Paralysis caused by acute myelitis in Theiler’s murine encephalomyelitis virus strain GD VII infection is induced by CD4⁺ lymphocytes infiltrating the spinal cord

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Intravenous infection by Theiler’s murine encephalomyelitis virus strain GD VII causes acute encephalomyelitis and paralysis in infected mice. However, nude mice and cyclophosphamide-treated ddY mice did not show paralysis when they were able to survive until day 20 post-infection (p.i.). Of ddY mice infected with 5 × 10⁷ p.f.u./mouse, 70–80% showed symptoms of paralysis on day 20 p.i. The viral titres in the brain and spinal cord in infected mice were not significantly different between paralytic and non-paralytic mice. In all of the mice infected with the virus, CD4⁺ lymphocytes and CD8⁺ lymphocytes had infiltrated the brain on days 10, 12, 14 and 20 p.i. as demonstrated by flow cytometric analysis. In contrast, few T lymphocytes infiltrated the spinal cord in the non-paralytic mice. Administration of an anti-CD4 monoclonal antibody (MAb) or anti-T cell receptor-αβ MAb on day 6 p.i. inhibited paralysis until day 20 p.i., though 20% of the MAb-treated mice and 80% of the control mice showed paralysis. Administration of anti-CD8 MAb was not effective in the suppression of paralysis. The MAb treatment did not significantly augment viral replication in the spinal cord, although the viral titres in the brain of the MAB-treated mice increased significantly. After the transfer of spleen cells from infected C3H mice, the recipient mice infected with a small amount of the virus showed paralysis, though uninfected mice did not. This transfer could be blocked by CD4⁺ lymphocyte depletion of the donor mice. These results indicate that paralysis caused by acute myelitis in Theiler’s virus strain GD VII infection is induced by CD4⁺ lymphocytes infiltrating the spinal cord.

Introduction

Theiler's murine encephalomyelitis virus is divided into two subgroups (strain GD VII and strain TO) on the basis of biological behaviour when mice are infected by intracerebral (i.c.) inoculation (Lorch et al., 1981). Subgroup TO is recognized to infect susceptible mice persistently and induce demyelination. Strain GD VII virus infection through the i.c. route causes fatal encephalitis (Lipton, 1980). It is reported that GD VII virus produces CNS disease following intraperitoneal (i.p.) or intravenous (i.v.) administration (Theiler & Gard, 1940). After inoculation with GD VII through the i.v. route, viral replication and elimination are observed in the central nervous system (CNS) and the infected mice show paralysis until day 20 post-infection (p.i.) (Kohanawa et al., 1993). Histological inflammatory findings and the infectious virus disappear in the CNS of the infected mice after day 30 p.i. (unpublished data).

T lymphocytes are important in both producing demyelinating lesions and eliminating the virus in Theiler's virus chronic strain infection (Borrow et al., 1992, 1993; Rodriguez & Sriram, 1988; Welsh et al., 1987). Nude mice infected with rabies virus do not show paralytic symptoms and die because of neuronal infection, these mice show paralysis as a result of the transfer of T lymphocytes (Sugamata et al., 1992). In strain GD VII infection, the CNS disease is thought to be induced by lytic infection (Lipton, 1994). However, it is not clear why GD VII virus infection through the i.v. route causes paralysis. Paralysis occurs after day 10 p.i., and apparent inflammatory cell infiltration in the spinal cord is also observed after that time (unpublished data), although T cell receptor (TCR) αβ⁺ cells do not infiltrate the spinal cord until day 9 p.i. (Kohanawa et al., 1995). Therefore, paralysis in strain GD VII infection might also be caused by inflammatory cell infiltration.

Methods

Mice. Female ddY mice (4 weeks old), male ICR nu/nu mice (6 weeks old), and C3H/HeN mice (5 weeks old) were obtained from SLC (Hamamatsu, Shizuoka, Japan).
Viral infection. Theiler's virus strain GD VII was grown on BHK-21 cell monolayers in serum-free RPMI 1640 medium. The ddY mice were inoculated i.v. with 1 × 10⁷ (cyclophosphamide-treated mice) or 5 × 10⁷ [anti-lymphocyte monoclonal antibody (MAb) treated mice] p.f.u./mouse. The nude mice were infected with 3 × 10⁶ or 1 × 10⁷ p.f.u./mouse.

Plaque assay. The brains and spinal cords of Theiler's virus-infected mice were removed aseptically and homogenized in RPMI 1640 medium with a Dounce tissue grinder. The homogenates of the brain and spinal cord were 30% (w/v) of the brain tissue and 20% (w/v) of the spinal cord tissue. The resulting tissue homogenates were frozen and thawed twice and centrifuged at 500 g for 30 min. The virus in the supernatant of the tissue homogenate was quantified by plaque assay of BHK-21 cells (Kohanawa et al., 1993).

Antibodies. Hybridoma cells secreting MAbs directed against CD4 (L3T4) (GK1.5; rat IgG2b; Dialynas et al., 1983), CD8 (Lyt-2) (53-6.72; rat IgG2a; Ledbetter & Herzenberg, 1979), TCR-αβ (H37-597; hamster IgG; Kubo et al., 1989) were used. H57-597 cells were kindly donated by Dr E. Nakayama, Department of Parasitology, Okayama University Medical School, Okayama, Japan.

These MAbs were prepared from ascites fluid in CD-1 mice. Partial purification by 50% (NH₄)₂SO₄ precipitation was followed by exhaustive dialysis against PBS, pH 7.4.

In vivo depletion of CD4⁺ cells, CD8⁺ cells, and TCR-αβ⁺ cells, and cyclophosphamide treatment. We gave each mouse a single 400 μg i.v. injection of anti-CD4 MAb, or anti-CD8 MAb (in 0.2 ml of pyrogen-free saline) on day 6 p.i. Normal rat globulin was injected as a control. Two hundred μg of anti-TCR-αβ MAb was injected i.v. on day 6 p.i. Normal hamster globulin was injected as a control.

Cyclophosphamide (150 mg/kg) was injected i.p. on day 6 p.i. PBS was injected as a control.

Isolation of mononuclear cells (MNC) from CNS tissue and flow cytometric analysis. The method of isolation of the MNC from brain and spinal cord was reported previously (Clatch et al., 1990). After the infected mice were sacrificed by perfusion with PBS through the heart, the brain and spinal cord were removed. The brain and the spinal cord tissues of 10 mice were dissociated by passage through 100-mesh stainless steel screens and resuspended in RPMI 1640 medium. Dissociated tissues were centrifuged for 10 min at 200 g and resuspended in 4 ml of 70% Percoll (Pharmacia) in PBS at 24 °C. Four ml of 30% Percoll was carefully overlaid over the 70% Percoll layer containing the dissociated tissues, and the gradients were centrifuged for 15 min at 500 g at 24 °C. Fractions from the gradients were collected by puncturing a hole in the bottom of the tube.

For flow cytometric analysis (FACScan, Becton Dickinson) of the distribution of the CD4⁺ and CD8⁺ cells, MNC were stained by phycoerythrin (PE) conjugated anti-L3T4 MAb (Becton Dickinson) and FITC-conjugated anti-Lyt-2 MAb (Becton Dickinson).

Adoptive transfer of the spleen cells. C3H mice infected with 1 × 10⁸ p.f.u. of virus were sacrificed on day 6 p.i., and the spleens were removed and minced in RPMI 1640 medium. Then they were washed and adjusted to the required concentration in PBS and 2 × 10⁶ cells in 0.2 ml were injected i.v. into each recipient. It was confirmed by plaque assay that the spleen cells did not contain infectious virus. The recipients were inoculated i.v. with 5 × 10⁸ p.f.u. of virus and the donor spleen cells were transferred on day 6 p.i.

Statistical analysis. The Wilcoxon test for paired samples was used to evaluate the statistically significant differences in virus titres between groups of mice that received MAb and normal globulin. In the experiment of the inhibition of paralysis by anti-CD4, anti-CD8, and anti-TCR-αβ MAb, the Wilcoxon and Cox-Mantel tests were used.

Results

Paralysis in nude mice and cyclophosphamide (CY) treated mice

We observed the appearance of paralysis in immunosuppressed mice. The CY-treated and nude mice showed high mortality after infection with a small amount of the virus, and half of the mice that died showed tetraplegia.

Table 1. Mortality and paralysis on day 20 p.i. in cyclophosphamide-treated ddY mice and nude mice*

<table>
<thead>
<tr>
<th>Group</th>
<th>Death</th>
<th>Paralysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0/20</td>
<td>8/20</td>
</tr>
<tr>
<td>CY-treated</td>
<td>12/20</td>
<td>0/8</td>
</tr>
<tr>
<td>Nude</td>
<td>3/10</td>
<td>0/7</td>
</tr>
<tr>
<td>CY-treated</td>
<td>10/10</td>
<td>-</td>
</tr>
<tr>
<td>(3 x 10⁶ p.f.u.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nude</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1 x 10⁷ p.f.u.)</td>
<td></td>
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</table>

* Control and CY-treated ddY mice were infected with 1 × 10⁷ p.f.u./mouse.

Fig. 1. Theiler's virus titres in the paralytic (black bars) and non-paralytic ddY mice (open bars). The mice were infected with 5 × 10⁴ p.f.u./mouse. Each value is expressed as the average of 10 mice, and standard deviation bars are shown.
Paralysis in Theiler's virus infection

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**Fig. 2. FACS analysis of the mononuclear cells infiltrating in the brain and spinal cord.** These cells were stained with PE-conjugated anti-CD4 (GK 1.5), FITC-conjugated anti-CD8 (Lyt-2). The cells separated from the brains of 10 mice were analysed in each experiment.

<table>
<thead>
<tr>
<th></th>
<th>Day 10 p.i.</th>
<th>Day 12 p.i.</th>
<th>Day 14 p.i.</th>
<th>Day 20 p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinal cord (not paralysed)</td>
<td>0.5%</td>
<td>0.4%</td>
<td>0.8%</td>
<td>0.2%</td>
</tr>
<tr>
<td>Spinal cord (paralysed)</td>
<td>6.7%</td>
<td>6.3%</td>
<td>7.1%</td>
<td>15.8%</td>
</tr>
<tr>
<td>Brain (not paralysed)</td>
<td>4.7%</td>
<td>7.8%</td>
<td>3.5%</td>
<td>6.6%</td>
</tr>
<tr>
<td>Brain (paralysed)</td>
<td>6.6%</td>
<td>5.5%</td>
<td>6.6%</td>
<td>7.1%</td>
</tr>
</tbody>
</table>

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for a short time before death (Table 1). However, the nude mice and CY-treated mice that survived did not show paralysis on day 20 p.i. (Table 1). All of the mice given $1 \times 10^5$ p.f.u. through the i.v. route showed evidence of viral replication in the CNS (data not shown). Therefore, the virus infected the CNS of all the nude and CY-treated mice.

**Viral replication in paralytic and non-paralytic ddY mice**

If paralysis in strain GD VII infection is induced only by the viral cytopathic effect on neural cells, it is possible that viral replication in the CNS of paralytic mice is greater than that in non-paralytic mice. However, there was no significant difference in the viral titres between the brains and spinal cords of the paralytic and non-paralytic ddY mice on days 10 and 15 p.i. (Fig. 1). The virus was detected (33 p.f.u./g to $2 \times 10^4$ p.f.u./g) in all of the mice in this experiment.

**FACS analysis of the cells infiltrating the brain and spinal cord**

We examined the correlation between T lymphocyte infiltration of the CNS and paralysis. CD4$^+$ lymphocytes and CD8$^+$ lymphocytes infiltrated the brain in both the paralytic and non-paralytic mice (Fig. 2). These cells also
infiltrated the spinal cord in the paralytic mice. However, few T lymphocytes infiltrated the spinal cord in the non-paralytic mice (Fig. 2).

Effects of depletion of TCR-αβ⁺ lymphocytes, CD4⁺ lymphocytes and CD8⁺ lymphocytes on the appearance of paralytic symptoms and viral replication

To evaluate the role of T lymphocytes in overt paralysis, we performed a T lymphocyte-depletion experiment using MAb administration. Anti-TCR-αβ MAb- or anti-CD4 MAb treatment suppressed the appearance of paralytic symptoms (Fig. 3). Eighty-five percent of the control mice and 15% of the anti-CD4 MAb- or anti-TCR-αβ MAb-treated mice showed paralytic symptoms (P < 0.01). Anti-CD8 MAb treatment showed no significant effect on the inhibition of these symptoms by the Wilcoxon test. However, in the Cox-Mantel test, the onset of paralysis was delayed significantly by anti-CD8 MAb treatment. In this case, anti-CD8 MAb treatment was not significantly effective in suppressing paralysis.

Depletion of CD4⁺ lymphocytes and CD8⁺ lymphocytes by the MAbs was confirmed by flow cytometric analysis (data not shown). Anti-TCR-αβ MAb treatment depleted CD4⁺ lymphocytes and CD8⁺ lymphocytes from the brain and spinal cord (data not shown). All of the paralytic mice treated with anti-CD4 MAb died. In contrast, only one mouse treated with anti-TCR-αβ MAb died.

Effect of depletion of CD4⁺ lymphocytes or CD8⁺ lymphocytes on the viral replication

To assess the effect of the depletion of CD4⁺ lymphocytes and CD8⁺ lymphocytes on viral replication, we assayed the amount of the virus in the brain and spinal cord. Anti-CD4 MAb and anti-CD8 MAb treatment significantly augmented the viral titre in the brain, but not in the spinal cord (Fig. 4).

Induction of paralysis by the transfer of spleen cells of mice infected with a high virus titre

To examine the ability of T lymphocytes to induce paralysis, we performed an adoptive transfer experiment. In uninfected recipient mice, paralysis was not induced
Table 2. Adoptive transfer-induced paralysis in the infected recipient mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Paralysis on day 20 p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection only</td>
<td>0/10</td>
</tr>
<tr>
<td>Spleen cell transfer from normal mice</td>
<td>0/10</td>
</tr>
<tr>
<td>Spleen cell transfer from infected mice</td>
<td>5/10</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;-depleted spleen cell transfer</td>
<td>2/10</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt;-depleted spleen cell transfer</td>
<td>5/10</td>
</tr>
</tbody>
</table>

* Donor mice were infected with 5 x 10<sup>7</sup> p.f.u./mouse, and recipient mice were infected with 5 x 10<sup>9</sup> p.f.u./mouse. Transfer to uninfected mice did not induce paralysis (data not shown).

Discussion

Theiler's virus strain GD VII could induce paralysis not only by lytic infection, but also by an immune-mediated mechanism. The viral titres of the paralytic and non-paralytic ddY mice were not significantly different (Fig. 1). This indicated that the degree of viral cytotoxicity for neural cells was not significantly different in the paralytic and non-paralytic mice. In flow cytometric analysis, CD4<sup>+</sup> lymphocytes and CD8<sup>+</sup> lymphocytes were found to have infiltrated the spinal cord in the paralytic mice (Fig. 2). Moreover, the CY-treated and nude mice that survived until day 20 p.i. were not paralysed, although high mortality occurred in these mice (Table 1). A similar result was observed in Theiler's virus chronic strain: demyelination was suppressed in the CY-treated mice that survived, and most of these mice died due to grey matter involvement (Lipton & Dal Canto, 1976). Anti-TCR-<sup>z</sup> cells and anti-CD4 MAb-treatments also suppressed paralysis (Fig. 3). Accordingly, paralysis induced by T lymphocytes could exist. On the other hand, some of these mice that received immunosuppressive treatment and died, showed severe wasting and tetraplegia for a short time before death. In GD VII strain infection, encephalitis preceedes myelitis (Kohanawa et al., 1993) and the elimination of the virus from the brain is dependent on T lymphocytes (Kohanawa et al., 1994). Therefore, some of the mice that die might be paralysed because of the deterioration due to encephalitis, and this paralysis would be caused by lytic infection.

GD VII virus infects and harms oligodendrocytes like strain DA virus <i>in vitro</i> (Ohara et al., 1990) and contains genetic determinants for late demyelination (Rodriguez & Roos, 1992). It is possible that the destruction of the infected glial cells by T lymphocytes and/or viral cytotoxicity induce paralysis. However, both Theiler's virus acute and chronic strains cause grey matter disease until 4 weeks after i.c. inoculation (Lipton & Dal Canto, 1975), and we observed inflammatory lesions histologically in grey matter, but not in white matter (data not shown).

Lipton (1975) reported that motor neurons in the brain stem and spinal cord are the main targets of Theiler's virus infection during poliomyelitis. GD VII virus infection is controlled by CD3<sup>+</sup>/TCR-<sup>z</sup> cells in the spinal cord (Kohanawa et al., 1995), although TCR-<sup>z</sup> cells eliminate the virus from the brain (Kohanawa et al., 1994). Our present study also shows that CD4<sup>+</sup> cells and CD8<sup>+</sup> cells do not suffice to eliminate the virus from the spinal cord in comparison with the brain on day 15 p.i. (Fig. 4). The infected cells in the spinal cord react with T lymphocytes with difficulty and it is possible that these cells are neurons, because neurons express few MHC molecules and TCR-<sup>z</sup> cells are not able to react with them (Joly & Oldstone, 1992). TCR-<sup>z</sup> cells might infiltrate the spinal cord to eliminate the virus from the infected glial cells, and harm the neurons by some cytotoxic mechanism. Anti-CD4 MAb treatment was able to prevent paralysis (Fig. 3), and the adoptive transfer of CD8<sup>+</sup> lymphocyte-depleted spleen cells was possible (Table 2). In Theiler's virus chronic strain, CD4<sup>+</sup> lymphocytes are also important in producing demyelination (Welsh et al., 1987). CD4<sup>+</sup> lymphocytes are well recognized as helper cells and produce cytokines (Ertl et al., 1989; Nakane et al., 1991). T lymphocytes are able to harm cells by cytokine production, the so-called bystander effect (Tite & Janeway, 1984; Tite, 1990). For example, tumour necrosis factor (TNF) is thought to be a cytotoxic factor in CNS inflammation, because it harms oligodendrocytes <i>in vitro</i> (Selma& Raine, 1988). We reported that CD4<sup>+</sup> lymphocytes and CD3<sup>+</sup>/TCR-<sup>z</sup> cells produced interferon-<sup>1</sup> (IFN-<sup>1</sup>) in the CNS of infected mice (Kohanawa et al., 1994). We also detected TNF-<i>x</i>, interleukin-4 (IL-4), IL-6 and IL-10 (unpublished data), and some of these cytokines might be involved in inducing paralysis. On the other hand, anti-CD8 MAb did not inhibit the induction of paralysis significantly (Fig. 3). CD8<sup>+</sup> lymphocytes might harm the infected cells directly as cytotoxic cells in an MHC class...
I-restricted fashion (Lindsley et al., 1991). If the paralysis is caused by the destruction of neurons, it is reasonable to conclude that a direct cytotoxic mechanism caused by CD8+ lymphocytes is not able to induce paralysis because of the limited MHC expression on neurons. However, anti-CD8 MAb administration has a tendency to delay the appearance of paralysis (Fig. 3), and induction of paralysis by the adoptive transfer of the spleen cells depleted of CD4+ lymphocytes was not inhibited completely (Table 2). CD8+ lymphocytes also produce cytokines (Benvenuto et al., 1992) and are important in inducing paralysis in Thielier's virus chronic strain infection (Rodriguez & Sriram, 1988). Therefore, it cannot be completely denied that CD8+ lymphocytes have an ability to induce paralysis. Moreover, macrophage-lineage cells might be concerned with inducing paralysis. In the infection by Thielier's virus chronic strain, the suppression of the macrophage function exacerbates grey matter disease and inhibits demyelination (Rodriguez & Quddus, 1986).

References


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