MATERIAL AND METHODS

Biochemical genetic monitoring of BALB/c mice

BALB/c mice used for all the experiments were bred and maintained at the Institute’s Animal Housing Facility. Genetic monitoring of these inbred mice was carried out through biochemical markers at the Advanced Centre for Treatment, Research & Education in Cancer (ACTREC) Mumbai, India. Most biochemical markers are isoenzymes. Markers are polymorphic and located throughout the genome. Homogenates of mice organs were applied to cellulose acetate gels for electrophoresis. Allelic forms of isoenzymes, termed cozymes, migrate at slightly different rates. Inbred strains should express only one form of each biochemical marker.

Preparation of virus pools and titration

JEV 733913, an Indian strain, isolated from Bankura district in West Bengal (1973) was used for all experiments. Phylogenetic analysis of the E gene sequence (Accession No. Z34095) placed this strain into Genotype III of JEV along with other well-known strains, P20778 and the Nakayama strain (46).

Virus was propagated in infant mice. Mice were obtained from the Institute’s animal housing facility and maintained according to the “Committee for Protection, Supervision and Control of Experiments on Animals” (CPSCEA) guidelines. Mice showed symptoms of infection on the 3rd day after IC inoculation of the virus. A 10% suspension of infected mice brain in 0.75% bovine albumin in phosphate saline (BAPS) was prepared, clarified of cell debris by low speed centrifugation, and aliquots of the supernatant were made and stored at -70°C.

Virus was then titrated in 14-day-old mice by inoculation via both the IP and IC routes, while adult mice were titrated by the IC route or by IP injection of virus followed by IC administration of 1% starch in phosphate buffered saline (PBS). The LD_{50} (Lethal Dose_{50}) was derived using the method of Reed and Muench (226).

For in vitro experiments, a tissue culture derived virus pool was prepared. Briefly, PS (Porcine Stromal Kidney) cells were seeded into a T-75 tissue culture flask (Corning). On attainment of a uniform cell monolayer, a 10^{-3} dilution of 10% mouse brain suspension of JEV was added and incubated until cytopathic effect (CPE) was observed. The supernatant containing virus was passaged 2 or 3 times in PS monolayers until adapted to the PS cell line. After observation of 80-90% CPE, 10% fetal calf serum (FCS) was added to the medium and centrifuged at 2000 RPM for 10 min. Aliquots of the tissue culture virus pool were made and
stored at -70°C. Virus was titrated by end point assay on PS monolayers in 96 well plates and by plaque assay in 24 well plates. TCID\textsubscript{50} (Tissue culture infective dose \textsubscript{50}) was calculated by the Reed and Muench method.

\[
\text{Reed and Muench Index} = \frac{\text{(% Mortality above 50%)} - 50\%}{\text{(% Mortality above 50%)} - \text{(% Mortality below 50%)} - 50\%}
\]

This index was applied to the virus dilution that produced the percentage of mortality immediately above 50%, giving the dilution that produced one LD\textsubscript{50} unit. For tissue culture, the mortality percentage was replaced by the cytopathogenic percentage to give the dilution that produced one TCID\textsubscript{50} unit.

**Preparation of JE antigens for in vitro assay**

**Purified virus antigen:** PS cells were grown on a large scale in Roux bottles and expanded into Roller cultures in Burlar bottles. On growth of a uniform monolayer, cells were infected with virus and incubated until 80% CPE was attained. Cells were scraped off and the supernatant was clarified by centrifugation for 45 min at 4500 RPM (Hettich). The supernatant containing virus was ultrapelleted by centrifugation at 45,000 RPM for 3 hrs (Sorvall). The pellet was dissolved in a minimum volume of 1X NaCl tris-EDTA (NTE) buffer and layered upon a linear density gradient of 10-40% sucrose. This was centrifuged on a swing bucket rotor at 27,000 RPM for 2 hrs and 30 min. 1ml fractions were collected and tested for virus by the Haemaglutination (HA) test. The fraction containing the purified virus was then pelleted by ultracentrifugation and resuspended in a minimum volume of NTE buffer. Antigenicity of the purified virus was then determined by enzyme linked immuno-sorbent assay (ELISA).

**Acetone extracted mouse brain derived antigen:** A 20% mouse brain suspension from infected mice was prepared in borate saline containing 8.5% sucrose (227). The solution was homogenized and inactivated with 0.01% BPL (β-propiolactone) for 48 hrs at 4°C, while constantly maintaining pH. The JEV antigen was then extracted twice with 20 volumes of acetone, reconstituted in borate saline, clarified by centrifugation at 10,000 RPM for 2 hrs and distributed into glass ampoules which were lyophilized and stored. The antigen was further tested for CPE and HA activity.

**Tissue culture derived JE antigen:** A confluent monolayer of PS cells in a Roux bottle was infected with JEV. On attainment of 50-60% CPE, cells were scraped off and sonicated on ice for 10 min (30” pulse on, 30” pulse off) with a sonicator (Sonics, Vibra Cell). The
suspension was centrifuged at 2500 RPM for 10 min in order to clear cell debris and the supernatant was inactivated with 0.01% BPL for 48 hrs at 4°C. The supernatant was tested for infectivity by adding onto a monolayer of PS cell line and checking for CPE. Control antigen of uninfected PS cells was also prepared. Protein was estimated by the Nanodrop 1000 spectrophotometer (Nanodrop technologies).

**JEV ELISA**

Microtitre wells (Nunc, Immunosorp) were coated overnight at 4 °C with a mix of JEV specific monoclonal antibodies (HS3+ HS4) in carbonate buffer (pH 9.6). Wells were blocked at room temperature (RT) for 1 hr with 1% gelatin in PBS. After removing the gelatin solution, 50μl of neat, 1:10 and 1:20 dilutions of JEV antigens in PBS + 0.05% Tween 20 (PBST) were added: a) Acetone extracted antigen, b) Tissue culture derived, inactivated antigen, and c) Sucrose density gradient purified antigen. Uninfected PS cell lysate and normal mouse brain antigen (N-antigen) were added as negative controls, while JEV infected mouse brain suspension was added as a positive control. Conjugate control and substrate control were used as reaction controls. The plate was incubated at 37°C for 1hr 30 min at the end of which wells were washed with PBST using an ELISA washer (Nunc Immuno Wash). 50μl of pre-titrated, biotinylated, flavivirus specific monoclonal antibody, HX-2 was added and incubated for 1 hr 30 min at 37 °C. Wells were washed with PBST and 50 μl of pre-titrated Streptavidin- horse radish peroxidase (HRP, Sigma) was added and incubated for 30 min at 37 °C. After thorough washing with PBST, the substrate H2O2 and colour developer O–phenylenediamine dihydrochloride (OPD, Sigma) were mixed in citrate phosphate buffer and 100μl of this solution was added to all wells. Once colour had developed, reaction was stopped by adding 100μl of 4N H2SO4. Plates were read at 492 nm in a spectrophotometer (Molecular devices). P/N ratios were calculated by dividing the optical density (OD) values of the sample by the OD value of the negative control. A ratio greater than 2 indicated that the sample tested was positive for the antigen (JEV).

**Mice immunization**

The immunization dose and schedule was standardized before the actual immunization procedure. Adult BALB/c mice were immunized by the IP route with 10^3 LD50 of the virus (10,000 PFU). Three doses of live JEV were administered on days 0, 14 and 28. Mice were bled at appropriate time points and sera was analysed for neutralizing antibodies. Immunized mice were challenged with a lethal dose (100 LD50) of virus by the IC route and
100 LD$_{50}$ of virus by IP virus followed by IC injection of 1% starch solution in PBS. Control mice were sham inoculated with normal mouse brain suspension in 0.75% BAPS (N-antigen). The dose and schedule which resulted in 100% survival of the immunized mice after virus challenge was selected.

In addition, adult, immunocompetent, BL6, heterozygous (nu/+ ) mice were immunized by the IP route with 10$^3$ LD$_{50}$ of the virus. Three doses of live virus were administered on days 0, 14 and 28. Splenocytes from immune animals were consequently used for cell transfer into immuno-deficient, homozygous (nu/nu) mice.

**Neutralization test**

The virus neutralization test (NT) was carried out to confirm the presence of virus specific antibodies in the sera of immunized mice. Mice were bled on the 13th day post every immunization by the retro-orbital route. Sera was collected and inactivated at 56°C for 30 min. Serial dilutions of sera in dulbecco’s minimum essential medium (DMEM) were then added to the wells of a round bottomed microtitre plate (Nunc). JEV immune peritoneal fluid (JEV IPF), JEV specific monoclonal antibody (HS-2) and normal mouse sera were used as controls. To this, an equal volume (60μl) of 100 TCID$_{50}$ of a tissue culture derived pool of JEV- 733913 in DMEM + 5% FCS was added and the plate was incubated at 37°C for one hour. 100μl of this pre-incubated mix was then added to a pre-formed monolayer of PS cells in a 96 well flat bottomed microtitre plate and incubated for 3 days. Serial dilutions of virus without sera were also added in order to determine the exact time for termination of the experiment. The plate was then washed with normal saline and stained with amido black. The NT titre was expressed as the reciprocal of the serum dilution at which 50% CPE was obtained.

**Lymphocyte Proliferation Assay**

**a)** Splenocytes from immunized mice were harvested and spleens were processed to make a single cell suspension. 2 x 10$^5$ splenocytes were plated/ well of a 96 well flat bottomed microtitre plate (Nunc). Cells were stimulated with live and inactivated JEV antigen. ConA (Sigma) was used as a positive mitogenic control while splenocytes from naïve animals stimulated with JEV antigen and unstimulated immune splenocytes served as negative control. Cells were incubated for 78 hrs and pulsed with 1 μCi/well of [$^3$H] thymidine (BRIT) for a further 18 hrs. Cells were then harvested on to GF/C (Whatman) filter disks. The filter disks were dried and 1 ml of scintillation fluid added, following which
the incorporated radioactivity was measured as counts per minute (cpm) in a β-counter (1209 LKB Rackbeta).

b) Naïve, non-immunized mice were harvested and spleens were processed as mentioned. Splenocytes were plated at a concentration of 2 x 10^5 cells/well of a 96 well flat bottomed microtitre plate (Nunc). After adherence for 2 hours at 37 ºC, plates were washed 2 times with DMEM to remove non-adherent cells. The adherent cells were stimulated with 5 and 10 multiplicity of infection (MOI) of live JEV and 10 and 20 μg/ml of mouse brain derived, acetone extracted JEV. Con A (1μg/ml) served as a positive mitogenic control. After overnight incubation, leftover antigen was washed off with DMEM. Immune splenocytes were harvested and CD4+ and CD8+ T cells were isolated as described below. 2 x 10^5 CD4+ or CD8+ cells were plated onto each well containing the antigen stimulated APCs. CD4+ and CD8+ T lymphocytes from naïve, non-immunized mice served as control. After 3 days in culture, wells were pulsed with 1 μCi/well of [3H] thymidine (BRIT) for 18 hrs. Cells were then harvested on to GF/C (Whatman) filter disks and the incorporated radioactivity was measured by liquid scintillation as cpm in a β-counter (1209 LKB Rackbeta).

**Cell separation**

Immunized mice were sacrificed 1-week post the last immunization dose by CO₂ asphyxiation. Blood was drained by cardiac puncture and spleens were harvested. A single cell suspension of splenocytes was prepared by teasing the spleen with forceps and passing through a nylon mesh. Red blood cells (RBCs) were lysed with RBC lysing solution (eBioscience), followed by 2 washes in MACS buffer (PBS + 0.5% BSA + 2mM EDTA). Viability staining was done with Trypan blue. Separation of various splenocyte subsets was then carried out according to the manufacturer’s protocol using the Miltenyi Biotec Midi MACS kit. In short, splenocytes were incubated with CD4+ or CD8+ specific magnetic bead conjugated antibodies in MACS buffer for 15 min at 4 ºC. CD4+ and CD8+ T cells were then either isolated or depleted from the splenocyte population by passing the labelled cells through a magnetic cell separator. Cell depletion was carried out by negative selection using LD columns (Miltenyi Biotec) while cell isolation was done by positive selection using LS columns (Miltenyi Biotec). Purity of the population (>94% for isolated cells and >98% for depleted cells) was confirmed by flow cytometric analysis using phycoerythrin (PE) conjugated rat anti-mouse CD4+ and CD8+ antibodies (BD Biosciences).
Cell staining for flow cytometric analysis

Flow cytometric analysis was done to confirm the purity of magnetically separated splenocytes and to test for trafficking of adoptively transferred Carboxy-fluorescein diacetate succinimidyl ester (CFSE) stained splenocytes into the spleens of recipient mice. A single cell suspension of splenocytes was prepared by filtering through a nylon mesh. This was layered carefully over 5 ml of Histopaque-1083 (Sigma) in a 15 ml centrifuge tube and centrifuged for 30 min, RT at 2300 RPM using a swing bucket rotor (International Centrifuge). The Buffy layer was collected and washed 2 times. 1 x 10^6 splenocytes or magnetically separated cells in 100μl of FACS buffer (PBS + 1% BSA + 0.01% Na azide) were taken in 1.5 ml tubes (Eppendorf) and blocked for 30 min at 4 ºC using 1 μl of FC block (antibodies to mouse CD16/ CD32, BD Biosciences) or 1% normal mouse sera. Cells were then stained with PE conjugated anti mouse CD4+ and CD8+ antibodies. Cells were also stained with fluorochrome conjugated isotype controls for all the antibodies. Stained cells were washed in FACS buffer and stored overnight in 500μl of 1% paraformaldehyde solution in PBS. Next day, cells were spun down and resuspended in 1 ml of FACS buffer, added into FACS tubes (Falcon), and acquired on a FACS calibur.

Adoptive transfer

A total of 1x10^7 whole splenocytes from immunized mice were transferred into naïve 14-day-old BALB/c mice by the IV route. Before transfer, reverse transcription – polymerase chain reaction (RT-PCR) for JEV was performed in order to verify that immune cells, infected with JEV, did not transfer the virus into recipient animals and thus initiate an immune response in recipient animals before actual virus infection. Primers specific for a stretch encompassing part of the C-PrM region of JEV (367 bp) were used. For other experiments, 1x10^7 a) CD4+ and b) CD8+ depleted or isolated cells were transferred. Control mice were transferred with splenocytes from normal, non-immunized animals. This was followed 24 hrs later by IP infection with 50 LD50 of JEV. Mice were monitored for 25 days for survival analysis. In another set of experiments, adoptively transferred mice were sacrificed every alternate day PI for cytokine analysis, determination of viral copies in various organs and antibody isotyping.

In addition, adult, athymic BL6 nude (nu/nu) mice were transferred with 1x10^7 whole splenocytes from JEV immunized heterozygous (nu+/) BL6 mice. This was followed 24 hrs later by virus challenge with 50 LD50 of JEV by the IP route followed by inoculation of 1%
starch solution by the IC route. Nude mice were housed in individual ventilated cages (IVC, Techniplast) and monitored for 25 days for survival analysis.

Table 3.1: Primer sequences from the C-PrM region of the JEV genome

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence (5’- 3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
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<tr>
<td>JEF</td>
<td>GCAGAAAGCAAAAACAAAAAGAG</td>
<td>367</td>
</tr>
<tr>
<td>JER</td>
<td>ACGGATCTCCTGCTGCTTGG</td>
<td>367</td>
</tr>
</tbody>
</table>

CFSE staining of splenocytes before adoptive transfer

CFSE staining of JEV immune splenocytes was done before adoptive transfer to confirm the trafficking of adoptively transferred cells into the spleen. A single cell suspension of splenocytes from immunized mice was prepared as previously described. 2x10^7 cells were stained with 8μM of the fluorescent vital dye CFSE in DMEM without FCS at 37°C for 15 min with intermittent shaking. The dye incorporation was stopped by adding an equal volume of ice cold DMEM with 10% FCS. Cells were washed extensively by centrifugation at 2000 RPM for 7 min in order to remove unbound dye. The CFSE stained cells were then injected by the IV route into adult, syngenic BALB/c mice that had been γ-irradiated with 500 rads. 24 hrs later, mice were primed by the inoculation of 100 LD50 of live JEV- 733913 by the IP route. Control mice were injected with an equal volume of PBS by the IP route. Mice were sacrificed 24, 48 and 72 hours PI and the population of splenocytes that were positive for CFSE was detected by flow cytometry. CFSE green fluorescence was collected by a 520 nm filter (FL1). The CFSE positive population was enumerated from a gated population of CD3 positive cells stained with PE-Cy5.5 (FL3).

In vitro stimulation of JEV immune splenocytes with T helper peptides of JEV

Immune splenocytes were expanded in vitro in the presence of peptides from T helper epitopes of JEV. These peptides were designated as peptide 1 (Envelop), peptide 4 (Envelop) and peptide 5 (Membrane), and were selected based on enhanced in vitro proliferation of JEV immune T cells upon stimulation with these peptides (228).
Table 3.2: Amino acid sequences of T helper peptides from structural proteins of JEV

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Peptide name</th>
<th>JEV gene</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Peptide 1</td>
<td>E glycoprotein</td>
<td>439-SIGGVFNSIGKAVHQ-455</td>
</tr>
<tr>
<td>2</td>
<td>Peptide 4</td>
<td>E glycoprotein</td>
<td>346-HVLGRLTTVN-355</td>
</tr>
<tr>
<td>3</td>
<td>Peptide 5</td>
<td>M protein</td>
<td>17-EAWLDSTKAT-26</td>
</tr>
</tbody>
</table>

1x10⁷ splenocytes/ml in DMEM + 10% FCS were seeded into T-75 flasks and cultured for 7 days in the presence of 10 μg/ml concentration of each peptide. During this period, antigen specific cells were seen to proliferate while non-antigen specific cells died out. This was followed by 3-4 cycles of restimulation after every 5 days in the presence of fresh APC’s from naïve, syngenic mice and 20 IU/ml of recombinant IL-2 and peptides in fresh medium. APC were γ- irradiated with a dose of 1500 rads. 1x10⁷ cells were then transferred by the IV route into the tail vein of 14 day-old BALB/c mice. This was followed 24 hrs later with IP challenge of 50 LD₅₀ dose of JEV- 733913. Mice were put under observation for 25 days and survival curves were plotted.

Mice were also immunized by the subcutaneous route w/”ith 3 doses of peptide 5 (50 μg in aluminium hydroxide gel). One week following the last dose, immune splenocytes were harvested and 1x10⁷ cells were transferred intravenously in 14-day-old mice. Mice were challenged with 50 LD₅₀ dose of JEV by the IP route. JEV immune splenocytes and non immune splenocytes were also transferred as controls.

**Kinetics of viral spread in control and experimental animals**

Mice that were adoptively transferred with splenocytes from control or immunized animals were sacrificed every alternate day PI for comparison of viral titres in various organs. Mice were euthanized by CO₂ asphyxiation and blood was drained by cardiac puncture. Fresh blood was immediately processed for RNA extraction while sera were used for plaque assay. Spleens and brains were excised, weighed, and a 20% suspension made in 0.75% BAPS. Tissues and cells were then disrupted using a tissue homogenizer.

a) Viral RNA from the supernatants of tissue homogenates was isolated by the QIAMP viral RNA kit (Qiagen), and virus titres were quantitated using the Geno Sen real time RT PCR kit for JEV (Genome Diagnostics), on an ABI 7300 real time PCR system (Applied Biosystems). Primers were specific for a 130 bp stretch of the E protein of JEV. The probe was labelled with the reporter dye FAM at the 5’ end and a quencher dye TAMRA.
at the 3’ end. Reaction conditions were 50 °C for 15 min, 95 °C for 10 min, 45 cycles of 95 °C for 20 sec, 50 °C for 30 sec and 72 °C for 20 sec. Viral copy numbers in the samples were calculated from the standard graph generated using pre-quantified JEV specific RNA standards with known copy numbers provided with the kit.

b) Serial dilutions of the organ homogenates were also added to BHK-21 cell monolayers for quantitation by direct plaque assay (229). Serum was separated before titration of blood samples. Samples were washed off after 1hr incubation at 37 °C and a 1:1 mix of MEM (containing 20% FCS and 10% tryptose phosphate broth, TPB) and 1% carboxy methyl cellulose (CMC) was added on to the monolayer and incubated for 72 hrs at 37 °C. Plates were then washed with normal saline, stained with amido black and plaques were counted. Virus titres were expressed as plaque forming units (PFU) per gm of tissue or ml of sera.

Cytokine RT-PCR
To detect cytokine mRNA expression in the brains of infected 14-day-old-mice, animals were sacrificed on days 2, 4, 6 and 8 PI, blood was collected by cardiac puncture and spleens and brains were excised. Brains were weighed and homogenized in buffer RLT (Qiagen) and total cellular RNA was isolated using the RNeasy kit (Qiagen). RNA from age matched naïve mice brain served as control. RNA was quantitated using a spectrophotometer (NanoDrop 1000) and 1μg of RNA from infected and control mice was reverse transcribed at 50 °C for 1hr using the Thermoscript RT-PCR System (Invitrogen) and 0.5μg random hexamers (Invitrogen). 2μl cDNA was then used for PCR amplification using gene specific primers for TNF-α, IFN-γ, IL-10, and the house keeping gene, β-actin, using PCR reagents from Invitrogen. Reaction conditions were: initial denaturation of 94 °C for 5 min, 30 cycles of 92 °C for 45 sec, primer specific annealing temperature for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. Products were analyzed on a 1.5 % ethidium bromide gel.
Table 3.3: Primers for RT-PCR analysis of cytokine genes in JEV infected mice brain

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence (5’- 3’)</th>
<th>Amplicon size (bp)</th>
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<tr>
<td>β-actin FP</td>
<td>GTGGGCCCGCTCTAGGCACCA</td>
<td>250</td>
</tr>
<tr>
<td>β-actin RP</td>
<td>TGGCCTTAGGGTTCAAGGGGG</td>
<td>250</td>
</tr>
<tr>
<td>IFN-γ FP</td>
<td>AACGCTACACACTGCATCTTTGC</td>
<td>350</td>
</tr>
<tr>
<td>IFN-γ RP</td>
<td>CTCATGAATGCATCCTTTTTCG</td>
<td>350</td>
</tr>
<tr>
<td>TNF-α FP</td>
<td>GTCTCAAAGACAACCAACTAGTC</td>
<td>300</td>
</tr>
<tr>
<td>TNF-α RP</td>
<td>CTCCAGCTGGAAGACTCTCCAG</td>
<td>300</td>
</tr>
<tr>
<td>IL-10 FP</td>
<td>GGAAGACAATAACTGCACC</td>
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</tr>
<tr>
<td>IL-10 RP</td>
<td>CATTTCGATAAGGCTTGG</td>
<td>180</td>
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</table>

Cytokine analysis by CBA

Th1 and Th2 cytokines in serum samples were estimated by the Cytometric Bead Array (CBA) assay. 2-fold serial dilutions of mouse cytokine standards, that covered a range from 20 - 5000 pg/ml, were prepared in the assay diluent. In a separate tube, individual capture beads were mixed together and 50 μl added to each assay tube. 50 μl of standards and test samples was added to individual tubes, followed by 50 μl of the PE detection reagent. Assay tubes were incubated for 2 hrs in the dark at RT at the end of which beads were washed and re-suspended in 300 μl of wash buffer. In separate tubes, cytometer setup beads were prepared. The instrument was then setup using the BD FACSComp software and setup beads. Following acquisition of standards and samples using multicolor flow cytometry on a FACS Calibur (Becton Dickinson), the cytokine concentrations of the samples were determined through the BD CBA software using the standard curves obtained for each cytokine. Levels of IFN-γ, TNF-α, IL-2, IL-4, and IL-5 were determined from pooled sera and cytokine levels from naïve mice sera were set as the threshold.

Antibody isotyping

The immunoglobulin subtypes of sera from mice adoptively transferred with control and immune splenocytes were measured by using an indirect isotyping ELISA using the Pierce Immunopure monoclonal antibody isotyping kit (Pierce). Briefly, microplates (Nunc) were coated overnight with 50 μl of a 1:50 dilution of sucrose density gradient purified,
ultrapelleted JEV. Plates were blocked with 125 μl of the blocking solution provided in the kit. Incubation was carried out for 1 hr at 37 ºC after each reagent addition step, and plates were washed with TBS+Tween-20 (TBST) after each incubation step. Following blocking, 50 μl of 2-fold serum dilutions starting at 1:10 dilution were added to the wells. A positive control provided in the kit and sera from uninfected mice were used as test controls. In the next step, 50 μl of normal rabbit sera or subclass-specific anti-mouse immunoglobulins were added to each antigen coated well. This was followed by the addition of 50 μl of HRP-conjugated goat-anti-rabbit IgG to each well. 100 μl of 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) substrate solution was then added and plates monitored for colour development. Absorbance was read at 405 nm. Ratios of the absorbance values of IgG1 and IgG2a were determined for control and experimental mice and antibody titres were expressed as the reciprocal of the highest dilution of sera that gave an absorbance 2 times to that of the negative control (uninfected mice sera).

**Histopathology of organs**

Mice lethally infected with JEV or adoptively transferred with JEV immune splenocytes before infection were sacrificed at various time points after infection. Blood was drained by cardiac puncture and brains were excised and fixed in 10% buffered formalin. Organs were dehydrated through increasing grades of ethyl alcohol for 1 hr each. Following 2 changes in absolute alcohol, organs were treated with 2 changes of xylene for 1 hr each. Organs were then impregnated with paraffin wax at 60 ºC for 90 min. The organs were embedded in warm paraffin wax and trimmed. 3-5 micron thick sections of the tissues were made and spread on microscopic glass slides.

For hematoxylin and eosin (H&E) staining, slides were dewaxed and dipped in 2 changes of xylene for 5 min each, followed by decreasing percentages of alcohol. Slides were then washed in tap water for 5 min and stained with hematoxylin for 5 min. After washing in running tap water for 5 min, slides were dipped in 1% acid alcohol followed by washing in running tap water and stained with eosin for 30 sec. Sections were finally dipped in xylene and mounted for observation.

**Immunohistochemistry of organs**

For immunohistochemistry, infected and protected mouse brain sections were taken on poly L-lysine (PLL) coated slides. Slides were de-waxed by heating, passed through 3 changes of xylene and 2 changes of absolute alcohol, then washed with distilled water.
Antigen retrieval was carried out by heating for 15 min in citrate buffer, following which slides were washed with Tris buffer and blocked with 3% H₂O₂. Slides were washed and blocked with 5% skimmed milk for 20 min and incubated with biotinylated mouse flavivirus cross reactive antibody (HX-B) for 40 min. Following 3 washes in Tris buffer, slides were incubated with streptavidin-HRP for 30 min. Slides were then washed and incubated with di- amino benzidine (DAB) chromogen solution for 3-5 min and then washed and counter stained with hematoxylin for 3 min. Slides were dipped in 1% acid alcohol, washed under running water, dried and then mounted for observation.
RESULTS

Genetic monitoring of BALB/c mice

Genetic monitoring of mice was performed at ACTREC, Mumbai, India, in order to confirm the inbred status of the BALB/c mice used for this study. Common biochemical markers were used (Table 3.4a), and migration patterns of different alleles of these markers were studied by electrophoresis. The allelic profiles of all the BALB/c mice tested were similar and confirmed their inbred status (Table 3.4b).

Table 3.4a): Common allelic markers used for genetic monitoring

<table>
<thead>
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<th>Chromosome</th>
<th>Gene</th>
<th>Description</th>
<th>Alleles detected</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Idh-1</td>
<td>Isocitrate dehydrogenase</td>
<td>a, b</td>
</tr>
<tr>
<td>4</td>
<td>Gpd-1</td>
<td>Gucose-6-phosphate dehydrogenase</td>
<td>a, b</td>
</tr>
<tr>
<td>7</td>
<td>Gpi-1</td>
<td>Glucose phosphate isomerase</td>
<td>a, b</td>
</tr>
<tr>
<td>7</td>
<td>Hbb</td>
<td>Hemoglobin beta-chain</td>
<td>d, s</td>
</tr>
<tr>
<td>9</td>
<td>Mod-1</td>
<td>Malic Enzyme</td>
<td>a, b</td>
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</table>

Table 3.4b): Allelic profile of BALB/c mice

<table>
<thead>
<tr>
<th>Allele</th>
<th>Idh-1</th>
<th>Gpd-1</th>
<th>Gpi-1</th>
<th>Hbb</th>
<th>Mod-1</th>
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<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>a</td>
<td>d</td>
<td>a</td>
</tr>
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</table>

Selection of immunization dose and schedule

Mice brain and cell culture derived virus pools of JEV- 733913 were prepared and titrated in mice of various ages and on cell lines respectively. The LD$_{50}$ titre as obtained by the method of Reed and Muench is shown in table 3.5. The appropriate immunization dose and number of immunizations were standardized in adult mice. Two doses of 1000 LD$_{50}$ administered 2 weeks apart by the IP route was seen to sufficiently immunize mice against JEV, as 100% of the immunized mice survived subsequent challenge with a lethal dose of JE-733913. Thus, adult mice were immunized with 2 doses of 100 LD$_{50}$ of JEV- 733913 at 14-day intervals by the IP route followed by a 3rd booster dose administered one week prior to the harvesting of splenocytes for adoptive transfer. The details of immunization and the percent survival of mice after virus challenge is shown in table 3.6.
Table 3.5: JEV- 733913 mouse brain and cell culture stock titrated in various systems

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Virus source</th>
<th>System used</th>
<th>Route of infection</th>
<th>Virus titre</th>
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<td>1</td>
<td>Mice brain</td>
<td>Infant mice</td>
<td>IC</td>
<td>$10^{6.8}$ (LD$_{50}$)</td>
</tr>
<tr>
<td>2</td>
<td>Mice brain</td>
<td>14 day old mice</td>
<td>IP</td>
<td>$10^{6.3}$ (LD$_{50}$)</td>
</tr>
<tr>
<td>3</td>
<td>Mice brain</td>
<td>Adult mice</td>
<td>IC</td>
<td>$10^{5.75}$ (LD$_{50}$)</td>
</tr>
<tr>
<td>4</td>
<td>Mice brain</td>
<td>Adult mice</td>
<td>IP + IC starch</td>
<td>$10^2$ (LD$_{50}$)</td>
</tr>
<tr>
<td>5</td>
<td>Cell culture</td>
<td>Cell culture</td>
<td>Virus overlay</td>
<td>$10^6$ (TCID$_{50}$)</td>
</tr>
<tr>
<td>6</td>
<td>Cell culture</td>
<td>Cell culture</td>
<td>Virus overlay</td>
<td>$10^{-8}$ PFU/ml</td>
</tr>
</tbody>
</table>

Table 3.6: Standardization of JEV immunization dose and schedule

<table>
<thead>
<tr>
<th>No. of immunizations</th>
<th>Immunization dose</th>
<th>Route of infection</th>
<th>Percentage of surviving mice after challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 doses</td>
<td>1000 LD$_{50}$</td>
<td>IP</td>
<td>100 %</td>
</tr>
<tr>
<td>2 doses</td>
<td>100 LD$_{50}$</td>
<td>IP</td>
<td>80%</td>
</tr>
<tr>
<td>1 dose</td>
<td>1000 LD$_{50}$</td>
<td>IP</td>
<td>80%</td>
</tr>
<tr>
<td>1 dose</td>
<td>100 LD$_{50}$</td>
<td>IP</td>
<td>60%</td>
</tr>
<tr>
<td>1 dose</td>
<td>N. Antigen</td>
<td>IP</td>
<td>0%</td>
</tr>
</tbody>
</table>

Adult mice were immunized with either 1 or 2 doses of live JE-733913. The virus titre administered was either 100 LD$_{50}$ or 1000 LD$_{50}$. Control mice were inoculated with normal mouse brain antigen (N. Antigen). 100% of mice immunized with 2 doses of 1000 LD$_{50}$ titre of virus, spaced 14 days apart, were seen to be protected from lethal challenge with JE-733913 by the IP route followed by the IC inoculation of 1% starch solution. JEV immunized mice were further tested for neutralizing antibodies against JEV and for JEV specific lymphocyte proliferation.

Neutralization test of immune mice sera

Mice were bled after immunization with 2 doses of JEV and the sera were tested for JEV specific neutralizing antibodies (Figure 3.1). All the sera tested showed neutralizing antibodies against JEV. On taking a mean of the neutralizing antibody titres for all the samples, the approximate neutralizing antibody titre in mice after 2 doses of immunization with JEV was estimated to be ~850.
Figure 3.1: Neutralization assay of sera from mice immunized with 2 doses of JEV. 14 serum samples were taken and analyzed for the presence of neutralizing antibodies to JEV. JEV immune peritoneal fluid (JEV-IPF) and a monoclonal antibody specific for JEV (HS-2) were used as positive controls. Peritoneal fluid from non-immunized mice (NPF) was used as the negative control. (CC-Cell Control, VC-Virus Control for serial 10 fold virus dilutions).

Lymphocyte proliferation assay of splenocytes from immunized mice

To determine whether JEV-specific lymphocyte proliferation was induced in immunized animals, spleen cells were harvested 1 week post the 2nd immunization dose. Immune spleen cells and control splenocytes from naïve animals were stimulated with live and a range of inactivated JEV antigens, the antigenic concentrations of which were pre-determined by JE-Antigen capture ELISA (Figure 3.2). Acetone extracted, mouse brain derived, and BPL inactivated JEV antigen showed a good P/N ratio as determined by antigen capture ELISA and also induced increased proliferation of JEV specific immune splenocytes. This antigen was used for further lymphocyte proliferation assays. Splenocytes from JEV immune mice proliferated in response to both live and inactivated JEV antigen (Figure 3.4). Maximum proliferation was observed in response to the positive control, ConA (Figure 3.3). Splenocytes from JEV immune mice, which were not stimulated with JEV antigen in vitro, and splenocytes from naïve, non-immunized animals, which were stimulated with inactivated JEV antigen were used as negative controls, and did not show increased proliferation as compared to experimental samples.
**Figure 3.2:** Antigen capture ELISA for JEV, using various dilutions of inactivated JEV antigens a) Acetone extracted antigen, b) Tissue culture derived antigen and c) Sucrose gradient purified antigen. The maximum P/N ratio was observed for acetone extracted JEV antigen and this antigen was used for the lymphocyte proliferation assay. (Ag=antigen).

**Figure 3.3:** Proliferation of lymphocytes from spleens of JEV immunized mice in response to 1, 5 and 10 μg/ml concentrations of the mitogen, ConA. Data represents a mean of 3 experiments. Values are expressed as counts per minute (CPM) and represent means ± S.D.
Figure 3.4: Proliferation of lymphocytes from spleens of JEV immunized mice in response to 5 and 10 MOI of live JEV- 733913 and 10 and 20 µg/ml concentrations of acetone extracted JEV antigen. Unstimulated splenocytes from JEV immunized mice and splenocytes from naïve, non-immunized animals stimulated with JEV antigen served as negative controls. Values are expressed as CPM and represent means ± S.D.

Lymphocyte proliferation assay of CD4+ and CD8+ isolated cells from JEV immunized mice

CD4+ and CD8+ T cells from immunized mice spleens were cultured with APC from the spleens of naïve, syngenic mice in an *in vitro* lymphocyte proliferation assay. Cells were stimulated with ConA, live JEV, inactivated JEV or medium alone. After 72 hrs, both CD4+ and CD8+ T cells showed the maximum proliferation in response to 1µg/ml concentration of ConA (Figure 3.5a, CD4+ T cells: CPM = 34088.38 ± 4353.26, CD8+ T cells: CPM = 39660.43 ± 11252.89). Primed CD4+ T cells were seen to proliferate more in response to 10 and 5 MOI of live JEV as compared to CD8+ T cells (Figure 3.5b, p = 0.001, p = 0.019 respectively). CD4+ T cells also showed increased proliferation in response to 20 and 10µg/ml of inactivated JEV antigen (Figure 3.5c, CPM = 10664 ± 212 and 9316 ± 116 respectively), as compared to CD8+ T cells (CPM = 8580 ± 548 and 6925 ± 116 respectively). CD4+ and CD8+ T cells from the splenocytes of control, naïve animals showed much lower levels of proliferation in response to both live and inactivated JEV.
Figure 3.5a

![Bar chart showing ConA 1ug/ml response in CD4+ and CD8+ T cells.](chart1.png)

Figure 3.5b

![Bar chart showing Live Ag 10 MOI and Live Ag 5 MOI responses in CD4+ and CD8+ cells.](chart2.png)
Figure 3.5: *In vitro* proliferation assay of CD4+ and CD8+ isolated T cells from immunized and naïve mice in response to different doses of a) ConA, b) live JEV and c) inactivated JEV antigen. Data represents a mean of 3 experiments. Immune CD4+ T cells showed a higher level of proliferation than CD8+ T cells to both live and inactivated JEV antigen. CD4+ and CD8+ T cells from naïve animals did not show enhanced proliferation in response to either live or inactivated JEV antigen. Significant differences between groups are denoted by an asterisk. Error bars indicate standard error of means (SEM).

**Selection of recipient mice age for adoptive transfer**

Initial adoptive transfer experiments were carried out in 10-day-old mice, but administration of cells into the tail vein of these animals proved cumbersome. Before deciding upon an older mouse model, it was important to determine the age up to which mice were susceptible to peripheral inoculation with JEV. 14, 17, and 19 day-old mice were inoculated with 100 LD50 of JEV by the IP route. While 100% of 14-day-old mice succumbed to infection, the percentage mortality was much lower for 17-day-old-mice, and 19-day-old mice did not succumb to IP challenge with 100 LD50 of JEV. 14-day-old mice showed symptoms of sickness, i.e. ruffled fur, hunched posture, lethargy, weight loss and hind limb paralysis, by day 5 PI and succumbed to JEV infection by day 8 PI. JEV was detected by RT-PCR in the brains of 14-day-old infected mice on day 6 and 8 PI and in the spleen on day 6 PI (Figure 3.6a, c), while virus was not detected in the blood at any time-point post infection (Figure 3.6b). 14-day-old mice were therefore selected as a model for adoptive transfer studies and infection with JEV.
**Figure 3.6a**: Detection of JEV-733913 in the brains of 14 and 17 day old mice at various time points PI. RT-PCR was performed for a 367 bp region encompassing the C-PrM region of JEV. Lanes 2, 3, 4 and 5 represent brain samples from 14-day-old-mice harvested on days 2, 4, 6 and 8 PI, respectively. Lanes 6, 7, 8 and 9 represent brain samples from 17-day-old-mice harvested on days 2, 4, 6 and 8 PI, respectively. Lane 10 represents the negative control (normal mouse brain) while lane 11 represents the positive control (JEV virus stock).

**Figure 3.6b, c**: Detection of JEV in the blood and spleen of 14-day-old mice at various time points PI. RT-PCR was performed for a 350 bp region encompassing the C-PrM region of JEV. Lanes 2, 3, 4 and 5 of Figure 3.6 (b) represent blood samples from 14-day-old-mice harvested on days 2, 4, 6 and 8 PI respectively. Lanes 1, 2, 3 and 4 of Figure 3.6 (c) represent spleen samples from 14-day-old-mice harvested on days 2, 4, 6 and 8 PI respectively.

**Adoptive transfer of JEV immune antibodies and cells**

**Passive sera transfer**

Passive protection studies were previously carried out in our laboratory using IPF from JEV immunized mice. It was seen that the passive transfer of neat and 1:10 diluted JE-IPF into adult mice by the IV route was capable of protecting 100% of the test animals from
lethal challenge with JEV (data not shown). Virus in this case was inoculated 24 hours after JE-IPF transfer by the IP route, followed by the injection of 1% starch by the IC route.

**Infective virus dose**

14-day-old naïve mice were titrated in order to determine the virus challenge dose to be given to mice that were subjected to adoptive transfer. Transfer of $1 \times 10^7$ splenocytes by the IV route followed 24 hrs by challenge with $100 \text{LD}_{50}$ titre of the virus resulted in approximately 50% survival of challenged mice. On reducing the virus dose to $50 \text{LD}_{50}$, approximately 95% of mice that were subjected to adoptive transfer were seen to survive virus challenge. A virus challenge dose of $50 \text{LD}_{50}$ was thus set for subsequent experiments.

**RT-PCR of JEV primed splenocytes before adoptive transfer**

Before adoptive transfer, it had to be determined that carry over of virus by transferred cells did not result in priming of the recipient mice before actual virus challenge. Total RNA from splenocytes of JEV immunized mice were reverse transcribed with JEV specific primers. No amplification for JEV was detected in the splenocytes from JEV immunized mice (Figure 3.7) and primed cells for adoptive transfer were determined to be free of JEV antigen.

![Image](image.png)

**Figure 3.7:** Total RNA from splenocytes of JEV immunized mice was reverse transcribed for a 367 bp stretch encompassing the C-PrM region of JEV. JEV specific amplification was not observed in the sample, suggesting that immune splenocytes were free of viral RNA.

**Depletion of APC from JEV primed splenocytes**

It was also important to ensure that activated APC in the immune splenocyte population did not present viral antigen to cells of the recipient mice before infection. Immune splenocytes were depleted of the adherent population, comprising mainly APCs and adherent cells from naïve, syngenic mice were added to the immune non-adherent splenocyte
population before adoptive transfer. Similar levels of protection were observed when recipient mice received splenocytes containing APC from either immune or naïve mice (Table 3.7). Thus, protection from JEV infection was seen to be solely mediated by immune T and B cells.

**Irradiation of recipient mice**

Earlier experiments by Pan et al., (2001) suggested that in sub lethally irradiated animals, cell mediated immunity did not play an important role. They also proposed that recipient immune cells from the host contributed to protection, resulting in faster kinetics of the secondary immune response. In order to determine the role of host immunity in adoptive transfer, 14-day-old recipient BALB/c mice were γ-irradiated with a dose of 250 rads before being subjected to adoptive transfer with splenocytes from immune mice. Mortality in these mice was compared with other animals that were not irradiated before immune cell transfer. It was seen that the survival rates of both irradiated and non-irradiated mice were similar after immune cell transfer when challenged with 100 LD₅₀ of JEV (Table 3.7). This suggested that JEV primed cells, adoptively transferred into the host recipient animals, were effective in clearing the host of virus infection.

**Table 3.7**

<table>
<thead>
<tr>
<th>Mice</th>
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<th>Challenge Dose (LD₅₀)</th>
<th>Surviving mice / Total mice used</th>
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<td>Irradiated</td>
<td>Immune spleen cells (non adherent cells) + adherent spleen cells from naïve mice</td>
<td>100</td>
<td>4/8</td>
</tr>
<tr>
<td>Irradiated</td>
<td>Immune splenocytes (whole)</td>
<td>100</td>
<td>3/7</td>
</tr>
<tr>
<td>Irradiated</td>
<td>Naive mice splenocytes (whole)</td>
<td>100</td>
<td>0/11</td>
</tr>
<tr>
<td>Non-irradiated</td>
<td>Immune spleen cells (non adherent cells) + adherent spleen cells from naïve mice</td>
<td>100</td>
<td>3/7</td>
</tr>
<tr>
<td>Non-irradiated</td>
<td>Immune splenocytes (whole)</td>
<td>100</td>
<td>5/8</td>
</tr>
<tr>
<td>Non-irradiated</td>
<td>Naive mice splenocytes (whole)</td>
<td>100</td>
<td>0/10</td>
</tr>
</tbody>
</table>

**Table 3.7**: JEV immune splenocytes were depleted of adherent cells and were reconstituted with adherent spleen cells from naïve, syngenic mice. Reconstituted splenocytes or whole splenocytes from either immunized or naïve mice were transferred into 14-day-old mice that were either γ-irradiated with 250 rads or non-irradiated. Mice were inoculated with 100 LD₅₀
of JEV and observed for survival. No significant difference was observed in the survival of either the irradiated or the non-irradiated groups of recipient mice. No difference in mortality was observed between mice that were subjected to adoptive transfer either with whole splenocytes from immunized mice, or with splenocytes containing adherent cells (APC) from naïve, non-immunized animals.

**CFSE staining of cells before adoptive transfer**

Splenocytes from immunized mice were stained with the fluorescent dye, CFSE and then adoptively transferred by the IV route into γ-irradiated adult mice. 24 hrs following transfer, mice were challenged by the IP injection of 100 LD$_{50}$ of live JEV-733913. Mice were sacrificed after 24, 48 and 72 hrs PI. Maximum CFSE stained T cells were recovered from CD3+ gated spleen cells at 48 hrs PI (Figure 3.8). It was seen that a greater population of JE specific CFSE stained T cells were recovered from spleens of mice that were challenged with JEV antigen (8%) in contrast to mice that were injected with only PBS (0.38%). Therefore, adoptively transferred splenocytes from JEV immunized mice were seen to traffic into the spleens of irradiated, recipient mice at 48 hrs PI, in response to stimulation with JEV antigen.

**Figure 3.8:** Splenocytes from JEV immunized mice were stained with CFSE and adoptively transferred into irradiated syngenic recipients, followed by the IP injection of live JEV. CFSE stained cells were recovered from the spleens of recipient mouse at 48 hrs PI (green peak, M1). The population of CFSE stained cells recovered was much lower in the spleens of mice that were not primed with JEV after adoptive transfer (black peak, M1).
Adoptive transfer into athymic nude (nu/nu) mice

In order to determine the role of recipient immune cells from the host in protection against JEV infection, athymic nude mice were used. Splenocytes from JEV immunized, immunocompetent, BL6 heterozygous (nu/+) mice were transferred into BL6 athymic (nu/nu) mice that lacked functional T cells, followed by lethal virus challenge. In addition, splenocytes from naïve BL6 (nu/+) mice were also transferred. It was observed that transferred JEV immune splenocytes were capable of protecting the host from lethal challenge with JEV, even in the absence of functional T cells in the host (70% survival, n=10). Nude mice receiving splenocytes from naïve animals were not protected against JEV challenge (0% survival, n=10). Control nude mice, infected with a lethal dose of JEV, mostly succumbed to infection (10% survival, n=10). These results suggested that protection against lethal JEV challenge in mice receiving immune cell transfer was mediated by the transferred immune cells and not by the host immune response.

![Graph showing survival curves](image)

**Figure 3.9:** Survival curves of athymic (nu/nu) mice transferred with a) JEV primed splenocytes (n = 10) and b) splenocytes from naïve mice (n = 10). c) Control mice did not receive any cells. Mice were challenged with JEV by the IP route followed by the administration of 1% starch solution by the IC route. Kaplan Meier survival curves were plotted. Mice receiving JEV primed splenocytes were found to be resistant to lethal challenge (70% survival) while mice receiving naïve splenocytes or control mice succumbed to JEV infection.
Adoptive transfer of CD4+ and CD8+ T cell depleted JEV immune splenocytes

To determine the role of CD4+ and CD8+ T cell subsets in protection from JEV infection, 1 x 10^7 spleen cells, depleted of either the CD4+ or CD8+ populations were transferred into naïve 14-day-old recipients followed 24 hrs later by challenge with 50 LD_{50} of JEV. The splenocyte population was seen to be more than 98% free of CD4+ or CD8+ T cells upon flow cytometric analysis (Figure 3.10a). Control mice, transferred with splenocytes from non-immunized animals began to succumb to JEV infection by day 7 PI, resulting in a percentage survival of 5% and an AST of 11 days, while 95% of mice transferred with JEV immune splenocytes were protected from lethal infection with JEV (p = 0.000). Mice receiving CD4+ depleted spleen cells showed a reduced survival percentage (34.62%) upon lethal challenge with JEV as compared to mice that received CD8+ depleted spleen cells (64.52%, p = 0.04) (Figure 3.11). The AST of mice receiving CD4+ depleted spleen cells was also reduced (16 days) as compared to mice that received CD8+ depleted cells (20 days).

Adoptive transfer of CD4+ and CD8+ isolated T cell subsets

Further studies were carried out using CD4+ and CD8+ isolated cells from immune splenocytes. Isolated cells were determined to be more than 94% pure by flow cytometric analysis (Figure 3.10b). Mice that were subjected to adoptive transfer with CD4+ isolated T cells showed an increased survival rate over mice injected with CD8+ isolated T cells (53.85% and 28.57% survival respectively), though the difference was not significant (Figure 3.12).
Figure 3.10: Flow cytometric analysis of splenocyte subpopulations for percentage purity of a) depletion and b) isolation. Cells were stained with PE conjugated CD4+/ CD8+ specific anti-mouse antibodies. Percentage of depletion was found to be >98% while the percentage purity of isolated cells was found to be >94%.

Figure 3.11: Pooled survival curves of mice transferred with a) JEV primed splenocytes (n = 47), b) CD4+ T cell depleted primed splenocytes (n = 26), c) CD8+ T cell depleted primed splenocytes (n = 31), d) control splenocytes (n = 18), following challenge with 50 LD₅₀ dose of JEV- 733913. Kaplan Meier survival curves were plotted, and the log rank test was used to determine significance. Survival of the CD4+ and CD8+ depleted groups were significant as compared to control (p = 0.017 and 0.00 respectively). The CD8+ depleted population showed a higher percentage survival as compared to the CD4 depleted population (p = 0.04).
Figure 3.12: Pooled survival curves of mice transferred with a) JEV primed splenocytes (n = 47), b) primed CD4+ T cell isolated cells (n = 13), c) primed CD8+ T cell isolated cells (n = 14), d) control splenocytes (n = 18), following challenge with 50 LD50 challenge dose with JEV- 733913. Kaplan Meier analysis was performed and the log rank test was used to determine significance. Survival of the CD4+ and CD8+ isolated groups were significant as compared to control (p = 0.0009 and 0.035 respectively). Mice transferred with CD4+ T cells showed a higher AST (19 days) as compared to mice transferred with CD8+ T cells (16 days), though the difference in percentage survival between the two groups was not significant.

Correlates of protection against JEV infection in mice receiving splenocytes from immunized mice

The mechanisms of protection from JEV infection in mice receiving primed splenocytes were further examined. Mice receiving either JEV primed splenocytes or splenocytes from naïve mice before JEV infection were harvested at specific time PI. Virus titres, cytokines produced, and antibody isotypes were compared between mice that succumbed to infection and mice that were protected from infection by the adoptive transfer of JEV primed splenocytes. Mice that were bled every alternate day for cytokine analysis and antibody isotyping studies were seen to succumb to JEV infection faster and were not included for survival analysis.
Virus burden in organs of mice receiving splenocytes from immune and non-immune animals

To determine if differences in the extent of viral replication in the peripheral organs and in the CNS of mice receiving immune or non-immune cells contributed to the difference in mortality in these animals, the time course of viral load in the blood, spleen and brains of these mice was measured. Viral loads were detected by plaque assay and by real time RT-PCR using JEV specific primers and probe.

JEV was detected on day 2 PI in the blood of acutely infected animals when quantitated by real time RT-PCR (Figure 3.13a). The maximum viral load was $10^5$ copies/ml, which gradually decreased thereafter. When estimated by plaque assay, virus was detectable in the sera of infected mice only on day 4 PI and the maximum viral load was $5 \times 10^2$ PFU/ml (Figure 3.14a). Virus titres determined by real time RT-PCR were seen to be more than 2 orders of magnitude higher than titres determined by plaque assay.

In the spleen, peak levels of virus replication were observed on days 4 and 6 PI by real time RT-PCR (Figure 3.13b). The highest viral load was $8 \times 10^6$ copies/gm, which was observed on day 6 PI. Virus levels were seen to decrease by day 8 PI, though virus was still detectable by real time RT-PCR in some samples. Virus was only detected on days 4 and 6 PI by plaque assay (Figure 3.14b). The highest viral load observed by plaque assay was $5 \times 10^4$ PFU/gm.

Virus levels in infected mouse brains increased exponentially, reaching peak levels on days 6 and 8 PI, as determined by both real time PCR and plaque assay. The maximum viral load in the brain observed by real time RT-PCR was $1 \times 10^9$ copies/gm (Figure 3.13c), while it was $3 \times 10^8$ PFU/gm by plaque assay (Figure 3.14c).

Viral replication in the organs of infected mice was thus seen to peak earlier in the peripheral sites during the course of infection. Virus could be detected in the brain from day 2, however. Once productive infection had been established in the CNS, virus titres reached extremely high levels there, with a corresponding decline in virus replication in the peripheral organs.

In mice that were subjected adoptive transfer with immune spleen cells before lethal infection, the course of infection was erratic both in the periphery and in the brain and did not follow the kinetics of viral replication observed in the organs of non-immune mice that succumbed to infection. Virus was undetectable by plaque assay in the sera and spleens of mice subjected to immune spleen cell transfer (Figure 3.14a, b). In the brains of these mice, virus was only detected on day 4 PI (Figure 3.14c). Although viral RNA was detected in the
peripheral organs of a few immune animals by real time RT-PCR, these reflected isolated cases and did not follow a trend parallel to that of infection in the blood and spleen of animals that succumbed to infection (Figure 3.13a, b). Viral RNA copies in the brains of mice receiving immune spleen cells were approximately 4 orders of magnitude lower than in mice receiving non immune splenocytes, as determined by real time RT-PCR (Figure 3.13c). This suggested that mortality from JEV infection was in some part due to accumulation of virus load in the CNS of infected mice.

**Figure 3.13a**

![Scatter plots showing viral RNA copies in the a) Blood, b) Spleens, and c) Brains of mice receiving either JEV non-immune or immune splenocytes, at various time points PI. The limit of sensitivity of the JEV real time RT-PCR assay is 48 viral RNA particles (represented by a horizontal bar). In mice receiving non-immune splenocytes, viral RNA copies in the blood were seen to be highest between day 2 PI and day 4 PI, with subsequent fall in virus titres at later time points. In the spleen, an increase in virus copies was observed till day 6 PI, followed by a subsequent fall, while peak virus levels in the brain were attained between 6 and 8 days PI. A continuous pattern of infection was not observed in organs of mice receiving immune splenocytes, with virus detected sporadically at certain time points.](image-url)

**Figure 3.13:** Scatter plots showing viral RNA copies in the a) Blood, b) Spleens, and c) Brains of mice receiving either JEV non-immune or immune splenocytes, at various time points PI. The limit of sensitivity of the JEV real time RT-PCR assay is 48 viral RNA particles (represented by a horizontal bar). In mice receiving non-immune splenocytes, viral RNA copies in the blood were seen to be highest between day 2 PI and day 4 PI, with subsequent fall in virus titres at later time points. In the spleen, an increase in virus copies was observed till day 6 PI, followed by a subsequent fall, while peak virus levels in the brain were attained between 6 and 8 days PI. A continuous pattern of infection was not observed in organs of mice receiving immune splenocytes, with virus detected sporadically at certain time points.
points in only a few animals. Virus copies in the brains of protected mice were almost 4 logs lower than in infected mice that succumbed to infection.

**Figure 3.13b**

![Figure 3.13b](image)

**Figure 3.13c**

![Figure 3.13c](image)
**Figure 3.14a**

![Graph showing the logarithmic scale of plaque-forming units (pfu/ml) vs. days post infection for infected sera and protected sera.](image)

**Figure 3.14b**

![Graph showing the logarithmic scale of plaque-forming units (pfu/gm tissue) vs. days post infection for infected spleen and protected spleen.](image)
Figure 3.14: Viral load in the a) blood, b) spleens, and c) brains of JEV infected mice and mice protected from infection by adoptive transfer of immune splenocytes, at various time points PI. Infectious virus was titrated by PFU assay on BHK-21 cells. Virus copies in the blood and spleen were seen to peak early during infection, while no virus was detected in the protected samples. In the brain, virus titres were seen to rise as the disease progressed. In brains of protected mice, virus was detected only on the 4th day PI. Data represents a mean of 3 experiments. Error bars indicate standard error of means (SEM).

Cytokine profiles from mice receiving either JEV immune or non-immune splenocytes

Cytokine mRNA expression from brains from JEV infected mice was examined by RT-PCR to detect pro and anti-inflammatory cytokines induced at various time points after infection. In addition, sera from mice receiving either non-immune or immune splenocytes were tested for Th1 and Th2 cytokines by the CBA kit.

Increased mRNA expression of the pro-inflammatory cytokines IFN-γ and TNF-α were observed in the brains of JEV infected mice as infection progressed (Figure 3.15). Bands showing TNF-α amplification were of maximum intensity on day 6 PI, which decreased thereafter. IFN-γ mRNA expression seemed to parallel the progression of disease in infected mice, and bands of the highest intensity were observed on day 8 PI, concurrent with the death of the animal. RT-PCR for IL-10, the anti-inflammatory cytokine, revealed
mRNA expression of this cytokine on day 8 PI. Amplification of the housekeeping gene, β-actin, showed no variation in expression at any of the time points examined.

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<th>Marker</th>
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<th>6 day</th>
<th>8 day</th>
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</tbody>
</table>

**Figure 3.15:** RT-PCR of cytokines from brains of mice harvested on alternate days PI. Total mRNA from the brains of infected and control animals were reverse transcribed and amplified with cytokine specific primers. Lane 1 refers to the 1KB+ marker. Lane 2, 3, 4 and 5 are samples from infected mice, while Lane 6 is from a control, naive animal. Data shown is the best representative of 3 experiments.

Quantitation of cytokine production was carried out by the CBA assay in the sera of mice receiving either non-immune or immune splenocytes. Similar levels of TNF-α and IFN-γ production was obtained as observed for gene expression by RT-PCR in mice that received splenocytes from non-immune animals.

Levels of TNF-α and IFN-γ, as determined by the CBA assay were seen to be higher in the sera of non-immune mice as compared to mice receiving immune splenocytes. (Figure 3.16a, 3.16b). TNF-α production in non immune mice was equal to that in mice receiving immune cells mice on day 2 PI, but showed an increase from day 4 PI, reaching peak levels of 75 pg/ml on day 6 PI (p = 0.014), and then decreasing slightly on day 8 PI. On day 8 PI, TNF-α levels were still higher in non-immune than in mice receiving immune splenocytes (p = 0.01). As observed by RT-PCR of brain tissue, IFN-γ expression levels were seen to
increase as disease progressed in non-immune mice, with baseline levels on day 2 PI (5 pg/ml) continuously increasing until day 8 PI (91 pg/ml). IFN-γ expression on the days 6 and 8 day PI in non-immune mice were significantly higher than in mice receiving immune splenocytes (p = 0.02, p = 0.005 respectively).

Expression of IL-2 and the Th2 cytokines IL-4 and IL-5, determined by CBA, were much lower in the sera of mice receiving either non-immune or immune splenocytes (Figure 3.16 c, 3.16d, 3.16e), as compared to levels of the pro-inflammatory cytokines, TNF-α and IFN-γ. Expression levels of IL-2, IL-4 and IL-5 was almost equal in both groups of animals. Late in the course of infection, expression of IL-4 and IL-5 was seen to decrease in the sera of mice receiving non-immune splenocytes (p = 0.014, p = 0.002 respectively).

**Figure 3.16a**
Figure 3.16b

[Graph showing cytokine levels over days post infection for mice receiving non-immune and immune splenocytes.]

Figure 3.16c

[Graph showing IL-2 levels over days post infection for mice receiving non-immune and immune splenocytes.]
**Figure 3.16d**

Induction of pro and anti-inflammatory cytokines a) TNF-α, b) IFN-γ, c) IL-2, d) IL-4, and e) IL-5 in the sera of infected and protected mice. Higher levels of pro-inflammatory cytokines TNF-α (p < 0.05) and IFN-γ were observed in the sera of infected mice as disease progressed. Levels of IL-2, IL-4 and IL-5 were much lower than the pro-inflammatory cytokines and peaked earlier during the course of infection (day 4 PI), reaching basal levels by days 6 and 8 PI. Cytokine levels from normal mouse sera were used as the threshold. The results are a mean of 3 datasets. Each dataset represents sera from a pool of 8 mice. Values represent means ± SD. Significant differences between groups are denoted by an asterisk.

**Figure 3.16e**

Figure 3.16: Induction of pro and anti-inflammatory cytokines a) TNF-α, b) IFN-γ, c) IL-2, d) IL-4, and e) IL-5 in the sera of infected and protected mice. Higher levels of pro-inflammatory cytokines TNF-α (p < 0.05) and IFN-γ were observed in the sera of infected mice as disease progressed. Levels of IL-2, IL-4 and IL-5 were much lower than the pro-inflammatory cytokines and peaked earlier during the course of infection (day 4 PI), reaching basal levels by days 6 and 8 PI. Cytokine levels from normal mouse sera were used as the threshold. The results are a mean of 3 datasets. Each dataset represents sera from a pool of 8 mice. Values represent means ± SD. Significant differences between groups are denoted by an asterisk.
**Antibody isotypes from mice receiving either JEV immune or non-immune splenocytes**

Induction of the antibody response after JEV infection in mice receiving splenocytes from either non-immune or immune animals was determined by the PIERCE antibody isotyping kit. Antibody isotyping of sera from naïve mice (normal mouse sera) was also done (Figure 3.17). Sera from naïve mice were seen to contain basal levels of IgG1, IgG2a, and IgG2b antibody isotypes.

Comparison of sera from mice after 1 and 2 doses of JEV immunization revealed an increase in JEV specific IgM antibody levels after the 1st immunization dose, while IgG1 and IgG2a levels did not increase in comparison to levels observed with normal mouse sera (Figure 3.18). IgG1 levels showed a substantial increase after the 2nd immunization dose, though levels of IgG2a remained low. IgM levels were elevated even after the 2nd immunization dose, though they were not as high as after the 1st immunization dose.

![Graph showing antibody subtypes](image)

**Figure 3.17**: Isotyping of sera from naïve mice (normal mouse sera). Minimum expression levels of IgG1, IgG2a and IgG2b antibodies were detected. IgM levels were lower than in JEV infected mice. Data represents pooled sera from 4 mice.
Figure 3.18: Isotyping of sera from mice after the 1st and 2nd immunization dose. An increase in IgM levels was observed after the 1st immunization dose, while IgG1 and IgG2a levels remained low. After the 2nd immunization dose, IgG1 levels increased, while IgG2a levels remained low. IgM levels were also high after the 2nd immunization dose. Data represents pooled sera from 4 mice per immunization dose.

In mice receiving splenocytes from non-immune animals, IgM antibody was detected early during JEV infection and showed an upward trend, reaching titres of 400 by day 8 PI (Figure 3.19). Mice that received immune splenocytes before challenge with JEV (Figure 3.20) showed an elevated level of IgM antibody at all time points PI, with titres that were higher overall (560) than those observed in mice receiving splenocytes from non-immune animals.

Antibody isotyping of IgG subtypes in mice receiving either non-immune or immune splenocytes revealed a significant increase in levels of IgG1 in the group that received immune splenocytes (Figure 3.20). IgG1 levels in mice receiving non-immune splenocytes were barely detectable at all time points PI, showing a constant titre of 40 (Figure 3.19). In mice receiving immune splenocytes, however, IgG1 levels were low on day 2 and 4 PI, but rose significantly by day 6 PI (0.01), reaching titres of 400 by day 8 PI (p = 0.007).
Figure 3.19: IgM and IgG1 titres from pooled sera of JEV infected mice collected at different time points PI. Titres were obtained from isotyping ELISA, using serial 2-fold dilutions of the sera. IgM titres in infected mice showed a noticeable increase after day 4 PI, while IgG1 titres remained almost constant, showing a slight increase on day 8 PI. Each dataset represents pooled sera from 8 mice, and the titres represented here are a mean of 2 independent datasets. Values shown represent means ± SD.

Figure 3.20: IgM and IgG1 titres from pooled sera of mice adoptively transferred with JEV immune splenocytes, collected at different time points PI. Titres were obtained from
isotyping ELISA, using serial 2-fold dilutions of the sera. IgM antibody titres in adoptively transferred mice showed an almost constant titre on various days PI, while IgG1 antibody titres from adoptively transferred mice increased from day 4 PI and kept increasing till the last time point considered. Each dataset represents pooled sera from 8 mice, and the titres represented here are a mean of 2 independent datasets. Values represent means ± SD.

The IgG1/ IgG2a ratio in mice receiving either non-immune or immune cells was calculated in order to determine the T helper antibody response induced upon infection with JEV (Figure 3.21). In contrast to non-immune mice, which showed a constant IgG1/ IgG2a ratio at all time points PI, mice receiving immune splenocytes showed an increase in the ratio by day 6 PI, indicating raised IgG1 titres that suggested a switch towards a Th2 response.

![Graph showing IgG1/IgG2a ratios](image)

**Figure 3.21:** IgG1/ IgG2a ratios from pooled sera in JEV infected mice and in mice protected from JEV infection by the adoptive transfer of immune splenocytes. Infected mice showed a constant IgG1/ IgG2a ratio that did not change as infection progressed. In contrast, IgG1 levels were seen to rise significantly by day 6 PI for protected mice, and this trend continued until the final time point at which samples were taken (day 14). For each group, a least square regression line was fitted and comparison of slopes, using uni-variate general linear model, showed a significantly higher trend for protected mice (p < 0.05) as compared to mice that
succumbed to infection. Each dataset represents pooled sera from 8 mice, and the ratios represented here are a mean of 2 independent datasets.

**Histological analysis of brain sections from mice receiving non-immune and JEV immune splenocytes**

Brains from mice receiving JEV non-immune or immune splenocytes were harvested and sectioned for histological analysis following hematoxylin and eosin staining. Sections were examined for histopathological changes following infection, at the histopathology laboratory of K. E. M. hospital, Pune, India. Brain sections from mice receiving JEV non-immune splenocytes revealed evidence of inflammation at all days PI. Major changes observed were the focal infiltration of lymphocytes in the meninges and cerebral tissues with congested blood vessels, polymorphic cells, perivascular cuffing and microgliosis. Necrosis and degeneration of neurons with chromatolysis were also seen. Immunohistochemistry (IHC) using a flavivirus cross reactive antibody showed detection of JEV antigen in mainly neurons. Though the staining by immunohistochemistry was faint, a cytoplasmic reaction was observed in several neuronal cell bodies, as has also been observed by others (230).

In mice receiving JEV immune splenocytes, mild congestion was observed in most of the brain sections. Focal polymorphic cell and mononuclear cell (MNC) infiltration was observed in the brain in some sections. Microglial activity was also seen. JEV antigen was also detected in the brain sections of mice that were protected from infection.

**Figure 3.22a**

**Figure 3.22b**
Figure 3.22 a, b, c, d, e, f: Histological analysis from brains of mice lethally infected with JEV, harvested at various time points post JEV infection. a) Nerve cell necrosis on day 4 PI, b) Degeneration of cerebral tissue and MNC infiltration on day 6 PI, c) Perivascular cuffing on day 8 PI, d) Inflammatory foci on day 8 PI, e) PMN cell infiltration on day 8 PI, f) PMN cell infiltration and neuronal shrinkage on day 8 PI.
Figure 3.22 g, h, i, j: Immunohistochemical analysis from brains of mice lethally infected with JEV, harvested at various time points post JEV infection. Sections were stained for JEV antigen using a biotinylated flavivirus cross-reactive monoclonal antibody raised in mice. g) Neuronal necrosis and shrinkage with surrounding PMN cells on day 6 PI, h) Perivascular cuffing with surrounding PMN cells on day 8 PI, i) IHC reaction in degenerated neurons on day 6 PI, j) IHC cytoplasmic reaction at margins on day 6 PI.
Figure 3.23 a, b, c, d, e, f, g, h: Histological analysis from brains of mice protected from lethal JEV infection by the adoptive transfer of JEV immune splenocytes. a) Slight congestion on day 2 PI, b) No abnormalities except for slight congestion on day 4 PI, c) Focal PMN cell infiltration on day 4 PI, d) Neuronophagia on day 8 PI, e) Necrosis and glial nodule gliosis on day 10 PI, f) Chromatolysis of neurons (40x) on day 12 PI, g) Neuronal chromatolysis (60x) on day 12 PI, h) MNC infiltration on day 14 PI.
Figure 3.23 i, j, k, l: Immunohistochemical analysis from brains of mice protected from lethal JEV infection by the adoptive transfer of JEV immune splenocytes. i) Faint IHC reaction in some cells on day 6 PI, j) Purkinje cell with cytoplasmic reaction at margins, on day 6 PI, k) Faint motor neuron reaction on day 12 PI, l) Motor neuron cytoplasmic reaction on day 12 PI.
Adoptive transfer with peptide stimulated T cells

It was observed in earlier studies that the contribution of T helper peptides incorporating immunodominant regions of JEV was an important addition to the induction of virus neutralizing antibodies against JEV. Neutralizing antibody inducing B cell and T helper epitopes from different structural and NS proteins of JEV were delineated. These T helper and B cell epitopes were incorporated in constructing chimeric T helper-B cell peptide immunogens. Immunization with these peptides was shown to induce protective ability in mice (228).

Priming with T helper peptides showed a substantial increase in the neutralizing antibody response against killed JEV antigen (228). In addition, *in vitro* stimulation of JEV immunized mice by T helper peptides for 3-4 rounds followed by the adoptive transfer of these stimulated cells into naïve, recipient mice resulted in protection of the recipient mice against lethal challenge with JEV (Figure 3.24). Enhanced protection was observed in mice subjected to adoptive transfer with splenocytes stimulated with Peptide 5, which was derived from the M protein epitope of JEV (100% survival, n=9).

![Figure 3.24](image)

**Figure 3.24:** Adoptive transfer of *in vitro* JEV T helper peptide stimulated splenocytes protected recipient mice against lethal infection with JEV. Transfer of splenocytes stimulated with Peptide 1 (E protein) and Peptide 4 (E protein) resulted in intermediate protection (~60% survival, n=13), while splenocytes stimulated with Peptide 5 (M protein) were capable of completely protecting naive recipient mice from infection with 50 LD$_{50}$ of JEV.
As Peptide 5 stimulated splenocytes were capable of protecting naïve mice against lethal challenge with JEV, in the next set of experiments, mice were immunized with 3 doses of 50μg Peptide 5 in aluminium hydroxide gel. Following immunization, splenocytes from Peptide 5 immunized animals were adoptively transferred into naïve mice followed by lethal challenge with JEV. It was seen that Peptide 5 primed splenocytes were capable of conferring protection against JEV infection in naïve animals (Figure 3.25).

**Figure 3.25:** Splenocytes from mice immunized with 3 doses of Peptide 5 were capable of protecting naïve, recipient mice against lethal infection with JEV (90% survival, n = 9). Mice subjected to adoptive transfer with JEV immune splenocytes (90% survival, n = 15) or non immune splenocytes (20% survival, n = 12) were used as controls.
DISCUSSION

Viral infection of the CNS presents a dilemma for the host, in that an appropriate antiviral response must be generated that clears the infectious agent without causing significant neuronal injury and loss, which otherwise would have catastrophic consequences. In flavivirus infection, the ratio of apparent to inapparent infection is quite low (1:100-1:300), implying that a number of host factors are involved in protection against CNS disease. Analysis of the host immune factors that determine the clinical course of JEV infection and confer life-long protection could aid in the rational development and introduction of new vaccines. In this study, the contribution of CD4+ and CD8+ T cell subsets in conferring protection against lethal challenge with JEV was examined in a murine model of infection. Analysis of the T helper response (Th1 or Th2) involved in protection against JEV infection was also carried out.

JE primarily affects young children, presumably because their developing immunity cannot mount an efficient immune response. Similarly, JEV is also more neurovirulent in the 14-day-old mouse model used in this study, probably due to lack of a well developed BBB and/or a better propensity of developing neurons to support viral growth than in adult mice, which have a well developed immune response (139-141). Studies have shown that in mice, BBB maturation occurs between 12 and 24 days (142). In these young mice, encephalitic flaviviruses can grow in diverse extraneural tissues and generate viremia. The strain of JEV used for our study (JEV-733913), is non pathogenic in adult animals when inoculated by the peripheral route. By infecting 14-day-old mice by the extraneural route, the involvement of peripheral immune responses against the infection could be better understood. Also, disease progression from the periphery into the brain could be studied.

In the study, it was seen that immunization of adult mice with live JEV by the IP route could confer adequate humoral and cell-mediated immunity in the immunized animal. Immunization was carried out with live JEV so that an immune response could be developed against both structural and NS viral proteins, as many T cell epitopes of JEV have been observed on NS proteins. Kumar et al., (2003) identified the NS3 protein of JEV as a dominant CD4+ as well as CD8+ T cell-eliciting antigen in a healthy JEV-endemic cohort, indicating that a Th1 immune response to the NS3 protein may be a critical determinant of immune control of JEV infection (231).

Sera from immunized animals had high titres of neutralizing antibodies to JEV (Figure 3.1), as determined in vitro, and were also capable of passively transferring immunity against the virus in naïve mice. In addition, splenocytes from immunized animals showed an
enhanced cellular proliferation in response to both live and inactivated JEV antigens (Figure 3.4). Earlier studies using PBMC from JE patients and vaccinees have shown that JEV proteins are presented by both MHC class I and class II pathways and stimulate strong CD8+ and CD4+ proliferative T cell responses (232). Our studies indicated an extensive proliferation of CD4+ T cells in response to both live and inactivated JEV antigen. Though CD8+ T cells were responsive to JE stimulation, the amount of proliferation was not as extensive as that shown by CD4+ T cells. A similar result was reported by Howe et al., (2005) where they found that CD4+ T cells from measles virus immune individuals proliferated maximally as compared to CD8+ T cells in response to live measles virus, suggesting the development of a Th1 like response at high antigen doses (233). Experiments with murine cytomegalovirus and MHV-68 highlight the evidence that CD4+ T cells are important effectors that operate, at least in part, via mechanisms involving IFN-γ. While the characterization of T cell responses in children with sub-clinical JEV infection showed the presence of strong Th1 responses against non-structural proteins of JEV (234), studies with CD4+ T cells from dengue immune donors indicate that these cells produce IFN-γ which may contribute to the pathogenesis of DHF and DSS (235).

The adoptive transfer of immune spleen cells into naïve 14-day-old mice had a protective effect against JEV challenge. In our results, almost complete protection was achieved in naïve mice by adoptively transferred immune splenocytes (95%). Earlier works imply that the protection is mediated by T lymphocytes (206, 207). Notably, Mathur et al., (1983) reported that depletion of B cells from the splenocytes using anti mouse IgG plus complement did not impact significantly on the outcome of adoptive transfer. Both CD8+ and CD4+ T cells have been shown to be involved in protection against JEV (207), while protection against WNV was mainly achieved by the adoptive transfer of CD8+ T cells, which can mediate their effector activity through perforin and granule exocytosis (157). Though earlier studies using a mouse model of adoptive transfer have been reported in JEV infection, these have been carried out in adult mice through the IC route of challenge, which allows the accumulation of high virus titres in the brain with little involvement of extraneural cells and tissues (206, 207).

In a study using sub-lethally irradiated mice, splenocytes from live JEV immunized donors conferred only a low level of protection to JEV challenge, suggesting that JEV challenge might stimulate faster kinetics of the host immune response with the help of transferred virus-specific T cells (196). We carried out various experiments to investigate if adoptively transferred cells were capable of functional activities against virus challenge in the
recipient animal. Adoptive transfer of cells into 14-day-old gamma irradiated mice resulted in a similar survival percentage as in non irradiated animals (Table 3.7). Correspondingly, the adoptive transfer of JEV immune splenocytes into athymic nude mice showed considerable protection against lethal JEV challenge, further demonstrating that transferred immune T cells were capable of functional activities in the recipient animal (Figure 3.9).

In our study, several issues remained unresolved regarding some of the basic features involved in effective adoptive transfer such as the trafficking of transferred T cells into lymphoid and non lymphoid tissues and the functional stability of recoverable CD4+ and CD8+ T cells. Bristol et al., (1999) showed that immune donor T lymphocytes were recoverable from the spleens and lungs of recipient mice and that functional activity was maintained at these sites after transfer (209). Recovery of adoptively transferred cells was greatest following antigen boost. The recovery and antigen specificity of JEV primed splenocytes was studied in recipient mice following incorporation of the vital dye, CFSE. It was seen that the recovery of CFSE stained, JEV primed splenocytes in the spleens of recipient mice was considerably higher following IP inoculation with JEV as compared to mice which did not receive JEV inoculation following adoptive transfer (Figure 3.8). Thus, JEV primed cells were seen to traffic to recipient mice spleen in response to antigen, probably in order to initiate a secondary immune response.

B cells, CD4+ T cells and CD8+ T cells have all been implicated in contributing to protection in flavivirus infection. Virus clearance from mouse brains acutely infected with JEV has been reported by virus specific CD8+ T cells adoptively transferred into the CNS; however, CD4+ T cells were also required (207). Our results indicate a major role for CD4+ T cells in protection from JEV infection. Depletion of CD4+ T cells before adoptive transfer resulted in a reduced level of protection against JEV infection (36% survival) as compared to mice that received CD8+ T cell depleted immune splenocytes (65% survival, Figure 3.11). Correspondingly, increased protection was observed when primed CD4+ isolated T cells were transferred as compared to CD8+ isolated T cells (Figure 3.12). Thus, CD4+ T cells seemed to play a definite role in the host immune response to JEV infection. However, some amount of protection was still observed when CD8+ isolated T cells were transferred, indicating that, to some extent, CD8+ T cells also were required for protection. Similar results have been obtained in measles virus infection, where CD4+ T cells in conjunction with CD8+ or B cells were required to control measles virus infection in mice (236). The importance of CD4+ T cells in conjunction with CD8+ or B cells have also been reported.
using knockout models in MHV and WNV infection, suggesting that CD4+ T cells function by regulating the antiviral activity of other cells.

In mice of different haplotypes, CD8+ CTLs have been readily induced in the spleen when inoculated with live flaviviruses by either the IP or the IV route (211, 212, 237). JEV specific CTLs were induced upon JEV infection or by immunization with extra cellular particle based or poxvirus based JEV vaccines (86, 87, 238). A need for CD8+ T cells in rotavirus infection was also observed, as depletion of CD8+ T cells abrogated the protective capacity of adoptively transferred splenocytes (205). CD8+ T cells were also found to exert effector function in WNV infection through perforin/ granzyme or Fas mediated virus clearance (157,216).

The importance of CD8+ T cells in flavivirus infection is probably as a result of the upregulation of MHC I molecules by these viruses (158). Infection with a high dose of WNV (Sarafend strain) resulted in greater mortality in Wt compared to β2M −/− mice, suggesting that under some conditions, CD8+ T cells might contribute to immunopathogenesis and disease (213). During infection with a neuroadapted strain of YF virus, CD8 −/− mice did not differ in survival from parental mice, while CD4+ knockout mice were unable to resist challenge.

The roles of CD4+ T cells in the response to flavivirus infection have been studied less extensively. The importance of CD4+ T cells in infection has been documented in experiments with WNV, where CD4+ or MHC II deficient mice showed higher mortality and persistent infection in surviving mice (104). The mechanism by which CD4+ T cells contribute to protection and virus clearance in the CNS is open to question. CD4+ T cells are required for APC maturation, and can mediate help for both primary and memory B and CD8+ T cell activation, and in class switching of antibodies to the IgG subclass (96, 225). CD4+ T cells are known to prime essential B cell responses following infection by measles virus, LCMV and rotavirus (104). They can also generate primary effector CD8 responses against HSV and MHV or memory CD8+ T cell responses against LCMV and influenza (105-108).

CD4+ T cells can exert multiple antiviral functions, including cytokine secretion (i.e., IFN-γ) and cytotoxicity, primarily via Fas-FasL interactions with infected targets or perforin mediated cytotoxic activity (222, 240). The potential role of Class II restricted cytolysis during JEV induced encephalitis has not been explored. Flavivirus specific, human CD4+ T cell clones were shown to have cytolytic activities in vitro (221, 241). However, no direct effector role for CD4+ T cells (perforin, FasL or IFN-γ) was observed during the adoptive
transfer of WNV primed CD4+ T cells into CD4-/- mice. CD4+ T cells may also contribute to protection by sustaining antibody production in vivo. Sitati et al., (2006) observed depressed levels of WNV specific IgM and IgG in CD4 deficient mice challenged with WNV. They also found that CD4+ T cells sustained antigen specific CD8+ T cells in the CNS. CD8+ T cells were also seen to rapidly undergo apoptosis in the CNS of CD4 deficient mice during MHV infection (106).

Little is known about the viral antigens recognized by T helper cells and the possible role of helper T cell responses in recovery/ resistance or pathogenesis of diseases. A strong anamnestic humoral response, absolutely dependent on T cell help, has been envisaged as the best defence strategy against JEV (242). We analyzed the contribution of Th cells to protection against JEV by using peptides from JEV- E and M proteins which contained Th epitopes of JEV. Three peptides were selected from a range of peptides which were tested for increased JEV neutralizing antibodies in mice following immunopotentiation studies. It was observed in a parallel study that the contribution by T helper peptides incorporating immunodominant regions of JEV was an important addition to the induction of virus neutralizing antibodies against JEV (228). Repeated in vitro stimulation of JEV primed splenocytes with Th peptides followed by adoptive transfer resulted in protection of recipient mice from lethal JEV challenge (Figure 3.24). In addition, splenocytes from mice immunized with peptide 5 (M protein derived) were seen to passively transfer immunity to naïve recipient mice and protect them from lethal challenge with JEV (Figure 3.25). Thus, the induction of T helper responses against JEV was seen to effectively contribute to immunity against JEV. Further, immune response against a single T helper epitope of JEV was enough to confer immunity in the host against JEV infection.

The nature of the T helper response (Th1 or Th2) elicited in mice that either underwent a progressive JEV infection resulting in death, or were protected from JEV infection by immune cell transfer was characterized. A progressive increase in levels of the pro-inflammatory cytokines IFN-γ and TNF-α was observed in mice acutely infected with JEV, which was in proportion to the rise in CNS virus titres. This increase in IFN-γ and TNF-α was observed in both the sera and brains of infected animals (Figures 3.15, 3.16 a, b). In comparison, mice that were protected from infection had depressed levels of IFN-γ and TNF-α in the sera, which was many fold lower than levels observed in acutely infected mice. A striking difference in levels of IFN-γ was observed between these two sets of mice on day 6, and especially on day 8 PI. Increased levels of pro-inflammatory cytokines, correlating with a poor prognosis, have been demonstrated in many other studies (167, 168, 170). In contrast,
IFN-γ or IFN-γ receptor deficient mice showed a greater peripheral viral burden, earlier virus entry into the CNS and increased lethality to WNV and dengue infection (182, 243). It was suggested that the dominant protective role of IFN-γ against WNV was antiviral in nature, occurred in peripheral lymphoid tissues and prevented viral dissemination into the CNS. IFN-γ was observed to have a less significant antiviral role in the CNS, indicating that this molecule may have a protective role in peripheral immune responses and a more pathogenic role in the CNS. An increase in IFN-γ levels in the CNS during progressive infection has also been observed during JEV infection (170).

IFN-γ is believed to contribute directly to the noncytolytic clearance of virus from neuronal cells and thus minimize host mediated tissue damage, as observed with LCMV and gammaherpesvirus infections (244). Neurons express IFN-γ receptors, providing support for the idea that they respond to this cytokine. However, results from a murine model of flavivirus encephalitis suggest that fatal disease is immunopathological in nature, with IFN-γ playing a crucial role (152). Thus, the unregulated production of proinflammatory cytokines in response to infection could have an adverse impact on the host. In fact, the proinflammatory cytokines TNF-α and IFN-γ are known to have a synergistic effect on each other’s signaling and may be involved in a positive feedback loop, thus aiding each other’s production. TNF-α has been linked to JEV triggered neuroinflammation and apoptosis of neurons (168). On the other hand, TNFR1 (-/-) mice had enhanced mortality and decreased survival time after WNV infection, and administration of a neutralizing anti TNF-α monoclonal antibody also resulted in decreased survival after WNV infection.

Concurrent with the rise of proinflammatory cytokines in the sera and brains of mice receiving JEV non-immune splenocytes, a corresponding decline in the production of the Th2 cytokines IL-4 and IL-5 was observed in these mice (Figure 3.16 c, d, e). While IL-2 levels were not significantly altered in either mice that succumbed to or were protected from lethal infection, IL-4 and IL-5 levels were seen to decrease in JEV susceptible mice as infection progressed. In contrast, mice that were protected from JEV infection had sustained expression of IL-4 and IL-5 at all time points PI. A significant difference in levels of IL-4 and IL-5 was observed between acutely infected and protected mice on day 8 PI. IL-4 has been identified as the dominant cytokine in Th2 development. Thus, Th2 cytokines, which are known to have immunomodulatory activities, probably promoted survival in mice receiving immune cell transfer, by reducing inflammation. Expression of IL-10 was also detected by RT-PCR in the brains of acutely infected mice late during infection (Figure 3.15). This detection of IL-10 may not be virus specific, but an immunoregulatory response in the brains of infected mice,
thereby preventing immune mediated injury caused by perpetuation of a proinflammatory cytokine environment, as has been observed in a model of YF virus infection (149). IL-10 is critical during most major CNS diseases and promotes survival of neurons and microglia (149, 245). Differential expression studies carried out by us revealed a considerable increase in the expression of immunomodulatory cytokines like IL-4, IL-10 and TGFβ in the brains of mice that were protected from JEV infection, whereas the converse was observed in mice that were lethally infected with JEV, thus lending support to our findings (Chapter 4).

The importance of an early antibody response in mediating recovery from JEV infection has been documented (190). Levels of IgM and IgG in plasma and CSF have been found to be higher in survivors of JEV infection (167). Mice deficient in B cells were more susceptible to WNV infection, with high viral titres in the CNS, and these mice could be protected by passive transfer with WNV immune sera (130). Administration of monoclonal antibodies to mice during infection with SLEV, MVEV and JEV also offered significant protection from otherwise lethal IP virus challenge (187, 188). In our study, the passive transfer of neat and 1:10 diluted JEV- IPF into adult mice by the IV route was capable of protecting 100% of the test animals from lethal challenge with JEV.

Virus infection induces a weak Th2 cytokine response, but this response can contribute to the production of neutralizing, virus specific IgG1 antibodies. Correspondingly, titres of JEV specific IgM and IgG1 antibodies were seen to be elevated in mice that were adoptively transferred with JEV immune splenocytes (Figure 3.20). IgG1 levels showed a rise from day 6 PI in protected mice, reaching titres that were 8 fold higher than in infected mice (Figure 3.19). Similarly, the IgG1: IgG2a ratio was increased in these animals, signifying a Th2 antibody response, while it remained constant in mice that received JEV non-immune splenocytes (Figure 3.21). This increase in IgG1 also correlated with the expression of IL-4 in protected mice, which is known to regulate the switch from IgM/D to IgG1 in activated B cells. IL-4 also has the ability to suppress IFN-γ driven Th1 antibody (IgG2a) responses. Our results contrasted with those of Ramakrishna et al., (2003) where immunization of C57BL/6J and BALB/c mice with both live and killed JEV induced a Th1 antibody response (200). However, in their studies too, outbred Swiss albino mice, despite high IFN-γ production by spleen cells, produced an initial Th2 type response, which reverted to a Th1 response after repeated immunization (200). In our study, mice receiving JEV non-immune splenocytes had low IgG1 titres (1:50), which did not increase as infection progressed, while IgM titres in these mice increased from the 4th day PI, reaching levels of 1:400 by day 8 PI. Despite an increase in IgM titres, infected mice were still found to succumb to infection.
The increase in IgG1 titres following adoptive transfer indicates a secondary response, probably due to memory CD4+ T cells in the transferred population. In a study using sub-lethally irradiated mice, splenocytes from live JEV immunized donors conferred only a low level of protection to JEV challenge, suggesting that JEV infection might stimulate faster kinetics of the immune response in the host, brought on by adoptively transferred JEV-specific T cells (196). We therefore carried out adoptive transfer studies in athymic nude mice, which have no functional T cells. We found a considerable rate of protection against lethal JEV challenge in nude mice following adoptive transfer with JEV primed splenocytes. Nude mice that received no splenocytes or splenocytes from JEV non-immune animals were seen to succumb to infection, demonstrating that anti JEV effector activities mediated by adoptively transferred immune T cells were capable of conferring protection in the recipient animal against lethal JEV infection.

It is not known which tissues are important in supporting peripheral virus growth and whether the magnitude of progeny virus produced in these tissues is a decisive factor that determines the incidence of CNS involvement. JEV titres in various organs from mice receiving JEV immune or non-immune splenocytes were compared by both real-time RT-PCR and plaque assay. Although both real-time RT-PCR and plaque assay showed similar trends for viral load in the blood, spleen and brain, PFU titers were found to be two logs less than those obtained by real-time RT-PCR. JEV was detected on days 2 and 4 PI in the blood of mice receiving non-immune splenocytes (Figures 3.13a, 3.14a). In the spleen, peak levels of virus replication were observed on days 4 and 6 PI (Figures 3.13b, 3.14b). Virus levels in the brains of mice receiving non-immune splenocytes increased exponentially, reaching peak levels on days 6 and 8 PI (Figures 3.13c, 3.14c). Viral loads in mice receiving non-immune splenocytes were thus seen to peak earlier in peripheral organs. Once productive infection had been established in the CNS, virus titres reached extremely high levels, with a corresponding decline in virus replication in the peripheral organs.

In mice that were subjected to adoptive transfer with immune spleen cells before infection, virus was detected in only a few animals. In those mice that had detectable virus titres, the course of infection did not parallel that observed in mice receiving non-immune splenocytes. Mice receiving immune splenocytes had viral titres that were four logs lower in the brain than mice receiving non-immune splenocytes. Thus, mortality due to JEV infection was, to some extent, determined to be due to the accumulation of virus load in the CNS of infected mice.
An examination of histopathological changes in the brains of mice receiving either JEV immune or non-immune splenocytes revealed changes that were indicative of cerebral inflammation. Major changes observed were the focal infiltration of polymorphonuclear cells, macrophages and lymphocytes in the meninges and cerebral tissues with congested blood vessels, and perivascular cuffing (Figures 3.22, 3.23). Most studies have detected JE viral antigen in the cytoplasm of neuronal and inflammatory cells, particularly in the hippocampus, perivascular tissue, cerebral cortex and the meninges, as was also observed in our case (230).

Histologic studies of patients who died of JEV and WNV infection have confirmed that vascular congestion in the cerebral cortex and perivascular cuffing often occurs with an acute inflammatory infiltrate of CD4+ and CD8+ T lymphocytes and moderate numbers of B cells and macrophages (68). CD8+ T cells have clearly been isolated from the brains of flavivirus infected mice and, in some cases, demonstrated to have cytolytic activity (239, 213). Virus clearance from mouse brains acutely infected with JEV has also been reported by virus specific CD8+ T cells adoptively transferred into the CNS; however, CD4+ T cells were also required (207).

In conclusion, protection from lethal JEV infection in naïve 14-day-old mice involved a CD4+ T cell mediated, Th2 immune response. Analysis of virus titres in the organs of mice that were susceptible to, or protected from lethal infection showed similar kinetics of virus replication at peripheral sites like the blood and spleen, while very high levels of virus were observed in the brains of acutely infected mice. Many pathogenic viruses are known to evade the host immune response, but in JEV, we notice an increased host inflammatory and antiviral response. In mice receiving JEV immune splenocytes, faster kinetics of Th2 antibody production resulted in higher levels of JEV specific antibody in the sera, which probably helped to reduce viral load in the CNS. Reduced levels of proinflammatory cytokines like IFN-γ and TNF-α in the sera of protected mice, combined with an increase in Th2 cytokines like IL-4 and IL-5 probably achieved an immunomodulatory effect that resulted in the enhanced survival of these animals. Thus we hypothesize that death following JEV infection is largely a consequence of immunopathological mechanisms in the host caused by an unregulated immune response against the virus.