with a mixture of all the primary antisera. For each histogram, the relative fluorescence is shown on a log scale on the x-axis and the number of cells on the y-axis. The shaded histogram shows the background level of fluorescence from these cells. (d) Visualization of intracellular Tat fusion proteins by confocal microscopy. PBMCs were incubated for 3 h with or without purified authentic GFP, Tat-GFP, Tat-NS3 (333 nM) and processed as above after fixing onto coverslips.

the above-mentioned four largest proteins of JEV. Fig. 3 shows the strength and percentage of the LPA responses to each of the individual viral antigens tested relative to the control protein, Tat-GFP. Overall, each and every individual responded to at least one of the four viral proteins (Fig. 3), with the magnitude of the positive responses ranging from 3.0 to 6.0, 23.2, 15.7 and 6.7 for the E, NS1, NS3 and the N-terminal half of NS5, respectively. In striking contrast, an LPA response to NS5C was noted only in donor V21, indicating a paucity of T cell epitopes in this region of NS5 (mean SI 1.3 ± 0.1). Tat-GFP did not stimulate PBMCs from any individual of either group, nor did the control donors respond to recombinant JEV antigens (data not shown), highlighting the specificity of the response to JEV antigens. As noted previously (Kumar *et al.*, 2003b), NS3 dominated as the most frequently recognized protein with nearly 96% of the cohort responding favourably to this protein as against 41.7, 62.5 and 50% for the E, NS1 and NS5 proteins, respectively. Moreover, the relative strength of the LPA responses elicited was significantly higher only for NS3 (mean SI 8.1 ± 0.8) in comparison to the other antigens ($P < 0.0001$, Friedman's test and Dunn's post test). The mean SI values observed with the other antigens did not significantly differ from each other (E, 2.7 ± 0.3 ; NS1, 4.9 ± 1.0 ; NS5N, 3.2 ± 0.3 ; $P > 0.05$, Mann-Whitney U-test).

Cytokine profile of Tat-JEV protein-stimulated lymphocytes

Our previous studies had already indicated that the baculovirus-expressed NS3 protein induced the synthesis of IFN- γ transcripts that mark a Th1 profile of lymphocyte response (Kumar *et al.*, 2003b). We carried out a comparative analysis of the secreted IFN- γ levels on stimulation with each of the JEV antigens being investigated. Fig. 4(a) depicts the breadth and magnitude of the IFN- γ secretion profile observed, and Fig. 4(b) the individual levels to each antigen tested. Tat-NS3 was the only JEV protein that stimulated the production of >500 pg IFN- γ ml $^{-1}$ in 66.7% of the donors tested (16/24, median 691 pg ml $^{-1}$; Fig. 4a) followed by Tat-NS1, Tat-NS5N and Tat-E inducing the above magnitude of responses in 33.3, 12.5 and 4.17% (8/24, 3/24, 1/24 and median levels of 304, 66, 169 pg ml $^{-1}$, respectively) of the JEV-exposed group. PBMCs from donor V21 alone, who incidentally also had the strongest responses to other JEV proteins, yielded 607 pg IFN- γ ml $^{-1}$ to the Tat-NS5C protein. Tat-NS3 was also the single largest contributor to the total viral antigen-specific IFN- γ produced by these individuals. In fact, in 15 out of 24 children studied, NS3 contributed more than 50% of the sum total of the IFN- γ produced by the four JEV antigens tested. The differences in mean IFN- γ produced in response to each of these antigens was highly significant

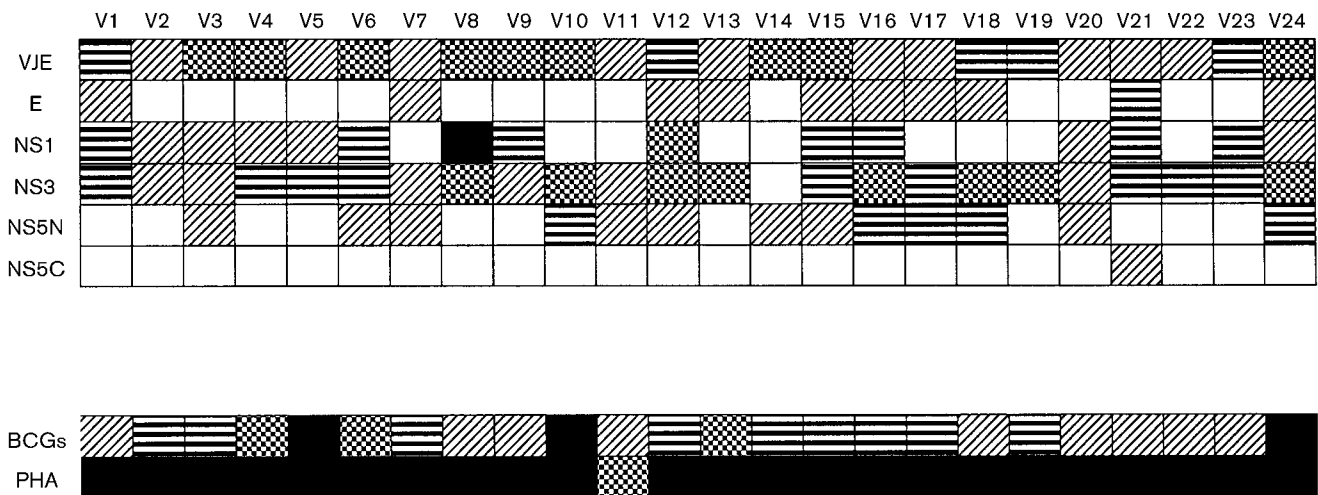


Fig. 3. Comparison of overall lymphoproliferative response to JEV proteins. Response of PBMCs to various stimulants in the 24 healthy JEV-exposed children (V1 to V24) have been graded according to the range of SI values obtained by dividing the counts incorporated with the test antigen (VJE/recombinant JEV proteins/PHA and recall antigens) by that with control antigen (VUI/Tat-GFP/no antigen). A cut-off value >3.0 decided a positive response to the individual antigens. Open boxes, negative responses (SI $<$ cut-off value); hatched boxes, positive SI values <5.0 ; horizontal-lined boxes, SI values between 5.0 and 10.0; checked boxes, SI values between 10.0 and 20.0; solid boxes, SI values >20.0 . SI for the control Tat-GFP antigen obtained as a ratio of counts incorporated in the presence of Tat-GFP to that in the absence of any antigen tested from 0.4 to 1.9 for both the test and control groups. The mean SI values in the control group for each of the antigens tested with were 1.1 ± 0.1 , 1.2 ± 0.1 , 0.9 ± 0.1 , 1.0 ± 0.1 , 1.2 ± 0.1 and 1.0 ± 0.1 for VJE, Tat-E, Tat-NS1, Tat-NS3, Tat-NS5N and Tat-NS5C, respectively.

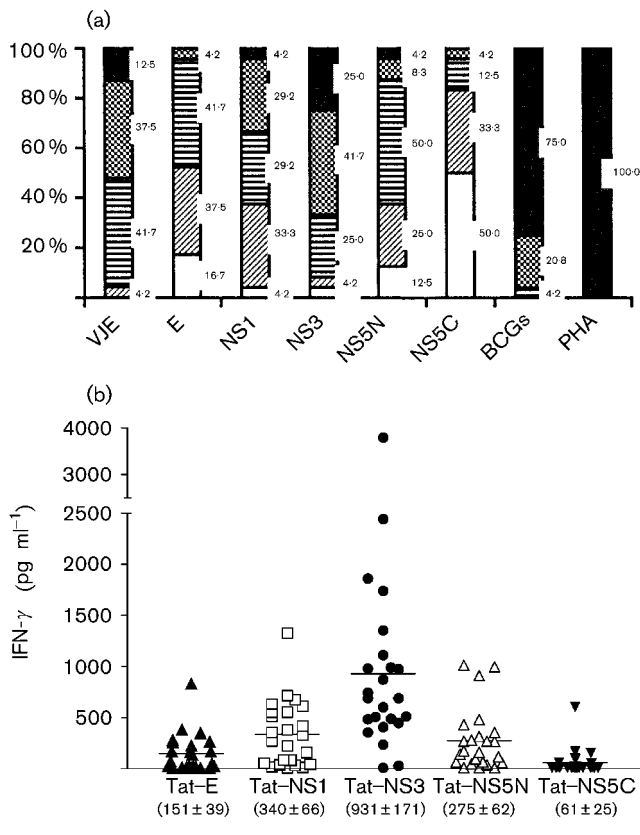


Fig. 4. Summary of IFN- γ production in response to Tat-JEV proteins. (a) IFN- γ secretion by PBMCs to various stimulants in the 24 healthy JEV-exposed children have been graded according to the range of IFN- γ levels obtained by subtracting the IFN- γ secreted in the presence of control antigen (VJI/Tat-GFP/no antigen) from that with the test antigen (VJE/recombinant JEV proteins/PHA and recall antigens). Open bars, undetectable IFN- γ (<15 pg ml⁻¹); hatched bars, IFN- γ levels between 15 and 100 pg ml⁻¹; horizontal-lined bars, IFN- γ levels between 100 and 500 pg ml⁻¹; checked bars, IFN- γ levels between 500 and 1000 pg ml⁻¹; solid bars, IFN- γ levels >1000 pg ml⁻¹. The numbers indicate the percentage of individuals whose PBMCs secreted the corresponding levels of IFN- γ in response to each antigen. (b) IFN- γ secretion in antigen-stimulated lymphocyte culture supernatants for each subject obtained after subtracting IFN- γ secreted in the presence of Tat-GFP is shown. The limit of detection was 15 pg IFN- γ ml⁻¹. The horizontal lines and values in parentheses indicate the mean levels of IFN- γ observed with each antigen. The levels of IFN- γ secreted in response to Tat-GFP ranged from below detection limits to 53 and 86 pg ml⁻¹ in the test and control groups, respectively. The mean values for secreted IFN- γ levels in the control group for each of the antigens tested were 14 ± 2 , 14 ± 2 , 18 ± 5 , 14 ± 1 , 12 ± 1 and 14 ± 2 pg ml⁻¹ for VJE, Tat-E, Tat-NS1, Tat-NS3, Tat-NS5N and Tat-NS5C, respectively.

($P < 0.0001$, Friedman's test and Dunn's post test), with only the NS3 protein eliciting significantly higher levels (mean 931 ± 171 pg ml⁻¹; Fig. 4b). Between proteins E,

NS1 and NS5N, the mean levels of IFN- γ induced were not statistically different (151 ± 39 , 340 ± 66 and 275 ± 62 pg ml⁻¹, respectively; $P > 0.05$, Mann-Whitney U-test). Tat-GFP was not an IFN- γ stimulating antigen in the cohort (mean 19 ± 3 pg IFN- γ ml⁻¹). IL-4 was undetectable (<15 pg ml⁻¹) on stimulation with any protein in all subjects, indicating a dominant Th1 profile of T cells responding to the recombinant proteins tested.

Phenotypic characterization of antiviral lymphocyte responses

An advantage of using proteins fused to the Tat PTD of HIV is the activation of both CD4⁺ and CD8⁺ T cells by presentation of processed peptide epitopes on both MHC class II and I, respectively (Shibagaki & Udey, 2002). We next sought to determine the subtype of T cells that served as the source of this cytokine in 10 of the 24 JEV-exposed donors by immunofluorescent staining and flow cytometric analysis of antigen-stimulated whole-blood cultures. Fig. 5(a) shows the activation of CD8⁺ T cells in response to each of the exogenously added soluble Tat-JEV fusion proteins in two of the best responders. While IFN- γ synthesis by both subsets of T cells was observed in several individuals in response to the antigens of JEV (Fig. 5a, b), there was an obvious difference in the subset of T cells activated by each antigen. Stimulation with Tat-E favoured activation of CD4⁺ T cells rather than CD8⁺ T cells in many individuals ($P = 0.0078$, Wilcoxon rank sum test) while a distinctly higher percentage of IFN- γ -positive CD8⁺ T cells in comparison to CD4⁺ T cells was visualized when cultures of whole blood were supplemented with Tat-NS3 ($P = 0.0137$, Wilcoxon rank sum test). A conspicuous absence of NS5C-responding T cells was noted in six of the 10 individuals tested (Fig. 5b). The NS1 and the NS5N proteins did not exhibit tendencies of preferential activation of a single subtype of T cells ($P > 0.05$, Wilcoxon rank sum test). As foreseen from the measurements of secreted IFN- γ , the percentages of NS3-responsive T cells, both CD4⁺ and CD8⁺, were maximal among the antigens tested. The stimulation of both subtypes of T cells by most of the non-structural proteins of JEV indicates that these proteins house both class I and class II epitopes while the class II epitopes of E are preferentially recognized during natural JEV infections.

Frequency of recognition of JEV antigens

The frequency of recognition of each of the JEV proteins based on SI values was undoubtedly the highest for NS3, with PBMCs from $>95\%$ of the study cohort responding to this protein. In order to adjust for the size difference of the proteins, we divided these values by the number of amino acids for each recombinant protein studied. The highest amino-acid-adjusted scores of 0.15 each retained by NS1 and NS3 following this treatment of data (Table 2) correlated well with their superior recognition frequencies (Spearman's $r = 0.9747$; $P = 0.0167$). NS5N followed with 0.09, a surprising observation when contrasted with the

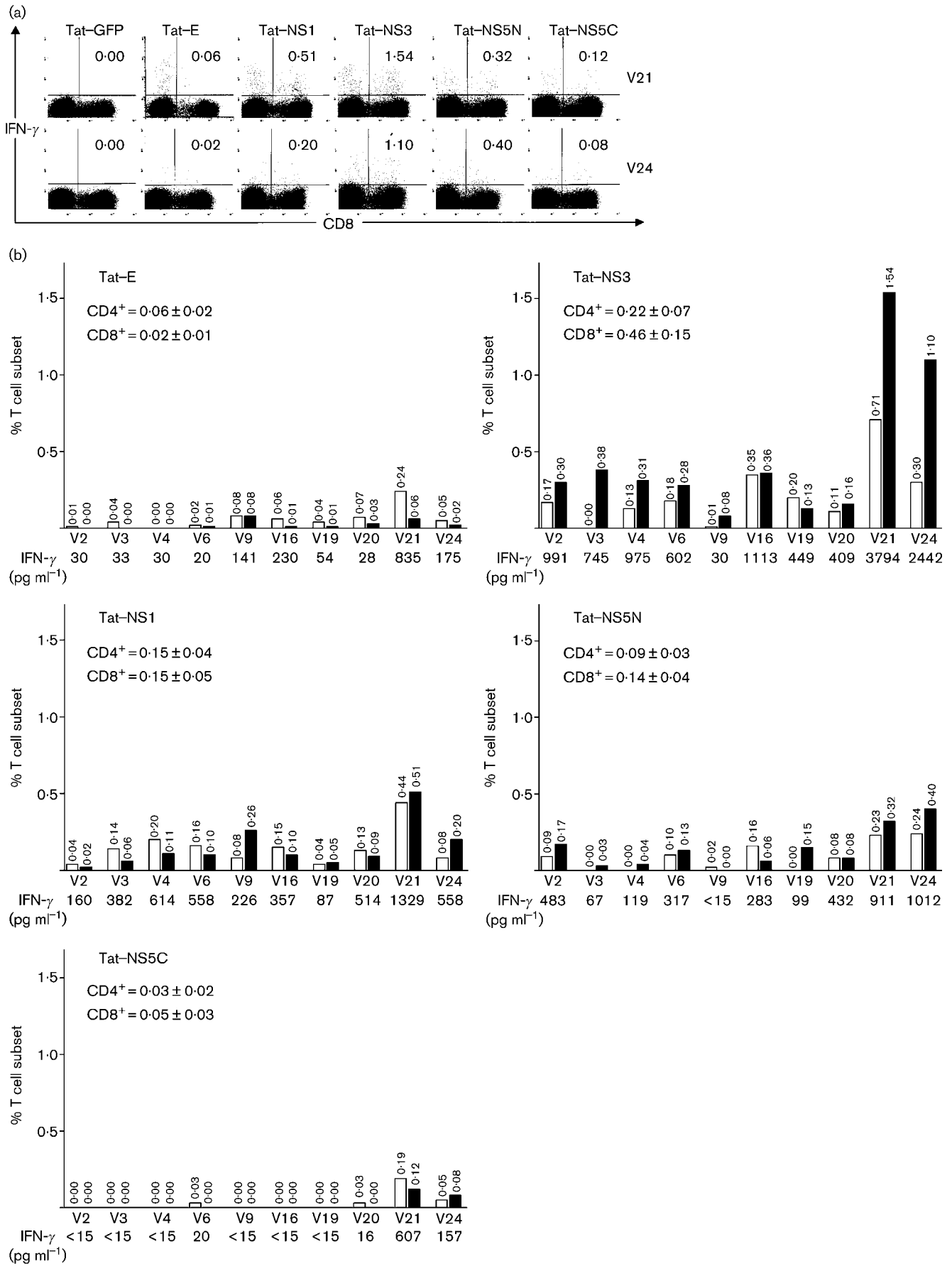


Fig. 5. Analysis of IFN- γ production at single-cell level by flow cytometry in children with JEV exposure. (a) Representative examples of the flow cytometric analysis of lymphocytes stained intracellularly for IFN- γ . Whole blood was stimulated with recombinant Tat fusion proteins for 6 h, fixed, permeabilized and stained with anti-human CD3 fluorescein isothiocyanate, anti-human CD8⁺ biotin and anti-human IFN- γ phycoerythrin followed by streptavidin Cychrome. Dead cells and debris were excluded by forward and side scatter. Cells were gated sequentially on lymphocytes, followed by CD3, and analysed for expression of CD8⁺ and IFN- γ . The frequencies in the upper quadrants are IFN- γ -producing cells as percentages of the total number of CD8⁺ T cells after subtracting the background frequencies observed with Tat-GFP. No positive staining was observed with matched isotype control antibodies. (b) Comparison of frequencies of CD4⁺ (open bars) and CD8⁺ (solid bars) IFN- γ -producing T cells to each of the Tat-JEV fusion proteins after independent staining for CD4⁺ and CD8⁺ T cells with corresponding antibodies. The numbers depict percentages of each T cell subtype responding with IFN- γ production after subtracting the background frequencies observed with Tat-GFP (mean frequencies 0.02 \pm 0.01 %, CD4⁺ as well as CD8⁺ T cells).

lack of response to the full-length NS5 protein tested previously as recombinant Sf21 lysates (Kumar *et al.*, 2003b). The E protein, incidentally the most dominant for humoral responses, scored the lowest with 0.08 while NS5C did not score (Table 2), indicating that these proteins were poor targets of human CMI responses. Despite the similar scores of 0.15, the vastly superior percentage of individuals responding to NS3 over NS1 (95.8 versus 62.5 %), as well as the magnitude of responses elicited by NS3, pointed to the obvious dominant immunogenicity of NS3 for CMI responses in a naturally immunized JE-endemic cohort. These observations could simply reflect a common HLA distribution of the responding volunteers; however, the observations on the highly immunogenic nature of NS3 from related flaviviruses in individuals with varied HLA haplotypes (Kurane *et al.*, 1991, 1995; Livingston *et al.*, 1995; Mathew *et al.*, 1996, 1998; Co *et al.*, 2002) prompts us to believe that NS3 could probably be the most immunodominant antigen among all the proteins of flaviviruses. Furthermore, typing of the class I alleles of some of the JEV-exposed donors in our cohort (Table 3) revealed an appreciable diversity in HLA distribution. The presence of CD8⁺ T cells recognizing a single protein (NS1, NS3 or NS5) in at least two volunteers with no common HLA class I alleles suggests the presence of a minimum of two epitopes on each protein recognized by independent HLA alleles.

Table 2. Frequency of recognition of individual JEV proteins adjusted for protein length in the 24 JEV-exposed individuals

Protein	Length of recombinant protein (aa)	Frequency of recognition (%)	Amino-acid-adjusted score*
E	500	41.7	0.08
NS1	412	62.5	0.15
NS3	619	95.8	0.15
NS5 (N)	564	54.1	0.10
NS5 (C)	403	0.0	0.00

*Frequency of recognition divided by the length of the recombinant protein.

DISCUSSION

Long-term protection from viral infections requires the development of virus-specific memory T cells that recognize viral antigens in association with class I or II molecules. Likewise, successful antiviral immune responses almost always rely on strong induction of IFN- γ , the cardinal cytokine that marks the Th1 type of T cell response. A Th1 cytokine profile is linked to good prognosis not only in viral but also in a number of parasitic, fungal and bacterial infections, while a Th2-type response leads to severe pathology and exacerbation of the disease (Mosmann & Sad, 1996). In this report, we characterized T cell responses prevailing in children from JE-endemic areas who had experienced subclinical infections and in whom virus replication was restricted to the periphery without progression to encephalitis, which would therefore provide important pointers to those arms of immunity required for self-limiting JEV infections. Thus, the presence of strong Th1 CMI responses directed to non-structural proteins in healthy individuals suggests their requirement for resistance against JEV.

Table 3. HLA haplotype of healthy JEV-exposed donors included in the study

Donor	HLA haplotype
V1	A1, A29, B7, B57(15), Cw6, Cw17
V2	A24(9), A1, B13, B35, Cw4, Cw6
V3	A2, A11, B7, B60(40), Cw6, Cw7
V4	A2, A24(9), B51(5)
V5	A2, A32(19), B51(5), B49(21), Cw7
V6	A32(19), A68(28), B51(5), B61(40)
V7	A2, A28, B35, B61(40)
V11	A31(19), B51(5), B8, Cw6, Cw7
V12	A2, A11, B35, B61(40)
V13	A24(9), B27, B13, Cw6
V15	A11, B35, Cw4
V17	A31(19), B13, B8
V18	A23(9), A24(9), B7, B44(12), Cw4
V23	A30(19), A33(19), B44(12), B13, Cw6, Cw7
V24	A32(19), A1, B49(21), B57(17), Cw6, Cw7

We had previously reported a marked tendency for the NS3 protein to dominate as a CD4⁺ T cell antigen in healthy individuals subclinically infected with JEV when exogenously added as lysates of recombinant baculovirus-infected Sf21 cells to PBMC cultures. These helper T cells could be envisaged to play a critical role in antiviral immunity, either indirectly, by providing helper function for neutralizing antibody and CTL responses, or directly, through the antiviral effects of IFN- γ produced by Th1 cells. The development of strong CD8⁺ cytotoxic T lymphocyte responses during acute infection has been demonstrated to be required for virus clearance and the persistence of these responses necessary to prevent disease progression in several important viral infections such as HCV, *Hepatitis B virus*, HIV, etc. (Chisari, 1997; Lechner *et al.*, 2000; McMichael & Rowland-Jones, 2001). Our choice of providing the four largest JEV proteins, representing nearly 71% of the coding potential of the JEV genome, as fusions to the Tat PTD of HIV was aided by the absence in any of the individuals under study, both in the test and control groups, of positive responses to either the Tat peptide or the GFP antigen. Several studies have identified proteins from various flaviviruses as targets of either CD4⁺ T cells when the protein used to establish the T cell clones was provided as a purified extracellular antigen (Kurane *et al.*, 1991; Zeng *et al.*, 1996; Konishi *et al.*, 1998; Aihara *et al.*, 1998) or CD8⁺ T cells following viral infection that provide antigens intracellularly (Livingston *et al.*, 1995; Mathew *et al.*, 1996; Co *et al.*, 2002). This study has resulted in the simultaneous detection of precursors of both T cell subsets activated by the Tat fusion antigens, without the involvement of long-term *in vitro*-generated T cell clones that may be biased in the relative recognition of proteins and not necessarily relevant *in vivo*.

We detected significant differences in the breadth and magnitude of LPA responses, cytokine profiles and phenotypes of responding T cells between the various antigens compared, despite all antigens stimulating a common dominant Th1 immune profile. The NS3 protein of JEV scored as the most frequently targeted antigen, eliciting the highest levels of proliferation and IFN- γ secretion. The presence of highest frequencies of circulating T cells, CD4⁺ as well as CD8⁺, recognizing NS3 in comparison to E, NS1 and NS5, as well as the ability of more than two independent HLA alleles to bind to and present epitopes from NS3, underscores the immunogenicity of this antigen during natural exposure to the virus. Incidentally, CMI responses to the NS3 protein have also been found to dominate in donors immune to DENV (Kurane *et al.*, 1991; Livingston *et al.*, 1995; Zeng *et al.*, 1996; Mathew *et al.*, 1996, 1998) as well as in patients responding to therapy during HCV infections (Diepolder *et al.*, 1995). In addition, epitopes on the NS3 protein have also been found to be serotype cross-reactive, with T cell reactivities most often directed towards epitopes conserved across flaviviruses (Kurane *et al.*, 1991, 1995; Livingston *et al.*, 1995; Mathew *et al.*, 1996; Zeng *et al.*, 1996).

E hardly stimulated CD8⁺ T cells even under conditions of *in vitro* antigen stimulation that promoted cytoplasmic localization of this protein for processing and presentation by class I MHC. Live-attenuated vaccines of DENV and YFV have, however, been shown to induce E-specific T cells (Mathew *et al.*, 1996; Co *et al.*, 2002). Based on previous observations (Konishi *et al.*, 1995; Desai *et al.*, 1995; Kumar *et al.*, 2003b), E of JEV, a strong antibody-inducing antigen, appears to poorly prime CMI responses when in the company of other dominant T cell-eliciting antigens of the virus, as happens during natural infection with JEV. The NS1 protein, which is about two-thirds the size of NS3, nevertheless displayed a rate of recognition equivalent to that of NS3. In fact, NS1 also induces cytolytic antibodies in the murine model of JEV (Lin *et al.*, 1998) and is a dominant target of IFN- γ -producing T cells in YFV-immune donors (Co *et al.*, 2002). Curiously, the N-terminal half of NS5 was fairly antigenic as a Tat fusion for T cells, although the full-length protein, provided as Sf21 lysates, was totally inept in this feature (Kumar *et al.*, 2003b). This could not be ascribed to an exclusive CD8⁺ T cell recruitment by NS5, though NS5N did activate considerable frequencies of CD8⁺ T cells. Thus, providing antigens as Tat fusion proteins appears to increase the sensitivity of detection of T cell antigens.

A strong anamnestic humoral response, absolutely dependent on T cell help, has been envisaged as the best defence strategy against JEV (Konishi *et al.*, 1999). In addition, a strong polyclonal and multispecific CD8⁺ T cell response with the production of the antiviral cytokine IFN- γ would eliminate virus-bearing cells, inhibit virus replication and thus limit virus persistence. The recent evidence of the requirement for robust CD4⁺ T cell help during priming for sustenance of a strong antiviral memory CD8⁺ T cell response (Shedlock & Shen, 2003) makes it essential for an efficacious vaccine to induce all the immune parameters described above. While E has been shown to be capable of eliciting helper T cells in individuals immunized with E-based vaccines (Aihara *et al.*, 1998), the potency of E as a T cell antigen appears to be eclipsed amidst the other strong T cell-recruiting non-structural proteins during natural infections of JEV. On the other hand, immune responses, presumably T cell-mediated, elicited by immunization with plasmids encoding NS1-2A, NS3 and NS5 of JEV were each by themselves not sufficient to induce protective immunity in the mouse model (Chen *et al.*, 1999). Thus, the operative mechanism for long-term protective immunity in adults from JE-endemic areas who are resistant to JEV infections is probably attributable to concomitant development of both humoral and CMI responses resulting in boosting of the E-specific antibody response-specific T cell help mediated by non-structural proteins. Thus, the short-term memory as well as the magnitude of neutralizing antibodies induced by the killed E-based vaccine could potentially be extended by complementing this neutralizing antibody recruiting element with T cell

antigens such as NS3 and NS1 that are capable of recruiting IFN- γ -secreting polyclonal helper and cytotoxic T cells.

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