INTRODUCTION

Japanese encephalitis is an acute flavivirus infection of the central nervous system that occurs over a wide geographic area in Asia, where it is a major public health problem in several countries (Monath & Tsai, 1997). An inactivated Japanese encephalitis virus (JEV) vaccine has been in use for over two decades. However, the complicated schedule of immunization and the lack of long-term immunity (Ku et al., 1994), as well as the recent reports of an increasing number of adverse effects with this vaccine (Andersen & Ronne, 1991), has limited its large-scale use. A stable neuro-attenuated strain of JEV (SA 14-14-2) is the only live vaccine available for human use at present in China (Eckels et al., 1988). This vaccine offers protective immunity in 98% of vaccinees, who receive two doses, and is devoid of side effects. Recombinant vaccinia viruses expressing precursor M (pre-M) and E proteins or E protein alone are highly effective at eliciting neutralizing antibodies and protection against JEV challenge in immunized mice (Mason et al., 1991; Chen et al., 1999; Pan et al., 2001). The NS1 protein also evokes a strong antibody response that protects the host against challenge (Lin et al., 1998). The recent spread of JEV to new areas, including the western half of India, Pakistan (Igarashi et al., 1994; Prasad et al., 1993) and Australia (Hanna et al., 1996), has resulted in the World Health Organization including JEV vaccine development as a priority (WHO, 1998).

The study of various factors that govern the generation of a specific and appropriate immune response against a pathogen is crucial to the development of an efficient and successful vaccine. Recently we have been focusing our efforts on the possibility of developing an oral vaccine for JEV (Ramakrishna et al., 1999). At present, there is very little information available on the type of T helper responses (Th0/Th1/Th2) generated, as well as the role of cytokines in JEV infection (Pan et al., 2001). In this study, we have investigated the T helper cytokine profiles produced in JEV-infected mice with special reference to the route of inoculation and the genetic background of the mice, as well as the type of immunogen used.

METHODS

Virus. An Indian strain of JEV (P 20778, a brain isolate of JEV obtained from a patient at Vellore) kindly provided by the National Institute of Virology, Pune, India, was used in all the experiments. Two types of live immunogen were prepared from this virus: mouse brain-derived immunogen (MBDI) and cell culture-derived immunogen (CCDI).
Mouse brain-derived immunogen. The procedure for the preparation of MBDSI has been described in detail elsewhere (Rao Bhao et al., 1988). Briefly, infected suckling mouse brains were homogenized in sucrose, treated with proteamine sulphate salt (0.09-0%, w/v; Sigma) and centrifuged at 10,000 g for 30 min to remove the precipitated non-viral proteins. The supernatant was then layered on to a 20% sucrose cushion, spun at 35,000 r.p.m. for 1 h (Sorvall) and the pellet resuspended in 1/100 of the original volume. This virus suspension was titrated using porcine kidney (PS) cells and the titre of the stock virus was adjusted to 4 x 10^8 p.f.u. ml^-1 and stored in aliquots at -70 °C.

Cell culture-derived immunogen (CCDI). The procedure for the preparation of CCDI has been described elsewhere (Gould & Clegg, 1991). The viral pellet obtained following PEG treatment of infected tissue culture fluid was resuspended and ultracentrifuged on a 10–40% sucrose gradient for 2 h at 100,000 g and the titre determined using PS cell lines. It was stored in aliquots at -70 °C after adjusting the concentration to 4 x 10^8 p.f.u. ml^-1.

Killed JEV vaccine. A commercial vaccine was obtained from the Central Research Institute, Kasauli, India. This vaccine is a formalin-inactivated product prepared from mouse brains infected with the Nakayama strain of JEV. The vaccine was received as a lyophilized preparation in a vial and was reconstituted in 5-4 ml of sterile distilled water provided by the manufacturers. This preparation was used immediately for immunization of mice.

Immunization of mice. Three different strains of mice (female, 15 g, 6 weeks old) were used in this study: random-bred Swiss Albino (New Zealand strain), C57BL/6J and BALB/c mice. The former was obtained from the Central Animal Facility, NIMHANS, Bangalore, while the other two strains were obtained from the National Institute of Nutrition, Hyderabad, India.

Three routes of immunization were used in this study, the intraperitoneal (IP), subcutaneous (SC) and peroral (PO) routes. Immunization was carried out as described previously by administering 250 μl of either MBDSI or CCDI (containing 1 x 10^6 p.f.u. JEV ml^-1) or the killed JEV vaccine. For each schedule of immunization, a minimum of 10 mice were used per route and they were divided into two groups of five each. One group (n=5) received a single dose of immunogen on day 0, while the other received three doses on days 0, 7 and 14. The spleen of each mouse was harvested 1 week after the last dose of immunization and the lymphocytes were separated using a Ficoll–Hypaque gradient. Cells (5 x 10^5) were seeded per well in a 96-well flat-bottomed tissue culture plate (Nunc). The lymphocytes were stimulated with 5 x 10^5 p.f.u. per well (suspended in 100 μl) for 48 h. The virus concentration required, as well as the duration of stimulation, was predetermined in separate experiments in order to obtain maximal secretion of the cytokines. The culture supernatants were harvested after 48 h and frozen in aliquots at -70 °C for estimation of interleukin-4 (IL-4) and interferon-γ (IFN-γ) levels by ELISA.

Cytokine assays. An ELISA using reagents from the Genzyme Duoset for IL-4 and IFN-γ was standardized to work with 50 μl of volumes of samples and reagents. The sensitivity of the assay for IL-4 and IFN-γ was 5 pg ml^-1. Briefly, the capture antibody for IL-4 and IFN-γ was diluted 1:200 in carbonate buffer (pH 9.6), coated on to Nunc Maxisorp strips in 50 μl volumes per well and incubated overnight at 4 °C. After washing, the strips were quenched with 2% milk powder (200 μl per well) in PBS, pH 7.2, containing 0.05% Tween-20 (PBST) for 2 h at 37 °C. The standards for IL-4 and IFN-γ (recombinant IL-4 and IFN-γ) and samples (50 μl undiluted sample per well) were then added to the respective wells. The standards of IL-4 and IFN-γ were serially diluted twofold in RPMI to provide a range from 1000 pg to 1 pg. Samples and standards were added in duplicate following the recommendations of the manufacturer. The plates were then incubated for 2 h at 37 °C. After washing three times with PBST, 50 μl of the respective biotinylated anti-mouse cytokine antibody (diluted 1:250 in PBST) was added to the respective wells and the plate incubated at 37 °C for 1 h. After washing the plates three times with PBST, streptavidin–horseradish peroxidase was added to the wells at a dilution of 1:2500 and the plate incubated at 37 °C for 15 min. After five washes with PBST, tetramethylbenzidine and H2O2 (Bangalore Genei) was added to the wells (diluted 1:20). Following incubation for 20 min in the dark at room temperature, the reaction was stopped by adding 50 μl 2 N H2SO4. Readings were taken at 450 nm and a standard curve was constructed using mean absorbance values with each concentration of the standard recombinant IL-4 and IFN-γ. The levels in samples were extrapolated using the standard curve.

Detection of anti-JEV IgG subtype antibodies. Anti-JEV-specific murine IgG subtype were detected in the pooled sera of the immunized mice in each group by ELISA using a mouse hybridoma subtyping kit (Boehringer Mannheim). Briefly, the procedure used was as follows: 50 μl purified JEV was coated at a predetermined concentration (200 ng per well) in carbonate buffer (pH 9.6) on to ELISA strips (Nunc) at 4 °C for 18 h. The plates were washed with sodium chloride (0.9%, w/v) containing 0.1% Tween 20 and quenched with 200 μl of the post-coating buffer provided by the manufacturer for 1 h at 37 °C. Following this, the plates were washed three times with sodium chloride solution and 50 μl of the 1/100-diluted sera was added to the wells. The plate was incubated for a further 2 h at 37 °C. After washing the plate three times with the wash solution, 50 μl of the corresponding conjugates for anti-mouse IgG subtypes IgG1, IgG2a, IgG2b and IgG3 provided by the manufacturer (diluted as recommended by the manufacturer) were added for each serum. After incubating the plate at 37 °C for 1 h, the plate was washed with the wash solution and reacted with the substrate solution provided by the manufacturer (50 μl) for a further 1 h. The plate was read in an ELISA reader using a wavelength filter of 405 nm. A positive anti-JEV monoclonal antibody and negative sera were also run in the test as controls. The T helper profile was deduced in a particular sample by determining the absorbance value ratio of IgG2a and IgG1 for that sample.

Statistical analysis. Statistical analysis was performed on the type of antibody, the level of protection offered by each schedule of immunization and the levels of cytokines produced by lymphocytes using the statistical package SPSS version 7.5 for Windows. A chi-square test was performed on the cytokine experiment data to study the degree of relationship between the T helper profile generated and the route of immunization, strain of mice and the type of vaccine used, while analysis of covariance (ANCOVA) was performed for the levels of IL-4/IFN-γ.

RESULTS

In this study, 270 mice were used for immunization by three routes using two different live antigenic preparations, MBDSI (n=90) and CCDI (n=90), as well as a commercially available killed vaccine (n=90), for each of three strains of mice. In addition, 36 mice were used as mock-immunized controls for each of the routes evaluated (four mice per route per strain), receiving PBS (pH 7.2) instead of JEV.

Cytokine profiles in the three strains of mice

The T helper profile in a given mouse was ascertained by determining the ratio of IFN-γ to IL-4 levels. If the ratio obtained was greater than 1, it was considered to be...
Table 1. IL-4 and IFN-γ responses noted in three strains of mice following immunization by various routes with one and three doses of cell culture-derived live Japanese encephalitis virus (CCDI)

IL-4 and IFN-γ levels indicated in the table are mean values for five mice in each group (except for the C57 group which has four) expressed in pg ml⁻¹. Numbers in parentheses indicate the SD.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dose</th>
<th>IL-4</th>
<th>IFN-γ</th>
<th>Ratio</th>
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<tr>
<td></td>
<td></td>
<td>Intrapерitoneal route</td>
<td>Subcutaneous route</td>
<td>Peroral route</td>
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<tr>
<td></td>
<td></td>
<td>IL-4</td>
<td>IFN-γ</td>
<td>IL-4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>56 (5-2)</td>
<td>201 (20-5)</td>
<td>3:60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>64 (1-6)</td>
<td>277 (52-6)</td>
<td>4:40</td>
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<td></td>
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<td>205 (36-9)</td>
<td>194 (34-5)</td>
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<td></td>
<td></td>
<td>37-4 (28-8)</td>
<td>76 (5)</td>
<td>2:05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>66 (15-5)</td>
<td>163 (22-2)</td>
<td>2:47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55 (4-5)</td>
<td>219 (99-2)</td>
<td>4:00</td>
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C57, C57BL/6J mice; SA, Swiss albino mice; BC, BALB/c mice.

indicative of a Th1 response. Ratios in the range 0.9–1 were considered to be an equivocal response and indicative of a Th0 response. All ratios less than or equal to 0.89 were considered to be indicative of a Th2 response. Table 1 depicts the mean IL-4 and IFN-γ levels and the IFN-γ/IL-4 ratios obtained in the supernatants of lymphocyte cultures obtained from mice immunized with one and three doses of CCDI. There were no detectable levels of IL-4 and IFN-γ in the supernatants of lymphocytes obtained from mock-immunized control animals. On the other hand, IFN-γ levels were found to be consistently higher than IL-4 levels in C57BL/6J immunized mice, irrespective of the route of immunization or the number of doses administered. While the IL-4 levels remained more or less constant over the three doses of immunization, IFN-γ levels showed a sharp increase between the first and third doses. A similar overall picture emerged with BALB/c mice immunized with CCDI. The mean IFN-γ levels were lower than those obtained with C57BL/6J mice, especially in culture supernatants obtained after a single immunization. The cytokine profiles obtained with Swiss albino mice were, however, different. High IL-4 levels were noted after the first dose of immunization for all three immunization routes and there was a steep decline in IL-4 levels (two- to threefold) following the third dose of CCDI. On the other hand, the IFN-γ levels were lower than IL-4 levels after the first dose of immunization with CCDI by all three routes and decreased after the third dose, but still remained slightly higher than IL-4 levels after the third dose. Table 1 shows the IL-4/IFN-γ ratio in the three strains of mice immunized with CCDI administered by the three routes. The C57BL/6J mice immunized with CCDI by the IP, SC and PO routes showed IFN-γ/IL-4 ratios above 3:5 with a single dose, increasing to 4:0–4:5 following three doses, indicating a Th1 profile. The BALB/c mice immunized by the IP and SC routes had similar ratios to the C57BL/6J mice. In addition, the ratios of IFN-γ to IL-4 were two- to fourfold higher in BALB/c mice following the third dose of immunization compared with a single dose of immunization. Swiss albino mice immunized with a single dose of CCDI by the IP and SC routes had ratios of 0.94 and 0.95, indicating a Th0 profile. However, after the administration of three doses, the IP and SC routes showed ratios of 2:05 and 4:37, respectively, indicating a Th1 response. On the other hand, the PO route showed ratios of 0.6 and 1:1 with one and three doses, respectively, indicating that an initial Th2 response had shifted to a Th1 profile after the third dose.

Table 2 portrays the cytokine profile of mice immunized with one and three doses of MBDI. The cytokine levels generated by MBDI were lower compared with CCDI in Swiss albino mice. However, the cytokine profiles of C57BL/6J and the BALB/c mice immunized with MBDI by the IP and SC routes were very similar to those obtained with CCDI. Similarly, there was no significant difference noted in the cytokine profiles of Swiss albino mice immunized with MBDI and CCDI. The only exception noted was with respect to BALB/c mice immunized by the PO route, which exhibited a Th2 profile after the first dose with MBDI.

Table 3 depicts the mean IL-4 and IFN-γ levels of Swiss albino mice following one and three doses of immunization with killed vaccine administered by the three routes. Swiss albino mice immunized with a single dose of killed vaccine by the IP and SC routes exhibited ratios of 0.24 and 0.58, indicating a Th2 profile. After the administration of three doses, the IP and SC routes showed ratios of 0.38 and 0.08, respectively, indicating that these mice were committed to a Th2 response. Similarly, the PO route showed ratios of 0.37 and 0.62 with one and three doses, respectively, indicating that a Th2 response was generated following one dose of immunization and that it remained throughout the full schedule of immunization.

**JEV-specific IgG subtype antibodies**

IgG subtypes could not be estimated on individual serum samples and hence they were estimated on the pooled serum samples (n=5) obtained from each group of mice immunized with either CCDI, MBDI or killed vaccine by the three routes. The absorbance (A) values (mean ±

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T helper profiles in JEV-infected mice
Table 2. IL-4 and IFN-γ responses noted in three strains of mice following immunization by various routes with one and three doses of mouse brain-derived live Japanese encephalitis virus (MBDI)

IL-4 and IFN-γ levels indicated in the table are in pg ml⁻¹. Numbers in parentheses indicate the SD.

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Dose</th>
<th>Intraperitoneal route</th>
<th>Subcutaneous route</th>
<th>Peroral route</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>IL-4</td>
<td>IFN-γ</td>
<td>Ratio</td>
</tr>
<tr>
<td>C57</td>
<td>1 (n = 4)</td>
<td>64 (2-8)</td>
<td>259 (9-8)</td>
<td>4-04</td>
</tr>
<tr>
<td></td>
<td>3 (n = 4)</td>
<td>68 (2)</td>
<td>253 (6-8)</td>
<td>3-73</td>
</tr>
<tr>
<td>SA</td>
<td>1 (n = 5)</td>
<td>44 (27-1)</td>
<td>61 (62-8)</td>
<td>1-4</td>
</tr>
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<td></td>
<td>3 (n = 5)</td>
<td>97 (54-9)</td>
<td>114 (13-5)</td>
<td>1-2</td>
</tr>
<tr>
<td>BC</td>
<td>1 (n = 5)</td>
<td>94 (57-4)</td>
<td>186 (131-1)</td>
<td>1-98</td>
</tr>
<tr>
<td></td>
<td>3 (n = 5)</td>
<td>67 (37-8)</td>
<td>287 (166-3)</td>
<td>4-28</td>
</tr>
</tbody>
</table>

*C57, C57BL/6J mice; SA, Swiss albino mice; BC, BALB/c mice.

2 SD) obtained with the sera of negative controls (mock-immunized with PBS) did not exceed 0-1 for IgG subtypes.

Fig. 1 depicts the JEV-specific IgG isotype profiles. It can be observed that in Swiss albino mice immunized with CCDI, IgG1 A values were higher than IgG2a following one and three doses by the IP and PO routes (Fig. 1a). However, no clear response was observed for Swiss albino mice immunized with MBDI by the IP route (Fig. 1b), while the SC and PO routes showed a predominant Th2 response. C57BL/6J mice immunized with CCDI had higher IgG1 isotype compared with IgG2a for the IP route indicating a significant increase in IgG1 for the IP route compared with IgG1 for the SC route. Such a phenomenon was not observed for the other routes (Fig. 1e). On the other hand, BALB/c mice immunized with MBDI uniformly showed an increase in A values for both isotypes between the first and third dose of immunization for the SC route. Such a phenomenon was not observed for the other routes (Fig. 1e). On the other hand, BALB/c mice immunized with MBDI uniformly showed an increase in A values for both isotypes between the first and third dose for all three routes.

Fig. 2, which depicts the IgG subtypes of Swiss albino mice immunized with killed vaccine by the three routes of immunization, indicates varying profiles. Following the third dose, the IP route displayed a Th1 profile, while the PO route had predominant IgG1. The SC route on the other hand did not display any major subtype after the third dose.

**DISCUSSION**

The immune system is characterized by a complex array of effector T cells that produce cytokines, which facilitate both cell-mediated and antibody responses to foreign antigen. It is now apparent that three functional classes of T helper cells exist: Th0, Th1 and Th2. These cells are characterized by the type of cytokine produced in vitro on stimulation with a specific antigen. Generally it is accepted that Th0 cells are the precursors and they can be driven towards either a Th1 response, which is predominantly cell-mediated, or a Th2 response, which is predominantly humoral (Romagnani, 1994).

The immune response to JEV infection in experimental animals as well as humans has been an active area of research in recent years (Desai et al., 1994, 1995; Mathur et al., 1983; Muralikrishna et al., 1996). Protection against JEV infection is probably determined largely by differential stimulation of CD4⁺ T cells. Recently it has been demonstrated that humoral immunity, particularly E-specific antibodies, plays a critical role in clearance of JEV infection, whereas CD8⁺ cytotoxic T cell activity may not be required for protective immunity. Induction of optimal antibody responses by DNA or live JEV vaccines is entirely dependent on the

Table 3. IL-4 and IFN-γ responses noted in Swiss albino mice following immunization by various routes with one and three doses of killed Japanese encephalitis virus (killed vaccine)

IL-4 and IFN-γ levels indicated in the table are in pg ml⁻¹.

<table>
<thead>
<tr>
<th>Dose</th>
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<tr>
<td></td>
<td>IL-4</td>
<td>IFN-γ</td>
<td>Ratio</td>
</tr>
<tr>
<td>1 (n = 4)</td>
<td>95</td>
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</tr>
<tr>
<td>3 (n = 4)</td>
<td>25</td>
<td>9</td>
<td>0-38</td>
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</table>
presence of CD4⁺ Th cells (Pan et al., 2001). Very few studies have, however, addressed the modulating influence of the genetic background (strain of mouse used), the type of immunogen and/or the route of inoculation of the antigen on the pattern of T helper cell induction. Th1 and Th2 cells produce distinct panels of cytokines, which can cross-regulate production and influence the activities of each other. It has been shown that Th commitment in mice infected with *Leishmania major* is determined by the cytokines produced in the first week of infection, during which the T cell priming occurs. Specifically, early production of IFN-γ and IL-12 leads to the production of Th1, while early production of IL-4 leads to Th2 development (Belosevic et al., 1989). We therefore felt it appropriate...
immunization, where both IFN-γ equivocal Th0 response with CCDI after the first dose of albino mice immunized by the IP and SC routes evoked an with these two strains of mice (Table 1). For instance, Swiss mice was significantly different (\(P<0.001\)). Route-specific Th responses were noted following a single dose of immunization. However, by the third dose, these mice had also reverted to a Th1 profile. Overall, there was no significant difference in the T helper profiles (\(P=0.89\)) between the IP and SC routes, which was true for all strains of mice as well as both the immunogens used. On the other hand, these two routes were significantly different from the PO route in the type of T helper profile generated (\(P<0.001\)) in all the strains of mice. With respect to the PO route, it was noted that, while C57BL/6J mice evoked a Th1 profile throughout the immunization regimen, a Th2 type of response was elicited in MBDI-immunized BALB/c mice initially (following the first dose). This response subsequently reverted to a strong Th1 response in these mice (by the third dose). An identical trend was also noted in PO-immunized Swiss albino mice. The reversibility of Th1 and Th2 populations has been extensively studied in mouse and human in vitro models of T cell priming. In an in vitro mouse model, using T cell receptor-transgenic mice derived on a different background, it was shown that fully differentiated Th1 cells were irreversibly committed, while Th2 populations could be induced to secrete IFN-γ under certain conditions (Hu-Li et al., 1997). IL-4-producing populations induced after 1 or 3 weeks of stimulation were not stable and were induced to produce IFN-γ in response to IL-12. In contrast, differentiated IFN-γ-producing cells could not be induced to produce IL-4 by further stimulation in the presence of IL-4. This could be due to the differentiation of uncommitted precursor cells. Such a phenomenon is likely to be operative to investigate whether such mechanisms were operative in JEV infection. The results of this study indicated that distinct patterns of cytokine production were observed in the three strains of mice immunized with JEV. Notable was the fact that T helper cells recruited were (i) strain-specific, (ii) route-specific and (iii) related to the type of immunogen and the number of doses administered. The absence of IL-4 and IFN-γ production in the splenocyte cultures of mock-immunized mice confirmed that the cytokine responses noted were indeed specific to JEV infection.

**Strain-specific Th responses**

The three strains of mice used in this study, C57BL/6J, BALB/c and Swiss albino mice showed variable T helper profiles in response to JEV immunization (\(P<0.001\)). C57BL/6J mice exhibited a typical Th1-type response soon after the first dose, irrespective of the immunogen used or the route of immunization. Very high ratios of IFN-γ to IL-4 were also noted in these mice (Tables 1 and 2). These results imply that there is an early commitment of the T cells to a Th1-type response in C57BL/6J mice. There was no significant difference between the cytokine profiles evoked by C57BL/6J and BALB/c mice (\(P=0.092\)). However, the T helper response elicited by the random-bred Swiss albino mice was significantly different (\(P<0.001\)) in comparison with these two strains of mice (Table 1). For instance, Swiss albino mice immunized by the IP and SC routes evoked an equivocal Th0 response with CCDI after the first dose of immunization, where both IFN-γ and IL-4 were secreted at equal levels. Yet another difference noted was the significantly higher levels of IFN-γ in C57BL/6J and BALB/c mice compared with Swiss albino mice after the first and third doses of immunization (\(P<0.001\)). On the other hand, splenocyte cultures obtained from immunized Swiss albino mice showed slightly higher levels of IFN-γ and IL-4 initially (day 7), which declined sharply (twofold or greater) by day 21. It is difficult to explain this paradoxical observation; nevertheless, it is tempting to postulate that the outbred nature of Swiss albino mice may account for this feature, since all three immunogens yielded identical results (i.e. CCDI, MBDI and killed vaccine). In fact, strain variation with respect to T helper responses has been reported in the literature in various other systems (Keane-Myers & Nickell, 1995; Scott, 1991). C57BL/6J mice have a H-2^b haplotype and BALB/c mice have H-2^d, while Swiss albino mice are random bred and would therefore probably produce heterologous responses. These differences in the genetic background could therefore have a critical bearing on the type of T helper response elicited. This may hold true for human immune responses to vaccines where the population being immunized is often genetically diverse.

Route-specific Th responses

Route-specific differences in T helper cell cytokine production were observed in this study in all three strains of mice tested (\(P<0.001\)). The IP and SC routes uniformly evoked a Th1 response in all the mice with high levels of IFN-γ and lower levels of IL-4, with the exception of Swiss albino mice immunized with CCDI (Table 1). In these CCDI-immunized Swiss albino mice, an equivocal Th0 response was noted following a single dose of immunization. Absorbance readings obtained by ELISA detecting JEV-specific IgG subtype antibodies in the pooled sera of Swiss mice was significantly different (\(P<0.001\)). On the other hand, these two routes were significantly different from the PO route in the type of T helper profile generated (\(P<0.001\)) in all the strains of mice. With respect to the PO route, it was noted that, while C57BL/6J mice evoked a Th1 profile throughout the immunization regimen, a Th2 type of response was elicited in MBDI-immunized BALB/c mice initially (following the first dose). This response subsequently reverted to a strong Th1 response in these mice (by the third dose). An identical trend was also noted in PO-immunized Swiss albino mice. The reversibility of Th1 and Th2 populations has been extensively studied in mouse and human in vitro models of T cell priming. In an in vitro mouse model, using T cell receptor-transgenic mice derived on a different background, it was shown that fully differentiated Th1 cells were irreversibly committed, while Th2 populations could be induced to secrete IFN-γ under certain conditions (Hu-Li et al., 1997). IL-4-producing populations induced after 1 or 3 weeks of stimulation were not stable and were induced to produce IFN-γ in response to IL-12. In contrast, differentiated IFN-γ-producing cells could not be induced to produce IL-4 by further stimulation in the presence of IL-4. This could be due to the differentiation of uncommitted precursor cells. Such a phenomenon is likely to be operative...
in the Swiss albino mice immunized by the PO route in the present study.

**Immunogen and dose-related Th responses**

The number of doses of immunogen administered was observed to have a significant influence on the IFN-\(\gamma\) and IL-4 levels in culture supernatants obtained from lymphocytes of immunized mice (\(P<0.01\)). Overall, it was noted that the level of IFN-\(\gamma\) was higher at the end of the third dose than the first dose (\(P=0.02\)). However, it must be emphasized that in the majority of the mice, high IFN-\(\gamma\) levels were noted at day 7 (i.e. after administering the initial dose) following immunization except in Swiss albino mice immunized by the PO route. This probably denotes that there is a strong commitment to a Th1 response in all strains of mice immunized with live JEV (either CCDI or MBDI). However, such a phenomenon was not noted in Swiss albino mice immunized with the killed vaccine, where a Th2 type of response was predominant (Table 3). It must be emphasized that several studies have indicated that the antigen dose can clearly have strong effects on the type of T helper response obtained (Seder & Mosmann, 1998). Thus, the induction of Th2 responses to killed JEV vaccine noted might be due to ‘low protein’ in vivo, resulting in preferential presentation by B cells. On the other hand, during infection with live organisms, particularly intracellular parasites, it has been noted that although antigen-presenting cells may be less frequent and/or localized to particular locations, each antigen-presenting cell is able to present relatively higher amounts of antigen to Th helper cells through active replication (Seder & Mosmann, 1998).

Unlike IFN-\(\gamma\), IL-4 secretion was significantly lower at the end of the third dose than at the end of the first dose (\(P<0.001\)). Cultures from C57BL/6J and BALB/c mice yielded lower levels of IL-4 to begin with, compared with cultures obtained from Swiss albino mice, where high levels of IL-4 were observed from the very first dose. While the C57BL/6J and BALB/c mice continued to express a Th1 profile by the end of the third dose with increased secretion of IFN-\(\gamma\), the Swiss albino mice had a significant fall in the IL-4 levels and reverted to a Th1 profile by the end of the third dose. This reversion to Th1-type noted in Swiss albino mice may be explained by the fact that hyper-immunization with an antigen can switch a response through a particular route if a few heterogeneous cells still remain in the draining nodes (Li et al., 1996). Alternatively, the degree of Th cell subset polarization could also influence the later responses to the antigen.

**Anti JEV-specific IgG isotypes**

Different patterns of cytokine production by Th1 and Th2 cells are known to contribute to distinct functions in the regulation of isotype-specific B cell responses (Finkleman et al., 1990). For instance, it has been shown that IFN-\(\gamma\) is the major Th1 cytokine, which is responsible for IgG2a production, and IL-2 synergizes this response (Snapper & Paul, 1987). In contrast, it has been shown that Th2 cells and IL-4 preferentially augment IgG1 synthesis in both LPS-triggered B cell cultures and in antigen-specific systems (Stevens et al., 1988; Keane-Myers & Nickell, 1995). In this study we investigated the pattern of anti-JEV IgG isotypes in the sera of JEV-immunized mice to ascertain whether it correlated with the cytokine profiles expressed in vitro. From the data presented here it can be observed that the IgG isotype profiles in the sera of immunized mice correlated well with the type of cytokine produced by lymphocyte cultures stimulated in vitro with JEV. The best correlation was obtained in BALB/c mice, where all three routes of immunization with both antigens (MBDI and CCDI) showed excellent correlation of in vivo isotype patterns with those of in vitro cytokine profiles. C57BL/6J immunized mice also showed a similar pattern of correlation. However, a discrepancy was noted between in vivo and in vitro functional correlates in mice immunized with MBDI by the SC and PO routes. In the Swiss albino strain, similar discrepancies were noted in mice immunized with CCDI by the SC route and with MBDI by the PO route. The interesting observations from these discrepant data show that, despite high in vitro IFN-\(\gamma\) production by spleen cells of these mice, serum JEV-specific IgG1 but not IgG2a remained high. It is possible that other non-T cell sources such as NK cells augment IFN-\(\gamma\)-levels in vivo in these mice. Chen et al. (1999) have also observed dissociation between the IgG isotypes generated in mice immunized by two different routes with recombinant plasmids containing the envelope gene of JEV. Intramuscular immunization generated high-avidity anti-\(E\) antibodies, predominantly of IgG2a isotype, while gene gun DNA immunization produced predominantly IgG1 anti-\(E\) antibodies of significantly lower avidity. It has been observed previously with other pathogens that, under certain conditions, antigen-induced cytokine profiles and isotypic responses may dissociate. Early cytokines have been suggested to play a determinant role in driving naïve T cells to polarized cytokine responses (Seder & Paul, 1994). Effector T cells, in contrast, are resistant to change in their initial cytokine profile (Seder & Paul, 1994). Thus, early signals presented to T cells during antigen presentation may determine their Th2 orientation, which persist even though IFN-\(\gamma\) is induced as in infection with Salmonella typhimurium (Comoy et al., 1997). In contrast, the late cytokine environment may influence the B cell isotype switching. Also, considering that the antigen was given by different routes, differences in homing and migration of T and B cells could be another reason for the differences. It has been noted in an earlier study (Thakare et al., 1991) that in most of the human JE patients (10/17) who recovered from acute viral encephalitis, JE-specific IgG1 subtype was predominant in their cerebrospinal fluid, and it was suggested that this could have a protective role in clearance of virus from the CNS. Indeed it has been shown that viruses induce mostly IgG2a in mice (Coutelier et al., 1988) and IgG1 and IgG3 in humans (Skvaril & Schilt, 1984). Thus, the observations of Thakare et al. (1991) are in agreement with the observations of this study.
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REFERENCES


