Cytotoxic T lymphocytes raised against Japanese encephalitis virus: effector cell phenotype, target specificity and in vitro virus clearance

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Several H-2 defined cell lines were examined for their ability to support infection and replication of Japanese encephalitis virus (JEV) before their use in in vitro and in vivo stimulation protocols for generating cytotoxic T lymphocytes (CTLs) against JEV. Among 11 different cell lines tested, two H-2k macrophage tumour lines (P388D1, RAW 264.7), an H-2k hybridoma (Sp2/0), an H-2KD neuroblastoma (Neuro 2a), and H-2K macrophage cell line (L929) were found to support JEV infection and replication. These cell lines were used to generate anti-JEV CTLs by using in vivo immunization followed by in vitro stimulation of BALB/c mice. We observed that not only syngeneic and allogeneic infected cells but also JEV-infected xenogeneic cells could prime BALB/c mice for the generation of JEV-specific CTLs upon subsequent in vitro stimulation of splenocytes with JEV-infected syngeneic cells. Although infected xenogeneic cells were used for immunization, the anti-JEV effectors that were generated lysed infected syngeneic targets but not JEV-infected xenogeneic or allogeneic target cells in a 5 h 51Cr release assay. These anti-JEV effectors recognized syngeneic target cells infected with West Nile virus to a lesser extent and were shown to be Lyt-2.2 T cells. The results of unlabelled cold target competition studies suggested alterations in the cell surface expression of viral antigenic determinants recognized by these CTLs. We further demonstrate that the JEV-specific CTLs generated could virtually block the release of infectious virus particles from infected P388D1 and Neuro 2a cells in vitro.

Introduction

Japanese encephalitis virus (JEV) is a positive-stranded RNA virus which belongs to the flavivirus group (Westaway et al., 1985) and causes acute encephalitis associated with damage to the central nervous system (Oyanagi et al., 1969). Its epidemiological and structural aspects have been extensively reviewed (Monath, 1986; Rosen, 1986; Umenai et al., 1985; Chambers et al., 1990). Passive administration of anti-JEV antibodies has been shown to confer protection against JEV in mice (Kimura-Kuroda & Yasui, 1988). However, the significance of this protection is unclear since the neurovirulence of JEV and yellow fever virus can be enhanced by the administration of virus-specific antibodies (Barrett & Gould, 1986; Gould & Buckley, 1989). The observation that adoptive transfer of immune T cells can protect mice from a subsequent challenge with JEV (Mathur et al., 1983) and the report that JEV-immunized normal nude (nu)/+ BALB/c mice were effectively protected from a subsequent intracerebral challenge with JEV whereas athymic nu/nu BALB/c mice were not (Miura et al., 1990) suggests that T cells play a role in host defence against JEV. It is not yet known whether T cells confer protection by cytotoxic T lymphocyte (CTL) generation in addition to antibody synthesis (Mori et al., 1970). These findings indicate the importance of further studies on cell-mediated immune responses against these viruses. Major histocompatibility complex (MHC)-restricted CTLs have been raised against West Nile virus (WNV)-infected and dengue virus-infected cells (Kesson et al., 1987, 1988; Bukowski et al., 1989; Pang et al., 1988). Both these viruses are flaviviruses.

However, there has been no report so far describing the generation of CTLs that specifically recognize JEV by immunization with JEV-infected cells. One of the reasons for this could be the difficulty in obtaining cells that are not only permissive to JEV infection and replication but are also syngeneic with responder mice (Hill et al., 1992). Several H-2 defined cell lines were therefore examined for their ability to support JEV infection and replication. Utilizing the JEV-infectible cell lines JEV-specific CTLs were generated by the immunization of BALB/c mice with virus-infected xenogeneic cells followed by in vitro stimulation with virus-infected syngeneic macrophage tumour cells. These effector cells are specific for JEV-infected syngeneic cells and are Lyt-2.2+. WNV-infected syngeneic targets were, however, recognized to a limited extent. In addition these CTLs recognized JEV-infected (H-2K*D) neuro-
blastoma cells and were also stimulated by them. We further demonstrate that these CTLs can block the release of infectious viral particles from JEV-infected target cells in vitro.

Methods

Mice. BALB/c (H-2^d^) mice were obtained from the Indian Institute of Science colony and 6- to 8-week-old male mice were used for all studies.

Stimulator and target cells. P388D1, P815 and L1210 cells (DBA/2 mouse origin, H-2^k^), Sp2/0, RAW 264.7, BALB/3T3 (BALB/c mouse origin, H-2^k^), YAC-1, Neuro 2a (A/J mouse origin, H-2K^d^D^b^), EL-4 (C57BL/6 mouse origin, H-2^b^) and L929 (C3H mouse origin, H-2^k^) cells were cultured at 37°C in RPMI 1640 medium supplemented with antibiotics and 5% fetal calf serum (FCS; Gibco) in a 5% CO_2_ atmosphere. All cell lines were obtained from the National Facility for Animal Tissue and Cell Culture Collection (Pune, India). Cells were used during their logarithmic growth phase in all experiments.

Virus growth and infection of cell lines. JEV strain P20778, an isolate from human brain (Kedarnath et al., 1986), was routinely grown in C6/36 cells grown to confluence in MEM supplemented with 10% FCS (Gibco). Infected C6/36 cell culture supernatants were used as the source of virus in all our studies. WNV mosquito isolate G22886 was obtained from the National Institute of Virology, Pune, India, and grown in the brains of suckling mice. Ten percent brain homogenate was used during their logarithmic growth phase in all experiments.

In vivo primary immunizations and secondary in vitro stimulations. Six- to eight-week-old male BALB/c mice were generally immunized intraperitoneally (i.p.) twice with 3 x 10^6 JEV-infected PS cells followed by 1 x 10^5 JEV-infected Sp2/0 cells at 3-day intervals on days 0, 3 and 6 respectively. The JEV-infected Sp2/0 cells were used after being infected at an m.o. of 1 and 10 p.f.u. per cell respectively. The mice were sacrificed on day 10 and 1 x 10^7 primed splenocytes (responder cells) were cultured in vitro in 24-well Limbro plates with 2 x 10^6 virus-infected syngeneic stimulator cells. P388D1 stimulator cells were prepared by infecting cells with JEV at an m.o. of 10 p.f.u. per cell for 24 h followed by irradiation at 3,000 rads using a ^60_Co irradiation source. Effector cells were generated at 37°C in a 5% CO_2_ atmosphere by in vitro culture in RPMI 1640 medium supplemented with antibiotics, 5 x 10^{-4} mg/ml mercaptoethanol, 0.32 mg/ml L-glutamine, 0.12 mg/ml non-essential amino acids, 0.12 mg/ml sodium pyruvate and 5% FCS. When Sp2/0 and Neuro 2a cells were used as stimulator cells, they were infected with JEV for 24 h and 6 h at an m.o. of 10 and 0.1 p.f.u. per cell respectively before irradiation at 3,000 rads. The number of virus units used to infect the cell lines and the times at which they were harvested were standardized to ensure that maximum possible infection at optimal cell viabilities was achieved.

CTL assays and target cell preparations. Various numbers of effector cells generated in mixed lymphocyte cultures (MLC) were incubated with 2 x 10^5 ^51_Cr-labelled virus-infected or uninfected cells for 5 h at 37°C after briefly centrifuging them together in 96-well V-bottom plates at 100 g for 5 min. Target cells were prepared by incubating them with 200 uCi of Na^251_CrO_4_ (BRIT, Bombay, India) for 60 min and subsequently washing them to remove free ^51_Cr. Virus infection of target cells was carried out as mentioned previously. P388D1 and Neuro 2a cells were harvested 48 h and 12 h respectively after being infected with JEV at an m.o.i. of 1 p.f.u. per cell as above. The conditions of viral infection were varied in initial experiments to optimize cell viability during ^51_Cr labelling and release assays. At the end of the incubation period, a sample of cell-free supernatant was removed and the ^51_Cr released was counted in an LKB gamma counter. Triplicate and quadruplicate examinations were routinely included in all assays and the percentage lysis of target cells was calculated as [(c.p.m. released in the presence of effector cells—c.p.m. released due to spontaneous leakage) / (total c.p.m. released by 0.2% Triton X-100—c.p.m. released due to spontaneous leakage)] x 100.

Effector cell depletion studies. Anti-JEV effector cells or allogeneic CTLs were treated with anti-Thy-1 monoclonal antibody (clone NIMR-1, Sera-Lab, 1:800 dilution), anti-Lyt-2.2 monoclonal antibody [clone AD4 (15), Cedareline, 1:5 dilution] or anti-L3T4 monoclonal antibody (clone YTS819, 1:2, Sera-Lab, 1:200 dilution) at 37°C for 15 min. The dilutions of anti-Thy-1 and anti-Lyt-2.2 were chosen based on manufacturers’ recommendations and an assessment of their ability to deplete completely the CTL activity of allogeneic BALB/c (H-2^k^) anti C57BL/6 (H-2^b^) effector populations. After removal of unadsorbed antibody, cells were incubated at 37°C for 30 min with fivefold diluted guinea-pig serum that had been specially screened for low toxicity on spleen cells before being used as a source of complement. After washing, the cytotoxic activity of surviving cells was tested by incubating them with 2 x 10^6 ^51_Cr-labelled target cells and assayed for ^51_Cr release as described above. Effector cells treated with guinea-pig serum alone or anti-mouse IgG antibody were used as controls.

Unlabelled target competition studies. Unlabelled virus-infected or uninfected competitor cells at various concentrations were mixed with 2 x 10^5 ^51_Cr-labelled P388D1 target cells and the mixture was incubated with anti-JEV effector cells at 37°C and monitored for ^51_Cr release as described above. Triplicate and quadruplicate samples were routinely assayed and the percentage lysis obtained at unlabelled
target cell ratios of 5, 10, 20, 40 and 80 were calculated. Results are calculated as a percentage of control lysis obtained with 51Cr-labelled JEV-infected P388D1 cells in the absence of unlabelled competitor cells.

Results

Virus infection of stimulator and target cells

The successful generation of MHC-restricted virus-specific CTLs in BALB/c mice (H-2^d) requires the co-culture of responder cells with virus-infected stimulator cells that are syngeneic to the responder mice. Hence different cell lines were examined for infection by JEV in vitro. Infection was monitored both by determining the amount of infectious virus release and immunofluorescence. As shown in Table 1, C6/36 and PS cells that are permissive to infection by JEV were used as positive controls and these were 100% positive for immunofluorescence at 24 h after virus infection. Neuro 2a cells were readily infectible with JEV and 73% cells were positive for immunofluorescence by 12 h after infection. When infected at an m.o.i. of 1 p.f.u. per cell, P388D1 cells were 58% positive for immunofluorescence at 48 h after infection. However when infected at an m.o.i. of 10, 40% of these cells were positive for JEV-specific immunofluorescence even at 24 h after infection. Sp2/0, RAW 264.7 and L929 cells were more than 40% positive only when infected at higher m.o.i.s.

To compare the actual number of viral particles produced by these cell lines, the infectious virus released into culture supernatants was measured by plaquing on PS cells. All cell lines showing positive immunofluorescence were also found to support viral replication. All other cell lines tested (P815, L1210, BALB.3T3, EL-4) were induced only in the presence of minor region disparity, this suggests that anti-JEV effectors produced by these cell lines were specific for JEV-infected P388D1 cells. Since the overall virus-specific lysis obtained was low when single immunizations were used, mice were subjected to multiple immunizations with the JEV-infected cell lines described above followed by secondary stimulation in vitro with JEV-infected P388D1 cells. As shown in Table 2, when BALB/c mice were injected i.p. twice with JEV-infected PS cells followed by a further injection with JEV-infected Sp2/0 cells at 3 day intervals, this served to prime mice for generation of anti-JEV effector cells to the optimum extent (Table 2, experiment 5). Immunization alone without in vitro secondary stimulation, however, failed to generate any JEV-specific effectors. All other multiple immunization protocols that utilized RAW 264.7 and L929 cells also resulted in the generation of effector cells that specifically lysed JEV-infected P388D1 target cells. It was observed that either the lysis of uninfected target cells was higher or that the percentage specific lysis of infected target cells was lower. Based on the observation that xenogeneic PS cells were able to prime for an antiviral CTL response, BALB/c mice were injected i.p. twice with JEV-infected PS cells and once with JEV-infected Sp2/0 cells for all other studies. The splenocytes obtained from such mice were stimulated in vitro with JEV-infected P388D1 cells.

Table 1. JEV infection of stimulator and target cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>H-2</th>
<th>Immunofluorescence- positive cells (%)</th>
<th>Infectious viral particles released† (p.f.u. × 10^-6 per 10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6/36</td>
<td>H-2</td>
<td>95 ± 5 (1)</td>
<td>10-00 ± 0.8</td>
</tr>
<tr>
<td>PS</td>
<td></td>
<td>95 ± 5 (1)</td>
<td>5-06 ± 1.4</td>
</tr>
<tr>
<td>Neuro 2a</td>
<td>K^d D^a</td>
<td>73 ± 4 (1)†</td>
<td>22-90 ± 100</td>
</tr>
<tr>
<td>P388D1</td>
<td>K^d D^a</td>
<td>58 ± 4 (1)§</td>
<td>1-75 ± 0.25</td>
</tr>
<tr>
<td>P388D1</td>
<td>K^d D^a</td>
<td>40 ± 3 (10)</td>
<td></td>
</tr>
<tr>
<td>Sp2/0</td>
<td>K^d D^a</td>
<td>27 ± 4 (10)</td>
<td>0-11 ± 0.02</td>
</tr>
<tr>
<td>Sp2/0</td>
<td>K^d D^a</td>
<td>42 ± 5 (50)</td>
<td></td>
</tr>
<tr>
<td>RAW 264.7</td>
<td>K^d D^a</td>
<td>44 ± 5 (50)</td>
<td>0-06 ± 0.09</td>
</tr>
<tr>
<td>L929</td>
<td>K^d D^a</td>
<td>86 ± 3 (50)</td>
<td>0-30 ± 0.016</td>
</tr>
</tbody>
</table>

* Cells were infected with JEV at an m.o.i. of 1, 10 or 50 p.f.u. per cell as indicated in parenthesis and cultured for 12 h (†), 24 h or 48 h (§) before the preparation of cell smears and further staining.

† Cells were infected with JEV at an m.o.i. of 1 p.f.u. per cell and cultured for 24 h. The number of viral units released into the cell culture supernatant is expressed as p.f.u. ± S.D. × 10^-6.

In vivo and in vitro priming requirements

In order to develop a proper immunization protocol for producing anti-JEV effectors, we initially injected BALB/c mice i.p. with JEV-infected syngeneic (Sp2/0, RAW 264.7, H-2^d), allogeneic (L929, H-2^d), xenogeneic (PS) cells or 1 × 10^7 p.f.u. of JEV in the form of infected mouse brain homogenate or infected C6/36 cell culture supernatant. Ten days after immunization, primed spleen cells were cultured in vitro with JEV-infected P388D1 (H-2^d) stimulator cells. Results of representative examples only are shown in Table 2. In vitro stimulation without in vivo priming did not generate effectors that significantly lysed infected P388D1 target cells. Since the overall virus-specific lysis obtained was low when single immunizations were used, mice were subjected to multiple immunizations with the JEV-infected cell lines described above followed by secondary stimulation in vitro with JEV-infected P388D1 cells. As shown in Table 2, when BALB/c mice were injected i.p. twice with JEV-infected PS cells followed by a further injection with JEV-infected Sp2/0 cells at 3 day intervals, this served to prime mice for generation of anti-JEV effector cells to the optimum extent (Table 2, experiment 5). Immunization alone without in vitro secondary stimulation, however, failed to generate any JEV-specific effectors. All other multiple immunization protocols that utilized RAW 264.7 and L929 cells also resulted in the generation of effector cells that specifically lysed JEV-infected P388D1 target cells. It was observed that either the lysis of uninfected target cells was higher or that the percentage specific lysis of infected target cells was lower. Based on the observation that xenogeneic PS cells were able to prime for an antiviral CTL response, BALB/c mice were injected i.p. twice with JEV-infected PS cells and once with JEV-infected Sp2/0 cells for all other studies. The splenocytes obtained from such mice were stimulated in vitro with JEV-infected P388D1 cells.

Specificity of effectors for syngeneic JEV-infected stimulator and target cells

Since BALB/c (H-2^d)-derived responders were stimulated by P388D1 (DBA/2-derived) cells with minor region disparity, this suggests that anti-JEV effectors were induced only in the presence of minor region disparity. We have hence studied the ability of JEV-infected RAW 264.7 and Sp2/0 cells to act as in vitro stimulators during the generation of anti-JEV effector
Table 2. Immunizations with virus-infected syngeneic, allogeneic and xenogeneic cell lines

<table>
<thead>
<tr>
<th>Injection i.p. with JEV-infected cells*</th>
<th>Lysis of targets (%)</th>
<th>Specific lysis (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control P388D1</td>
<td>Infected P388D1</td>
</tr>
<tr>
<td>Day Injection</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>1 None</td>
<td>39 ± 14</td>
<td>66 ± 0.7</td>
</tr>
<tr>
<td>2 Sp2/0</td>
<td>46 ± 14</td>
<td>21 ± 12</td>
</tr>
<tr>
<td>3 L929</td>
<td>10 ± 0.9</td>
<td>31 ± 0.8</td>
</tr>
<tr>
<td>4 PS</td>
<td>6 ± 0.4</td>
<td>17 ± 12</td>
</tr>
<tr>
<td>5 PS Sp2/0</td>
<td>61 ± 18</td>
<td>37 ± 18</td>
</tr>
<tr>
<td>6 PS Sp2/0</td>
<td>8 ± 3.1</td>
<td>28 ± 3.2</td>
</tr>
<tr>
<td>7 L929 L929 Sp2/0</td>
<td>21 ± 0.8</td>
<td>32 ± 2.8</td>
</tr>
</tbody>
</table>

* PS cells were infected at an m.o.i. of 1 p.f.u. per cell. P388D1 cells at 10 p.f.u. per cell and Sp2/0, RAW 264.7 and L929 cells at 50 p.f.u. per cell. Male mice were injected i.p. with 24 h JEV-infected PS (3 x 10⁶), Sp2/0, RAW 264.7 or L929 cells (1 x 10⁷) on the days indicated. All mice were sacrificed on day 10 and spleen cells obtained were stimulated in vitro with JEV-infected P388D1 cells. No lysis of infected or uninfected target cells was observed when the secondary in vitro restimulation step was omitted.

† Specific lysis (%) was calculated by subtracting percentage lysis of uninfected target cells from lysis obtained for JEV-infected target cells at an E:T ratio of 40:1.

cells. RAW 264.7 is a macrophage cell line and Sp2/0 is a hybridoma cell line derived from BALB/c mice and these are therefore completely syngeneic to the BALB/c-derived responder spleen cells. As shown in Fig. 1(c), culture in vitro of such primed splenocytes with JEV-infected RAW 264.7 cells stimulated production of effector cells that could recognize JEV-infected P388D1 cells specifically. The use of infected Sp2/0 cells instead of infected P388D1 cells as stimulators also resulted in effectors that exhibited specific lysis of infected P388D1 target cells although the extent of lysis was low (Fig. 1a). Similarly, anti-JEV effectors generated using the optimal protocol also lysed infected Sp2/0 cells in a virus-specific manner although the percentage specific lysis of infected Sp2/0 cells was substantially lower than that of infected P388D1 target cells (Fig. 1b). The extent of lysis of uninfected P388D1 cells was found to be similar to that obtained when using YAC-1 target cells suggesting that virus-specific lysis was not due to natural killer cell activity.

The cross-reactivity of effector cells towards another flavivirus was determined by examining their ability to lyse P388D1 cells previously infected with WNV. As shown in Table 3, 30% of WNV-infected P388D1 target cells were lysed at an effector to target (E:T) ratio of 80:1, whereas 52% of JEV-infected P388D1 target cells were lysed at the same E:T ratio. However, P388D1 cells were not lysed in a virus-specific manner when infected with an unrelated virus such as bovine rinderpest virus.

![Fig. 1. Generation of anti-JEV effectors. Spleen cells from mice that had been primed in vivo as described in Methods were restimulated in vitro with JEV-infected Sp2/0 (a), JEV-infected P388D1 (b) and JEV-infected RAW 264.7 (c) cells. Effector cells generated after 5 days of culture were assayed on ⁵¹Cr-labelled JEV-infected P388D1 (▲), normal P388D1 (▲), JEV-infected Sp2/0 (■), normal Sp2/0 (□) and YAC-1 (○) target cells. Spontaneous release of ⁵¹Cr was 15% (P388D1), 29% (JEV-infected P388D1), 12.8% (Sp2/0), 27% (JEV-infected Sp2/0) and 10% (YAC-1) cells. Values are expressed as mean percentage lysis ± s.d. Data represent one example of two similar experiments. In a separate experiment neither JEV-infected PS nor uninfected PS cells were lysed by the anti-JEV effectors generated.](image-url)
Table 3. Specificity of anti-JEV effector CTLs

<table>
<thead>
<tr>
<th>Expt.</th>
<th>E: T ratio</th>
<th>Lysis of P388D1 targets (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninfected</td>
<td>JEV-infected*</td>
</tr>
<tr>
<td>1</td>
<td>80:1</td>
<td>78±0.3</td>
</tr>
<tr>
<td>1</td>
<td>40:1</td>
<td>41±0.7</td>
</tr>
<tr>
<td>1</td>
<td>20:1</td>
<td>27±0.4</td>
</tr>
<tr>
<td>1</td>
<td>10:1</td>
<td>11±0.1</td>
</tr>
<tr>
<td>2</td>
<td>80:1</td>
<td>23±3.2</td>
</tr>
<tr>
<td>2</td>
<td>40:1</td>
<td>12±1.8</td>
</tr>
<tr>
<td>2</td>
<td>20:1</td>
<td>14±1.3</td>
</tr>
<tr>
<td>2</td>
<td>10:1</td>
<td>6±3.2</td>
</tr>
</tbody>
</table>

* Fifty-eight percent of cells were positive for JEV-specific immuno- fluorescence.
† Under these conditions 7 x 10^3 p.f.u. of WNV was released per 1 x 10^6 cells into culture supernatant at 48 h after infection as assayed by plaquing on PS cells.
‡ P388D1 cells were infected with rinderpest virus (RPV) at an m.o.i. of 20 p.f.u. per cell and used as targets at 36 h after infection. Immunofluorescence analysis using antiserum specific for RPV showed 56% of cells positive for fluorescence.
§ ND, Not determined.

Table 4. Phenotype of anti-JEV effector CTLs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lysis (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>75±3.1</td>
</tr>
<tr>
<td>Anti-Thy-1+ complement</td>
<td>44±2.6</td>
</tr>
<tr>
<td>Anti-Lyt-2.2+ complement</td>
<td>63±4.1</td>
</tr>
<tr>
<td>Anti-L3T4+ complement†</td>
<td>68±4.3</td>
</tr>
<tr>
<td>Anti-Lyt 2.2 alone</td>
<td>0</td>
</tr>
</tbody>
</table>

* Values are expressed as percentage lysis ± S.D. of JEV-infected P388D1 targets at an E:T ratio of 40:1.
† Addition of anti-L3T4 (1:200) antibody caused 86% inhibition of concanavalin A-induced [3H]thymidine incorporation in splenocyte cultures.

Phenotypic characterization of anti-JEV effector cells

The cell surface phenotype of anti-JEV effector cells that were generated was characterized by depletion of T cells or Lyt-2.2⁺ cells from the effector cell population. As shown in Table 4, both Thy-1⁺ and Lyt-2.2⁺ cell depletion resulted in reduced lysis of JEV-infected P388D1 cells. Also, addition of anti-Lyt-2.2⁺ monoclonal antibody (ATCC TIB 210) alone to anti-JEV effector cells during 51Cr release assays completely inhibited lysis of target cells. This antibody inhibits lysis mediated by MHC class I-restricted Lyt-2.2⁺ cells even in the absence of complement (McCarthy & Singer, 1989; Sarmiento et al., 1980). However, depletion of L3T4⁺ cells by incubating effector cells with anti-L3T4 monoclonal antibody followed by complement or B cell depletion using anti-mouse IgG and complement had no effect on specific lysis of infected target cells.

H-2K⁺D⁺ neuroblastoma cells are lysed by anti-JEV CTLs

Since JEV replication was high in Neuro 2a cells and JEV predominantly infects the central nervous system, the ability of this neuroblastoma cell line to be lysed by anti-JEV CTLs was examined. No specific lysis of these cells was observed at 0 and 6 h post-infection, as shown in Fig. 2(a), but 58% of Neuro 2a cells infected with JEV for 12 h were efficiently lysed by anti-JEV effector cells at an E:T ratio of 80:1. The degree of lysis observed was lower than that seen on infected P388D1 cells (79%) at

Fig. 2. Recognition of JEV-infected neuroblastoma cells. (a) Anti-JEV effector cells were obtained by in vitro restimulation of primed mouse spleen cells with JEV-infected P388D1 cells. After 5 days of culture effector cells were assayed on JEV-infected Neuro 2a target cells that had been infected for 0 h (O), 6 h (■) or 12 h (▲) with JEV. Controls included uninfected (▲) and JEV-infected (▲) P388D1 target cells. Spontaneous release of 51Cr for 0 h, 6 h and 12 h Neuro 2a infected targets was 14%, 29% and 29% respectively. Values are expressed as mean percentage lysis ± S.D. of triplicate assays. (b) BALB/c spleen cells of animals that had been primed i.p. as described in Methods were restimulated in vitro with JEV-infected Neuro 2a cells. After 5 days of culture, effector cells were assayed on JEV-infected Neuro 2a target cells (▲) or normal P388D1 (▲) target cells in a 5 h 51Cr release assay. Spontaneous 51Cr release was 20% from normal P388D1 targets and 31% from JEV-infected P388D1 targets. Percentage lysis values are expressed as mean ± S.D. of triplicate assays. Data represent one example of three similar experiments.
Unlabelled target competition studies

The specificity of anti-JEV effector cells generated in primed BALB/c mice was further analysed by examining the competitive inhibition effects of unlabelled target cells added during the assay. As shown in Fig. 3(a), addition of unlabelled JEV-infected P388D1 cell competitors significantly inhibited the lysis of labelled JEV-infected P388D1 target cells even at an unlabelled to labelled target cell ratio of 10:1. However, addition of unlabelled infected RAW 264.7, Sp2/0 and Neuro 2a cell competitors inhibited lysis of labelled JEV-infected P388D1 cells only at higher unlabelled to labelled target cell ratios (Fig. 3b, c and d).

Anti-JEV effector cells inhibit virus production

Lysis of cells by CTLs could lead to release of virus already formed before the lytic event, leading to further infection of neighbouring cells. The ability of virus-specific CTLs to mediate protection in vivo would, therefore, depend also on their ability to prevent further release of infectious viral particles efficiently. Therefore we examined virus production from JEV-infected cells in continuous cell cultures with antiviral CTLs. Since \( ^{31}\text{Cr} \)-labelled P388D1 target cells were lysed maximally at 48 h after JEV infection, P388D1 cells infected with JEV for 48 h were harvested, washed and further cultured with anti-JEV effector cells, which were generated as mentioned previously. As shown in Fig. 4(a) virus production in infected P388D1 cells that were infected with JEV for 48 h and then washed and recultured progressively increased, reaching a maximum at 48 h after reculture (96 h after infection). Relatively few virus particles were produced in cultures, however, when CTLs were added. Similarly, incubation of P388D1 cells that were infected with JEV for 24 h and then washed and recultured with antiviral CTLs also blocked virus production (Fig. 4b). Inhibition of virus production was apparent only at 12 h after the addition of CTLs. The effect of the addition of antiviral CTLs to P388D1 cells 1 h after JEV infection followed by continuous culture for a further period of 48 h was then examined. As shown in Fig. 4(c), whereas virus production progressively increased in normal cultures reaching a maximum after 48 h, substantial
inhibition of virus production was seen only at 24 h after antiviral CTL addition. Since JEV is neurotropic, a similar experiment was carried out with infected Neuro 2a cells and results are shown in Fig. 4(d). Antiviral CTLs blocked further virus release 18 h after addition to these cells. It should be noted that the residual increase in viral titres occurring up until 18 h after CTL addition could be due to the relatively lower lysis of infected Neuro 2a cells by anti-JEV effector cells as compared to infected P388D1 cells that was observed in 51Cr release assays.

**Discussion**

A variety of humoral and cellular effector mechanisms facilitate host defence against viral infections (Allan & Doherty, 1986; Gajdosova et al., 1981). Similarly T cell responses of helper and cytotoxic types occur both in humans and mice infected with flaviviruses (Hill et al., 1992; Kulkarni et al., 1991). Human CD8^+ and CD4^+ cytotoxic T cell responses to dengue virus-infected cells have been reported (Kurane et al., 1989; Rothman et al., 1989). CTL responses to another flavivirus, WNV, have also been demonstrated. While this manuscript was in preparation the priming of CBA/H mice with JEV in vivo and in vitro to generate effector cells that lysed macrophages infected with WNV was reported (Hill et al., 1992). However, these workers were unable to infect syngeneic macrophages with JEV. Hence it was not known whether these effector cells could have lysed JEV-infected syngeneic target cells. This needs to be determined since JEV does not share complete amino acid identity with the sequences of WNV and Kunjin viruses (Coia et al., 1988). As a prerequisite for the generation of anti-JEV CTLs we screened a number of cell lines for their ability to support JEV replication. Utilizing these permissive cell lines (P388D1, L929, Sp2/0 and RAW 264.7) several protocols were designed for the purpose of generating anti-JEV effector cells.

Immunization of mice in our initial experiments with JEV-containing brain homogenate or C6/36 culture supernatant samples resulted in a CTL response upon
secondary in vitro stimulation with JEV-infected P388D1 cells. However, these effector cells exhibited higher non-
specific lysis of uninfected target cells (data not shown) possibly owing to the presence of extraneous proteins in
the brain homogenate or FCS in the cell culture supernatants used for immunization. Priming of the immune system to MHC class I-mediated responses requires intracellular replication of the virus. Therefore
the use of infected cells as immunizing agents could prime the immune system to produce antiviral CTLs more efficiently, as has been shown by Green et al. (1979) who reported that immunization of mice with virus-
producing allogeneic cell lines facilitated the generation of CTLs raised against endogenous AKR/Gross murine leukaemia viruses that are otherwise weakly immuno-
genic.

All immunization protocols were performed with JEV-
infected cells rather than with purified virus to facilitate
the generation of CTLs not only against virus-specific proteins but also against viral proteins such as prM and NS1 which are present only in infected cells but not in mature viral particles (Russell et al., 1980; Chambers et al., 1990). In the event of poor surface expression of viral determinants, successful in vivo priming may occur due
to simultaneous cell surface display of both virus-
associated determinants and allo-antigens (Green et al.,
1979). In addition to the strong primary responses raised
against allogeneic MHC antigens, efficient recognition of
HLA xenoantigen structures has also been reported
(Kievits et al., 1990). Hence we examined the ability of infected virus-allogeneic and xenogeneic cells to prime mice. The CTLs generated in this study were not only
JEV-specific but also specific for self H-2 antigen (Table
3). Although mice were primed with infected xenogeneic
cells, neither infected allogeneic L929 cells nor infected
PS cells were lysed to any significant extent. This may be
due to expansion of H-2-restricted, virus-specific pre-
cursor CTL populations upon secondary in vitro culture
with JEV-infected P388D1 cells. The mechanism whereby xenogeneic cells prime BALB/c mice to generate a self-
restricted antiviral CTL response is as yet unknown. However, fixation of PS cells with paraformaldehyde did
not alter their ability to prime BALB/c mice when injected i.p. demonstrating that this effect was not due to virus release in vivo (data not shown). PS cells, being of
non-murine origin, could also lead to stronger stimu-
lation in vivo by the stronger generation of lymphokines.

In addition, we observed that these anti-JEV effector
cells lyse WNV-infected P388D1 target cells (Table 3).
Lysis values for 51Cr-labelled infected P388D1 target
cells reached a maximum of approximately 80% (Fig.
1b, c) although cells positive for JEV envelope-specific immunofluorescence reached a maximum value of 50 %
at the time of CTL assay. This could be accounted for if
either antigenic specificity of CTLs might be different
from that of the antibody or alternatively that the effectors efficiently recognized extremely low concentra-
tions of viral antigenic determinants not expressed on the
cell surface.

The positive lysis observed in Neuro 2a cells suggests
the absence of H-2 class II-restricted CTLs since the class
II allotype of Neuro 2a is H-2k (Morgeaux et al., 1989).
In addition, T cell depletion studies also demonstrate
that the effector cells generated were Lyt-2.2+, and hence
class I-restricted. The observation that infected Neuro 2a
cells were recognized by anti-JEV effector cells (Fig. 2)
suggests that the viral antigens were at least in part
recognized in association with H-2Dk antisgens. Although
Neuro 2a cells were more permissive to JEV as compared
with P388D1 cells the lysis of P388D1 cells was greater
than that of Neuro 2a cells. This could be due to the
recognition of H-2Kd-associatetd viral antigen in P388D1
cells in addition to H-2Dk-associated antigen. Both
uninfected as well as infected P388D1 and Neuro 2a cells
were found to be positive for H-2 class I antigen
expression when analysed for class I-specific immuno-
muno-fluorescence (data not shown).

Our unlabelled target competition experiments demon-
strate qualitative differences in the recognition of
different JEV-infected syngeneic target cells by CTLs.
This could be due to (i) preferential association of
antigenic peptides with either H-2K or D antigen in
different cell lines and (ii) expression of the CTL-
recognizable viral determinants may vary over time and
from cell line to cell line (Mullbacher & King, 1989).

Addition of anti-viral CTLs to infected Neuro 2a and
P388D1 cell cultures effectively blocked further release of
virus particles. Although only 33 to 36% lysis of target
cells was observed in 51Cr release assays, the reason for
the virtually complete blockage of virus release from
infected cells (Fig. 4) at a corresponding E:T ratio is
unknown. This could also be due to the inactivation of
existing virus particles by granule exocytosis from
antiviral CTLs as suggested earlier (Anderson et al.,
1985). Alternatively liberation of cytokines may also
block virus release. It has been reported that WNV
infection can upregulate expression of H-2 class I
antigens in primary astrocyte cultures (Young et al.,
1989). This observation along with our data that antiviral
CTLs can inhibit ongoing virus production in JEV-
infected syngeneic P388D1 and Neuro 2a cells will assist
in future examinations of the potential of such CTLs to
protect mice from lethal intracerebral challenge by

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