Protection of adult but not newborn mice against lethal intracerebral challenge with Japanese encephalitis virus by adoptively transferred virus-specific cytotoxic T lymphocytes: requirement for L3T4+ T cells

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The protective ability of cytotoxic T cells (CTL) raised in vitro against Japanese encephalitis virus (JEV) was examined by adoptive transfer experiments. Adoptive transfer of anti-JEV effectors by intracerebral (i.c.) but not by intraperitoneal (i.p.) or intravenous (i.v.) routes protected adult BALB/c mice against lethal i.c. JEV challenge. In contrast to adult mice, adoptive transfer of anti-JEV effectors into newborn (4-day-old) and suckling (8–14-day-old) mice did not confer protection. However, virus-induced death was delayed in suckling mice compared to newborn mice upon adoptive transfer. The specific reasons for lack of protection in newborn mice are not clear but virus load was found to be higher in newborn mice brains compared to those of adults and virus clearance was observed only in adult mice brains but not in newborn mice brains upon adoptive transfer. Specific depletion of Lyt 2.2+, L3T4+ or Thy-1+ T cell populations before adoptive transfer abrogated the protective ability of transferred effectors. However, when Lyt 2.2+ cell-depleted and L3T4+ cell-depleted effectors were mixed and transferred into adult mice the protective activity was retained, demonstrating that both Lyt 2.2+ and L3T4+ T cells are necessary to confer protection. Although the presence of L3T4+ T cells in adoptively transferred effector populations enhanced virus-specific serum neutralizing antibodies, the presence of neutralizing antibodies alone without Lyt 2.2+ cells was not sufficient to confer protection.

Introduction

Major histocompatibility complex (MHC)-restricted, virus-specific cytotoxic T lymphocytes (CTL) are important in protective immunity against viral infections. Adoptive transfer of polyclonal or cloned populations of in vitro generated virus-specific cytotoxic effector cells has been shown to confer protection to the host and reduce viral titre in infected tissues in the case of several viral infections such as lymphocytic choriomeningitis, influenza, herpes simplex, rabies, rotavirus, murine cytomegaloviruses and others (Joly et al., 1991; Cossins et al., 1993; Sethi et al., 1983; Kawano et al., 1990; Offit & Dudzik, 1990; Hom et al., 1991; Reddehase et al., 1988). CTL and natural killer (NK) cells can be induced against flaviviruses and the immunodominant cytotoxic T cell determinants in Kunjin virus have been identified in mice using a panel of vaccinia virus–Kunjin virus recombinants. These determinants were present in the non-structural proteins NS3 and NS4A. Modest cross-reactive recognition of these vaccinia virus–Kunjin recombinants by anti-JEV cytotoxic lymphocytes has also been reported (Rothman et al., 1993; Kesson et al., 1988; Parrish et al., 1991; Hill et al., 1995). The action of cytotoxic macrophages may be important during the early stages of Kunjin virus infection (MacFarlan & White, 1980; Rodda & White, 1976). While virus-immune spleen cells have been shown to participate in protective immunity against Japanese encephalitis virus (JEV) and Banzi virus infections (Mathur et al., 1983; Jacoby et al., 1980), the actual role of antiviral CTL in conferring protective immunity against JEV is not known. Our previous studies reported the generation of JEV-specific Lyt 2.2+ CTL in BALB/c mice (Murali-Krishna et al., 1994). The results presented in this paper deal with the ability of adoptively transferred anti-JEV polyclonal effectors to confer protection against lethal intracerebral (i.c.) challenge with JEV, the relative
contribution of Lyt 2.2+ and L3T4+ cells in protective activity and the influence of recipient mouse age on the protective ability of adoptively transferred effectors.

Methods

Virus. JEV strain P20778, an isolate from human brain was routinely grown at 28 °C in C6/36 cells that were grown to confluency. Infectious virus was titrated by plaque assay as described elsewhere (Murali-Krishna et al., 1994) or by LD50 measurement. LD50 of JEV was calculated by inoculating groups of adult (above 40-day-old), weanling (22–40-day-old), suckling (5–21-day-old) and newborn (up to 4-day-old) mice with 0.1, 1, 5, 10, 100, 500 and 1000 p.f.u. of JEV by i.c. or intraperitoneal (i.p.) routes in a volume of 30 μl and monitored for mortality over a period of 15 days. The LD50 value by i.c. challenge for weanling and adult mice was 10 p.f.u. per mouse whereas for newborn mortality over a period of 15 days. The LD50 value by i.c. challenge for newborn mice was 1000 p.f.u, whereas i.p. challenge did not cause mortality in the case of adult, weanling and suckling groups of mice.

Generation of anti-JEV effectors. Anti-JEV effectors were generated according to the optimal in vivo immunization and in vitro stimulation protocol as described previously (Murali-Krishna et al., 1994). Briefly 6- to 8-week-old male BALB/c (H-2d) mice were primed by i.p. immunization with JEV-infected PS cells on days 0 and 3 and with JEV-infected Sp2/0 cells on day 6. Three days after the last immunization, primed mouse spleen cells were restimulated in vitro with JEV-infected P388D1 (H-2d) cells in a 4 day culture and the virus-specific CTL activity was monitored by chromium release assay.

Adoptive transfer of anti-JEV effectors. Anti-JEV effector cells generated in vitro as described were harvested on 5th day of culture and washed once with RPMI 1640 medium (Sigma) containing 0.2% fetal bovine serum (FBS) (Gibco). Viable effector cells were isolated by layering on Ficoll hypaque (Sigma; density 1.119 g/cm3) to remove dead cells and red blood cells (RBC), washed three times with medium containing plus 0.2% FBS and were resuspended in RPMI 1640 medium supplemented with 5% FBS before transfer into mice. Unless otherwise mentioned, mice were usually injected i.c. with a mixture of effector cells along with 10 LD50 JEV in a volume of 30 μl using a sterile 26 gauge needle. Controls included injection of 10 LD50 dose of JEV only, 10 LD50 JEV plus naive mouse spleen cells, 10 LD50 JEV plus anti-H-2d effectors or only RPMI 1640 medium supplemented with 5% FBS.

Depletion of lymphocyte subpopulations before adoptive transfer. Depletion of Thy-1+, Lyt 2.2+ and L3T4+ lymphocyte subpopulations was carried out in RPMI 1640 medium supplemented with 5% FBS. Anti-JEV effector cells that were separated from dead cells and RBC using Ficoll hypaque were suspended at a final concentration of 1 x 108 cells/ml and incubated for 20 min at 37 °C with 1:800, 1:5 and 1:200 diluted anti-Thy-1 (clone NIMR-1; Sera-Lab), anti-Lyt-2.2 [clone AD4(15); Cedarlane] and anti-L3T4 (clone YT8191.2; Sera-Lab) monoclonal antibodies, respectively. These dilutions were previously confirmed to completely deplete the respective lymphocyte subpopulations (Murali-Krishna et al., 1994). After incubation, the cells were washed once with ice-cold medium and resuspended in the original volume containing 1:5 diluted pre-screened guinea-pig serum as a source of complement. The suspension was incubated at 37 °C for 30 min and then washed once. After three such consecutive cycles of depletion, the remaining cells were washed and suspended in RPMI 1640 medium supplemented with 5% FBS before transfer into mice. The number of cells adoptively transferred after depletion was based on the original viable cell count before depletion. Thus the depleted cells injected into each mouse were derived from 8 x 106 or 16 x 106 undepleted effectors as stated in the legends. Controls for each mouse included the appropriate number of undepleted effectors used for depletion. T cell enrichment of separated anti-JEV effectors (4 x 1010 cells) was carried out by nylon wool column separation according to manufacturer’s recommendations (Polysciences, Warrington, USA). The recovery of non-adherent cells was 56% of the original number of viable cells loaded on the column. The non-adherent cells were washed once, resuspended in RPMI 1640 medium supplemented with 5% FBS before injection into the mice. The concentration of cells for injection was calculated based on the original cell count before depleting the nylon wool-adhering population.

Virus titration in infected mouse brains. JEV-infected adult mice were sacrificed by etherization, whereas newborn mice were sacrificed by freezing them at −20 °C for 30 min. Brains were dissected out, frozen in liquid nitrogen and stored at −70 °C. The tissues were homogenized in PBS to obtain a 10% w/v homogenate using a Potter–Elvehjem homogenizer fitted with a teflon pestle at 4 °C. The homogenate was centrifuged at 10000 g for 10 min and the sterile filtered tissue homogenate was titrated by plaque assay on PS cells.

Neutralizing antibody titration. Mice were bled through the retro-orbital plexus, the serum was sterilized by filtration and stored at −70 °C until use. Neutralizing antibody titre was measured using 50% plaque reduction end-point assay (Kimura-Kuroda & Yasui, 1988). Various dilutions of immune serum ranging from 1:5 to 1:1600 were incubated with 100 p.f.u. of JEV at 37 °C for 1 h in 1 ml of MEM supplemented with 5% FBS. After incubation, 300 μl of the immune serum plus virus mixture was inoculated onto monolayers of PS cells that were grown in Linbro plates and incubated at 37 °C for a further period of 1 h. The unadsorbed inoculum was removed and cells were overlaid with MEM containing 0.55% agarose, cultured for 3 days and stained for plaques. The neutralizing antibody titre (NT Ab titre) was expressed as the reciprocal of the serum dilution required to cause 45–65% reduction in the number of plaques obtained with the control inoculum. Control inoculum contained 100 p.f.u./ml of JEV pre-incubated at 37 °C for 1 h. The number of plaques obtained with control inoculum usually ranged between 20–30 in different experiments. Negative controls included inoculation of PS cells with immune serum alone which never formed plaques. Each serum sample was assayed for its neutralizing antibody titre in triplicate or quadruplicate. The standard deviation (s) for triplicate or quadruplicate titres never exceeded 22% of the mean neutralizing antibody titre.

Statistical analysis. Data used to represent survival curves were statistically analysed by the 'G' test as recommended by Sokal & Rolf (1981) while MDD (mean day of death) values and NT Ab titres were analysed for significance by Student’s ‘t’ test.

Results

Protective ability of adoptively transferred anti-JEV effectors and the influence of recipient mouse age

JEV strain P20778 did not cause mortality of adult mice by any route other than i.c. In addition, in the case of a neurotropic virus such as lymphocytic choriomeningitis virus (LCMV), co-inoculation of antiviral CTL along with a lethal dose of virus i.c. was shown to efficiently clear infectious virus in the CNS (Klavinski et al., 1989). Hence our initial studies employed a similar approach in which anti-JEV effectors were adoptively transferred by
Table 1. Protection of adult BALB/c mice against lethal JEV challenge by adoptively transferred anti-JEV effectors

<table>
<thead>
<tr>
<th>Expt</th>
<th>Age of mice (days)</th>
<th>Route of JEV challenge*</th>
<th>Route of cell transfer†</th>
<th>Percentage of mice surviving (dead/total)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30–50</td>
<td>i.c.</td>
<td>None</td>
<td>0 (10/10)</td>
</tr>
<tr>
<td>2</td>
<td>30–50</td>
<td>i.c.</td>
<td>i.v.</td>
<td>0 (9/9)</td>
</tr>
<tr>
<td>3</td>
<td>30–50</td>
<td>i.c.</td>
<td>i.p.</td>
<td>0 (7/7)</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>i.c.</td>
<td>None</td>
<td>0 (6/6)</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>i.c.</td>
<td>i.c.</td>
<td>0 (5/5)</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>i.p.</td>
<td>None</td>
<td>0 (5/5)</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>i.p.</td>
<td>i.p.</td>
<td>0 (5/5)</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>i.p.</td>
<td>i.c.</td>
<td>0 (6/6)</td>
</tr>
</tbody>
</table>

* Young adult (30–50-day-old) or newborn (4-day-old) BALB/c mice were injected with 10 LD₅₀ JEV by i.c. or i.v. routes as indicated. Injection of only medium did not cause mortality. (Adult i.c., n = 5; newborn i.c., n = 10; newborn i.p., n = 15).
† 8 x 10⁶–16 x 10⁶ anti-JEV effectors were injected per mouse by i.c., i.v. or i.p. routes. In each experiment prior to adoptive transfer an aliquot of anti-JEV effectors was examined for virus-specific lytic activity on ¹¹⁵Cr-labelled JEV-infected P388D1 targets. Specific lysis of infected targets ranged from 53–65% in different experiments at an E:T ratio of 40:1. Transfer of 70 x 10⁶ anti-JEV effectors into adult mice by i.v. or i.p. routes was also attempted in a separate experiment without successful protection.
‡ Percentage of total mice survived at day 30, post-challenge/adoptive transfer.
§ When mice were challenged with 100 LD₅₀ instead of 10 LD₅₀ JEV only 2/10 mice (20%) survived. Protection was not observed when mice were adoptively transferred with a decreased number (8 x 10⁵–8 x 10⁶) of effectors.

The i.c. route along with simultaneous lethal challenge by JEV as described in Methods. As shown in Table 1, transfer of 8 x 10⁶–16 x 10⁶ anti-JEV effectors i.c. protected 61–5% of adult BALB/c mice against lethal JEV challenge i.c. during the observation period of 30 days. In contrast to the significant protection observed in mice challenged with 10 LD₅₀ of virus, adoptively transferred effectors protected only 20% of the mice when subjected to a relatively higher challenge dose of 100 LD₅₀ of JEV. While the mice that were challenged with 10 LD₅₀ JEV alone exhibited 100% mortality, those which were injected with medium alone did not die suggesting that the death was not caused by the injection procedure. Although i.c. transfer of effectors conferred protection, intravenous (i.v.) or i.p. transfer did not protect the mice from mortality induced by i.c. injection of virus. In addition, transfer of effectors into 4-day-old mice either by i.c. or i.p. routes did not protect them from simultaneous lethal challenge with JEV, demonstrating that newborn mice were not protected by adoptively transferred cells. The death of newborn mice owing to injection procedures could be ruled out because injection of medium either by i.c. or i.p. routes did not cause mortality.

The influence of recipient mouse age on the protective ability of adoptively transferred anti-JEV effectors is shown in Fig. 1. Cumulative survival curves were obtained from several separate adoptive transfer experiments involving mice of different ages (based upon their availability). Controls used each time included mice belonging to the same age group used in that particular experiment. As shown in Fig. 1, more than 55% of the adult (70–115-day-old) mice survived beyond 100 days after receiving anti-JEV effectors. In the case of the weanling (22–39-day-old) group, although statistically insignificant (P > 0.05) compared to the adult mice, only 35% of the mice survived beyond 100 days. However, none of the mice in 4–14-day-old (newborn and suckling) age group survived beyond 12 days. Attention is drawn to the observation that the time taken for death of newborn and suckling mice to die (12 days) was more than the time taken for death of age-matched control mice that were challenged with JEV alone (7 days). The 4–14-day-old age group comprised mice of essentially three different ages, namely, 4 days, 8 days and 14 days. When the mean time taken for 100% mortality (MDD) of 4-, 8- and 14-day-old mice was calculated, it was observed that there was a gradual increase in the MDD from 5.25 ± 0.43 to 10.3 ± 0.74 days with increasing age of recipient mice (Table 2) (P < 0.005). However, the MDD for 4-, 8- and 14-day-old control mice that received only JEV challenge did not markedly increase with age (P > 0.05). These results clearly demonstrate that the duration and percentage of protection conferred by adoptively transferred anti-JEV effectors increased with the increasing age of recipient mice.

Lymphocyte subpopulations responsible for protection

Both CD4-positive and CD8-positive T cell-mediated virus clearance have been demonstrated during several viral infections (Binder & Kundig, 1991; Pearce et al., 1994). To address the potential role of L3T4⁺ (CD4⁺) and Lyt 2.2⁺ (CD8⁺) T cells in protection of mice from JEV-induced mortality, anti-JEV effectors were preferentially depleted of one or the other lymphocyte subpopulations prior to adoptive transfer. The effector cells remaining after depleting each of the cell subsets were transferred into BALB/c mice along with a lethal i.c. challenge of JEV. As shown in Fig. 2(a) depletion of Lyt 2.2⁺ T cells prior to adoptive transfer abrogated the protective activity of anti-JEV effectors. However, it should be noted that the time taken for death of these mice (11–22 days after challenge) was delayed (P < 0.001) compared to the time taken for death of control mice that were injected with JEV alone (6–8 days after challenge). The delay in death after transfer of Lyt 2.2⁺ cell-depleted anti-JEV effectors suggested the possible complementary
involvement of cells other than Lyt 2.2+ lymphocytes in protection. This was in fact proved by the observation that depletion of L3T4+ cells prior to transfer also abolished the protective ability (Fig. 2b). The death of these mice was also delayed compared to JEV-challenged controls (P < 0.001). In addition, depletion of total T cells (Thy-1+ cells) prior to adoptive transfer completely abrogated the protective ability of effectors (Fig. 2a). The delayed death that was evident by transfer of Lyt 2.2+ cell-depleted or L3T4+ cell-depleted effectors was not observed with the total T cell-depleted population of effector cells. These results demonstrate that both Lyt 2.2+ and L3T4+ cells were necessary to confer protection of mice from lethal JEV challenge. This was also supported by the observation that a T cell-enriched population of anti-JEV effectors obtained by passing them through a nylon wool column retained their protective activity upon adoptive transfer (Fig. 2b).

Further, as shown in Table 3, 86% of the mice survived JEV challenge when Lyt 2.2+ and L3T4+ cell-depleted effectors were mixed together in equal proportions and then transferred i.c. into adult mice. However, individual transfer of either Lyt 2.2+ cell-

Table 2. Effect of increasing age on duration of protection

<table>
<thead>
<tr>
<th>Expt</th>
<th>Age of mice (days)*</th>
<th>Mean day of death (MDD)†</th>
<th>JEV + CTL i.c.‡</th>
<th>JEV alone i.c.§</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>5.25 ± 0.43 (4)</td>
<td>5.25 ± 0.82 (4)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>7.60 ± 1.01 (5)</td>
<td>5.00 ± 0.80 (5)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>10.30 ± 0.74 (6)</td>
<td>6.16 ± 0.68 (6)</td>
<td></td>
</tr>
</tbody>
</table>

* Age of mice at the time of adoptive transfer/virus challenge. Mice were returned to their mothers for suckling after injection.
† Mean time taken for the death of 100% of the mice. The differences in the MDD for 4-, 8- and 14-day-old animals that received anti-JEV effectors were significant (P < 0.005). The differences in the MDD for control age matched groups were not significant (P > 0.05).
‡ Mice were injected with 8 x 10^6 anti-JEV effectors and 10 LD_{50} JEV i.c.
§ Mice were injected with only 10 LD_{50} dose of JEV i.c.
Protection of adult but not newborn mice

Fig. 2. Requirement for both Lyt 2.2\(^+\) and L3T4\(^+\) T cells in protection. Anti-JEV effectors generated as described in Fig. 1 were subjected to specific depletion of Lyt 2.2\(^+\), L3T4\(^+\) and Thy-1\(^+\) T cells by complement-mediated lysis or subjected to enrichment of T cell population by passing through nylon wool columns before transfer i.c. into mice as described in Methods. Groups of male BALB/c mice aged 90-130 days were coinoculated i.c. with 10 LD\(_{50}\) JEV + 16 x 10\(^6\) undepleted (○), Lyt 2.2\(^+\) cell-depleted (■), Thy-1\(^+\) cell-depleted (∆), L3T4\(^+\) cell-depleted (■) or T cell-enriched (○) populations of anti-JEV effectors. The number of cells used to transfer i.c. was based on initial viable cell count before specific cell-subset depletion. The percentage of cell loss by Lyt 2.2\(^+\), L3T4\(^+\) and Thy-1\(^+\) cell depletion was 58\%, 32\% and 72\%, respectively. The recovery of effector cells after passing through nylon wool columns was 56\% of the total cells. Values are expressed as cumulative percentage of surviving mice in each group at the indicated day after transfer. Differences in the cumulative survival on day 30 post transfer as examined by G test were statistically significant with P values < 0.001 between groups (○) and (■); (∆) and (○) (a) and between groups (■) and (○) (b). The differences in the MDD between (○) and (∆) were not statistically significant whereas the differences in the MDD between (○) and (∆) (a); between (○) and (■) (b) were significant with P values < 0.001.

depleted effectors or L3T4\(^+\) cell-depleted effectors did not confer significant protection from JEV challenge in the same experiment. These results also substantiate the observation that both Lyt 2.2\(^+\) and L3T4\(^+\) T cells were necessary to confer protection of adult mice from JEV challenge.

Virus clearance in protected mice

Since infection with JEV in vivo has been demonstrated to decrease with increasing neuronal maturity (Ogata et al., 1991), it was possible to have relatively higher virus production in the brains of newborn mice, which in turn may contribute to the lack of protection. Alternatively the lack of protection in newborn mice could also arise due to virus elimination and subsequent immunopathology. To examine the differences in virus load between adult and newborn mice and to examine whether adoptively transferred effectors cleared infectious virus in the CNS, virus titre was examined in the brains of newborn and adult mice with and without adoptive transfer of anti-JEV effectors.

Initially the time course of virus production was examined in newborn and adult mice brains at different
Table 3. Requirement for Lyt 2.2* and L3T4 cells together

<table>
<thead>
<tr>
<th>Adoptively transferred anti-JEV effectors*</th>
<th>Percentage survival (dead/total)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>53 (18/19)</td>
</tr>
<tr>
<td>Lyt 2.2* cell depleted</td>
<td>42 (23/24)</td>
</tr>
<tr>
<td>L3T4* cell depleted</td>
<td>13 (20/23)</td>
</tr>
<tr>
<td>Lyt 2.2* cell depleted + L3T4* cell depleted</td>
<td>86 (2/15)</td>
</tr>
<tr>
<td>Undepleted</td>
<td>85 (2/14)</td>
</tr>
</tbody>
</table>

* Mice were adoptively transferred with the indicated effector cells along with 10 LD50 dose of JEV i.c.
† The mortality was recorded for a period of 30 days after adoptive transfer. Data was pooled from 3 experiments.
‡ 16 x 10⁶ anti-JEV effectors were depleted of Lyt 2.2* cells or L3T4* cells separately followed by mixing and injection into mice.

Table 4. Virus titre in adoptively transferred newborn and adult mice

<table>
<thead>
<tr>
<th>Age of mice (days)*</th>
<th>Treatment (i.c.)†</th>
<th>Log10 p.f.u./brain‡</th>
<th>Protection§</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>JEV</td>
<td>8.06 ± 0.208</td>
<td>Not protected</td>
</tr>
<tr>
<td>4</td>
<td>JEV + anti-JEV effectors</td>
<td>8.38 ± 0.425</td>
<td>Not protected</td>
</tr>
<tr>
<td>90</td>
<td>JEV</td>
<td>5.06 ± 0.970</td>
<td>Not protected</td>
</tr>
<tr>
<td>90</td>
<td>JEV + anti-JEV effectors</td>
<td>&lt; 2</td>
<td>Protected</td>
</tr>
</tbody>
</table>

* Age of mice at the time of adoptive transfer/challenge.
† BALB/c mice were injected i.c. with 10 LD50 JEV along with or without 8 x 10⁶ anti-JEV effectors.
‡ Five days after JEV-challenge/adoptive transfer, 3 mice from each group were sacrificed and the viral titre in their brains was examined.
§ Remaining mice that were not sacrificed on 5th day were observed for mortality over a period of 30 days.
¶ The difference of viral titre between this group and JEV-injected adult mice was statistically significant (P < 0.005).

In order to examine the virus clearance upon adoptive transfer, groups of adult and newborn mice were adoptively transferred with anti-JEV effectors along with lethal JEV challenge i.c. as described earlier. Based upon the previous result that virus production started from day 3 and reached a maximum by days 6–8 post-challenge (Fig. 3), an intermediate time point of day 5 post-challenge/adoptive transfer was selected and 3 mice from each of the above groups were sacrificed for subsequent evaluation of infectious viral titre in their brains. As shown in Table 4, 4-day-old mice that received anti-JEV effectors along with virus challenge exhibited significant titres of infectious virus (P > 0.005 compared to virus-challenged age-matched controls) in their brains and were not protected. However, adult mice that received anti-JEV effectors along with virus challenge were protected and did not exhibit a detectable titre of virus in their brains. In addition, the differences in the load of infectious virus between JEV-challenged newborn and adult mice (P < 0.005) were evident in this experiment also.

Anti-JEV neutralizing antibodies and virus titre in adoptively transferred mice

Since L3T4* T cells are generally known to provide help for antibody-producing cells it was possible that L3T4* population among the transferred effectors can

**Fig. 3.** Comparison of virus production in newborn and adult mice brains. Groups of 4-day-old (newborn; ○) and 90-day-old (adult; △) mice were injected with 10 LD50 JEV by i.c. route and three mice from each group were sacrificed every day until the onset of deaths due to infection. Brains collected from individual mice were homogenized and infectious virus was titrated by plaque assay on PS cells. Data represents individual values of virus titre in the brains (open symbols) and mean ± so of virus titre in triplicate mouse brains (closed symbols).
Protection of adult but not newborn mice

Fig. 4. Neutralizing antibody and virus titres in adoptively transferred mice. Adult BALB/c mice were co-inoculated i.c. with 10 LD₉₀ JEV + 8 x 10⁶ undepleted (undepleted), Lyt 2.2⁺ cell-depleted (Lyt-2⁺ dep), L3T4⁺ cell-depleted (L3T4⁺ dep), Thy-1⁺ cell-depleted (Thy-1⁺ dep) and nylon wool adherent cell-depleted (nylon wool passed) anti-JEV effectors as described in Fig. 2. Controls included mice that were preimmunized i.p. with JEV-infected cells followed by i.c. challenge with 10 LD₉₀ JEV (immunized and challenged), naïve mice that were challenged with 10 LD₉₀ JEV (JEV alone) and naïve mice that did not receive any challenge (normal mice). Four mice from each of these groups were sacrificed on day 5 post-challenge and serum neutralizing antibodies or brain virus titres were measured as described previously. Data represents mean virus titre ± so of quadruplicate mice brains (histograms) and mean neutralizing antibody titre ± so of quadruplicate mice sera (closed circles). The sensitivity of virus titration assay is represented as a dotted line. Individual values for neutralizing antibody titres of each mouse are also expressed in the form of open circles.

potentiate their protective activity by enhancing the titre of virus-specific neutralizing antibodies in vivo. Hence it was of interest to examine the neutralizing antibody titres in mice that received anti-JEV effectors. Anti-JEV effectors were separately depleted of different lymphocyte subpopulations as described earlier. After depleting each subpopulation of cells, the remaining effectors were transferred into groups of BALB/c mice along with lethal JEV challenge i.c. Five days after adoptive transfer four mice from each of these groups were sacrificed, neutralizing antibodies and virus titres were examined as described in Methods. The results shown in Fig. 4 demonstrate that detectable infectious virus titres were not found in the brains of mice that were adoptively transferred with undepleted or T cell-enriched population of effectors. It should be noted that these groups of mice were protected from JEV challenge as described in previous experiments (Fig. 2). In addition to their complete clearance of infectious virus, the above two groups of mice exhibited significantly higher titres of virus-specific neutralizing antibodies in their sera (P < 0.005 compared to mice that received only JEV challenge). Similarly, preimmunized and virus-challenged mice also exhibited high titres of neutralizing antibodies and no infectious virus. However, mice that were adoptively transferred with Lyt 2.2⁺ cell-depleted, L3T4⁺ cell-depleted or Thy-1⁺ cell-depleted effectors were not protected and also exhibited infectious virus in their brains. When neutralizing antibody titres were compared in these three groups, it was observed that only mice that were transferred with Lyt 2.2⁺ cell-depleted effectors contained similar titres of neutralizing
antibodies compared to protected groups of mice ($P > 0.1$). In contrast to mice that received Lyt 2.2$^+$ cell-depleted effectors, the neutralizing antibody titres were relatively low in mice transferred with L3T4$^+$ cell-depleted or Thy-1$^+$ cell-depleted effectors ($P < 0.01$) suggesting that adoptively transferred L3T4$^+$ T cells were responsible for the enhanced titre of virus-specific neutralizing antibodies in these mice. Yet, infectious virus in the brain was not cleared in mice transferred with Lyt 2.2$^+$ cell-depleted effectors despite the presence of high neutralizing antibody titres. This suggests that mere transfer of L3T4$^+$ cells and consequent enhancement of neutralizing antibodies in the absence of Lyt 2.2$^+$ CTL was not sufficient to protect naive mice from i.c. challenge with JEV. Thus, although neutralizing antibody titres were enhanced in the presence of L3T4$^+$ cells, the absolute requirement for antibodies in protection from i.c. JEV challenge was not clear.

**Discussion**

Mortality caused by the neurotropic virus, JEV, usually depends upon a complex interaction of both host and viral factors such as status of the immune system, age (Ogata et al., 1991) or species and strain of the infected animal (Miura et al., 1990; Sangster et al., 1994). Protection of mice by preimmunization with flavivirus or JEV-derived proteins has been associated with elevated levels of virus-specific neutralizing antibodies (Putnak & Schlesinger, 1990; Yasuda et al., 1990; Konishi et al., 1992). While passive administration of anti-JEV monoclonal antibodies confers protection from lethal JEV challenge by the i.v. route (Kimura-Kuroda & Yasui, 1988) it has also been shown to enhance neurovirulence with a different panel of monoclonals when challenged by i.c. route (Gould & Buckley, 1989). Hence, the relative contribution of humoral and cell-mediated immune mechanisms in protection remains unclear. In addition, i.e. challenge experiments with nude mice (Miura et al., 1990; Lad et al., 1993) and passively transferred virus-immune spleen cells (Mathur et al., 1983) suggest a T cell involvement in protection against i.c. challenge with JEV. The results presented in this paper demonstrate that in vitro generated anti-JEV effectors can protect adult, but not newborn mice from i.c. lethal challenge with JEV and that complete protection can be achieved only by simultaneous presence of both Lyt 2.2$^+$ (CD8$^+$) and L3T4$^+$ (CD4$^+$) virus-specific T cells.

It has been reported that both NK and Lyt 2$^+$ cells can be isolated from the brains of mice infected with West Nile virus (WNV) (Liu et al., 1989). However, we observed protection of adult mice from JEV-induced mortality only by transferring the effectors by i.c. route, but not by i.v. or i.p. routes. The lack of protection by i.v. or i.p. transfer of effector cells in our study could possibly arise due to inefficient migration of peripherally inoculated effector cells in a timely manner into the site of rapid virus replication in the CNS. Similar observations were reported during LCMV infection in which complete clearance of LCMV from the CNS required transfer of LCMV-specific CTL clones by i.c. route (Klavins et al., 1989). Such a co-inoculation of virus and effector cells directly into the brain may provide the transferred cells an opportunity to bypass barriers that are otherwise encountered with peripheral routes of inoculation.

Our adoptive transfer studies demonstrated that both L3T4$^+$ and Lyt 2.2$^+$ T cells are necessary to achieve efficient protection of mice against lethal JEV challenge i.e. Such adoptively transferred helper T cell-mediated protection from i.c. inoculation has also been demonstrated for another neurotropic positive-stranded RNA virus, JHM strain of mouse hepatitis virus (JHMV) (Sussman et al., 1989; Pearce et al., 1994). The mechanism by which L3T4$^+$ cells contribute to protection and virus clearance in the CNS is open to question. Since elevated titres of neutralizing antibodies have been demonstrated during protective immunity against JEV (Yasuda et al., 1990; Konishi et al., 1992) we examined virus-specific neutralizing antibodies in adoptively transferred mice. The decreased neutralizing antibody titres in mice that received L3T4$^+$ cell-depleted or Thy-1$^+$ cell-depleted effectors in comparison to mice that received Lyt 2.2$^+$ cell-depleted or undepleted effectors suggests that transfer of L3T4$^+$ cells may indeed help in enhancing neutralizing antibody titres (Fig. 4). Unlike L3T4$^+$ cell depletion, depletion of antibody-producing cells by nylon wool columns prior to transfer did not decrease neutralizing antibody titres in the recipient mice. Since preimmunized mice cleared infectious virus in the brain and also exhibited elevated serum levels of virus-specific neutralizing antibodies (Fig. 4), the transferred L3T4$^+$ cells are likely to have enhanced the neutralizing antibody titres by interacting with the host humoral immune system. However, it is not clear whether the L3T4$^+$ cells contribute to protection only by increasing neutralizing antibody titres or by other mechanisms. Although our direct i.c. cell transfer experiments demonstrate that both L3T4$^+$ and Lyt 2.2$^+$ cells are necessary for virus clearance and protection, it should be noted that both virus and effector cells were administered directly into the brain. The relevance of these observations under a situation wherein a peripherally inoculated virus strain enters the CNS is not clear. Intracerebral challenge experiments were resorted to only because the strain P20778 failed to cause death when injected through other routes. Despite this, we consider our observation about recipient age-dependent protection conferred by adoptively trans-
ferred effectors to be interesting since virus-induced immunosuppressive defects have been shown to vary with the age at which infection occurs (Tishon et al., 1993).

In addition to enhancing antibody titres, L3T4+ cells may also possibly exert their protective effect by production of lymphokines. Secretion of interferon-γ (IFN-γ) can upregulate MHC expression and antigen presentation in infected brain cells as shown for mouse hepatitis virus and WNV (Pearce et al., 1994; Liu et al., 1989). Infections with Theiler’s virus and mouse hepatitis virus are also known to upregulate class I expression in the CNS (Suzumura et al., 1986; Massa et al., 1986). Although L3T4+ cells may not be obligatory for induction of Lyt 2+ cell responses (Ahmed et al., 1988), L3T4+ cell-derived lymphokines may aid in the clonal expansion of Lyt 2+ CTL.

Our experiments demonstrate that adoptive transfer of anti-JEV effectors results in virus clearance and protection only in adult mice and not in newborn mice. Several viral and host factors may contribute to the recipient mouse age-dependent protective activity of transferred cells. Viral titres were 100–1000-fold higher in newborn mouse brains than in adult brains. This difference was 102–104-fold when expressed as p.f.u./g tissue. Such virus loads could be too high for effector cells to eliminate. This was supported by our observation that adoptively transferred effector cells did not protect adult mice efficiently when challenged with 100 LD50 instead of 10 LD50 JEV. Injection of effector cells at different times after virus administration was not attempted since this would have required several i.c. insults. In addition to differences in virus load, the relative proportion of various cell types that are infected with JEV could also differ with age. JEV predominantly infects the CNS and developing neurons are primary targets for JEV infection (Kimura-Kuroda et al., 1992). Our LD50 values also demonstrated that an inoculum of only 1 p.f.u./mouse was sufficient to cause 50% mortality of newborn mice, whereas adult mice required 10 p.f.u./mouse. MHC class I antigen expression in the CNS may be regulated differently in different neural cell types. Hence such age-dependent differences may also influence the recognition and subsequent elimination of virus by transferred effector cells. Another possible reason for lack of protection in newborn mice could be due to delayed antibody responses as well as defective macrophage and T cell function. (Berkowitz & Becker, 1992; Sarmiento, 1988). Even though all the above listed differences between newborn and adult mice logically appear to be possible reasons for the lack of protection in newborn mice, we do not have at this stage any experimental evidence to pinpoint the actual reason. However, since JEV is known to cause mortality predominantly in infected children (Umenai et al., 1985), further studies on these aspects would throw some light on the age-dependent pathogenesis by JEV.

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