Role of T Lymphocytes in Recovery from Murine Cytomegalovirus Infection

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Congenitally athymic nude (Nu/Nu) mice inoculated intraperitoneally with murine cytomegalovirus (MCMV), in doses as low as 1.3 × 10⁴ plaque-forming units succumbed to the infection. In contrast, the mean lethal dose for heterozygous euthymic (Nu/+ ) littersmates was 4 × 10³ plaque-forming units. Though histopathological changes consistent with MCMV infection were found in the spleen, lungs, and adrenals of nude mice, there were only small focal areas of involvement in the liver. In contrast, Nu/+ mice dying from infection had pathological evidence of severe hepatitis. Spleen cells from immune and control BALB/c mice were injected intravenously into syngeneic mice that had been inoculated previously with lethal doses of MCMV intraperitoneally. Mice receiving 1 × 10⁷ or more immune spleen cells were protected against the infection, whereas mice receiving 1 × 10⁶ control spleen cells or immune serum were not. Treatment of immune spleen cells with anti-theta serum and complement significantly reduced their protective effect. Immune mechanisms associated with T lymphocytes appear to be critical for recovery from MCMV infection.

Cytomegaloviruses (CMV) may cause severe infections in immunocompromised patients, particularly those with certain types of cancer and those receiving immunosuppressive therapy in conjunction with transplantation procedures. Understanding of the immunological mechanisms involved in recovery from CMV infections might suggest new ways of preventing or treating severe manifestations in such patients. This would be particularly desirable since therapy of CMV infections with antiviral agents has been so far disappointing (9).

With a murine cytomegalovirus (MCMV) model, Brody and Craighead reported that the majority of mice treated with anti-lymphocyte serum and inoculated with small doses of virus develop fatal infections with extensive pulmonary pathological changes (3). Since anti-lymphocyte serum has been shown to suppress both cell-mediated and antibody-mediated immunological responses (6), these results do not indicate which immunological impairments are responsible for enhanced susceptibility. The observation of Selgrade and Osborn that administration of silica enhances the susceptibility of mice to infection with MCMV suggests that macrophages, which are selectively inhibited by silica (2), play an important role in the recovery process (13).

To define further the role of immune cells in recovery from MCMV infection, we inoculated congenitally athymic nude (Nu/Nu) mice (10) and also studied the effects of transfers of immune cells or serum in BALB/c mice.

MATERIALS AND METHODS

Mice. Nude mice of the CBA/CaCrc strain and heterozygous (Nu/+) littersmates were obtained from the barrier-maintained breeding colony of the Clinical Research Centre, Harrow, England. Mice were transferred directly from the breeding unit to isolators and provided with sterile food and water. BALB/c mice were provided by Steven Shore, Center for Disease Control, Atlanta, Ga. and were housed under standard conditions. Salivary gland homogenates prepared from selected mice of each strain did not produce a cytopathic effect in mouse embryo fibroblast (MEF) cultures, indicating that the mice were free of chronic MCMV infection.

Virus. The Smith strain of MCMV was provided by C. A. Mims, Guy's Hospital Medical School, London, England. Four- to 6-week-old BALB/c mice were inoculated with 2 × 10⁶ plaque-forming units (PFU) intraperitoneally (i.p.). Three weeks later the mice were killed, and 10% (wt/vol) homogenates of their salivary glands were prepared with Ten-Brock tissue grinders (Wheaton Scientific, Millville, N.J.). Ten percent dimethyl sulfoxide (Fisher Scientific Company, Fair Lawn, N.J.) was added, and portions were stored at −70°C until used. The mean lethal dose (LD₅₀) for 6- to 8-week-old BALB/c mice inoculated i.p. was 8 × 10⁴ PFU.

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Virus titrations. Organ homogenates were serially diluted with L-15 (Leibovitz medium, Grand Island Biological Company, Grand Island, N.Y.) medium and 0.1-ml aliquots of each dilution were added to monolayers of MEF in tissue culture trays with 16-mm-diameter wells (Linbro Scientific Co., New Haven, Conn.). After the trays were incubated for 1 h at 37°C, a 0.75% methyl cellulose (Fisher Scientific Company, Fair Lawn, N.J.) overlay containing 2% fetal calf serum (FCS) was added, and the trays were incubated for 5 to 7 days at 37°C in a humidified atmosphere containing 5% CO₂. When plaques were visible, the monolayers were stained with 1% crystal violet.

Histopathology. Organs were fixed in 10% Formalin, and sections were stained with hematoxylin and eosin.

Spleen cell suspensions. Immune spleen cell suspensions were prepared from 6- to 8-week-old BALB/c mice that had been inoculated 6 days previously with 4.8 × 10⁵ PFU i.p. These mice and age-matched uninoculated controls were killed by cervical dislocation. Spleens were aseptically removed, placed in L-15 medium, and gently teased apart with 21-gauge needles. The cells were washed twice with L-15 medium containing 1% FCS. Viability, as determined by trypan blue exclusion, was 60 to 80%.

Treatment of spleen cells with anti-theta serum. Immune spleen cells were suspended at a concentration of 1 × 10⁶ cells/ml in either mouse anti-theta serum (Bionetics, Kensington, Md.) diluted 1:10 or Hanks balanced salt solution (HBSS; Flow Laboratories, Rockville, Md.) plus 5% FCS or similarly diluted normal BALB/c mouse serum. After 1 h of incubation at 4°C, the spleen cells were washed twice with HBSS plus 5% FCS and suspended in guinea pig complement (Cordis Laboratories, Miami, Fla.) diluted 1:5 with HBSS at a concentration of 1 × 10⁶ cells/ml. After incubation at 37°C for 1 h, the spleen cells were washed twice with HBSS plus 5% FCS, and the number of viable cells was counted. Treatment with anti-theta serum and complement produced lysis of 32 and 46% of immune spleen cells in two separate experiments. Similar treatment of BALB/c femoral bone marrow cells produced lysis of <5% of cells.

Immune serum. Serum obtained by cardiac puncture of BALB/c mice inoculated 6 days previously with 5.8 × 10⁶ PFU was stored at −20°C prior to use. This serum, tested by the method of Overall et al. (9), had a complement-requiring neutralizing antibody titer of 1:160.

Transfer of spleen cells or immune serum. Adult 6- to 8-week-old BALB/c mice inoculated with lethal doses of MCMV i.p. received immune or control spleen cells suspended in 0.2 ml of L-15 medium, or 0.2 ml of undiluted immune serum, by tail vein injection, 24 h later. The mice were observed daily for illness or mortality. At 2, 4, and 6 days after virus inoculation, randomly selected mice were sacrificed for virological and histological studies.

RESULTS

Mortality of nude and Nu/+ mice inoculated with MCMV. Four- to 6-week-old nude and Nu/+ mice were inoculated with various doses of MCMV by the i.p. route. As shown in Table 1, 100% of Nu/+ mice survived inoculation with 1.3 × 10⁶ PFU or less. In contrast, all of the nude mice inoculated with 1.3 × 10⁹ PFU or less succumbed, including those receiving as few as 1.3 × 10⁸ PFU. The nude mice died within 2 to 3 weeks after inoculation, the interval being longer with smaller doses of virus. Nude and Nu/+ mice receiving 1.3 × 10⁴ PFU died at about the same time after inoculation.

Histopathological findings in nude and Nu/+ mice. The histopathological findings at the time of death in nude mice inoculated with 1.3 × 10⁶ PFU or less were similar, regardless of virus dose, and consisted of a mild interstitial pneumonitis, focal areas of degenerating cells, some containing intranuclear inclusions in the spleen and adrenals, and small foci of mononuclear inflammatory cells in the liver, whereas most of the liver parenchymal cells appeared normal. No histopathological changes were noted in the brain, pancreas, and kidneys. In nude and Nu/+ mice receiving 1.3 × 10⁶ PFU, pathological changes in the lungs, adrenals, and spleen appeared to be of similar severity; however, extensive changes were noted in the liver parenchymal cells of Nu/+ mice (Fig. 1a), whereas the livers of nude mice contained only small focal areas of inflammation (Fig. 1b).

Effect of transfer of spleen cells or immune serum in BALB/c mice. When BALB/c mice were inoculated with 4.8 × 10⁵ PFU i.p. (6 LD₅₀) and given 1 × 10⁶ immune spleen cells intravenously (i.v.) 24 h later, only two out of seven mice survived. In subsequent studies with mice inoculated with 1.2 × 10⁶ PFU (1.5 LD₅₀), survival of those receiving 1 × 10⁶ immune spleen cells 24 h later was complete (26/26); it was significantly higher (P < 0.001, chi-square test) than that of mice receiving 1 × 10⁴ control spleen cells (5/17) or no treatment (5/18), as shown in Fig. 2. Increased survival was also

<table>
<thead>
<tr>
<th>Virus dose (PFU)</th>
<th>Nu/Nu or heterozygous (Nu+) littermates inoculated with various doses of murine cytomegalovirus i.p.</th>
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</thead>
<tbody>
<tr>
<td>Nu/+</td>
<td>Nu/Nu or heterozygous (Nu+) littermates inoculated with various doses of murine cytomegalovirus i.p.</td>
</tr>
<tr>
<td>1.3 × 10⁴</td>
<td>7/7 (7.3d)⁴</td>
</tr>
<tr>
<td>1.3 × 10³</td>
<td>0/6</td>
</tr>
<tr>
<td>1.3 × 10²</td>
<td>0/3</td>
</tr>
<tr>
<td>1.3 × 10¹</td>
<td>ND⁵</td>
</tr>
<tr>
<td>1.3 × 10⁰ or less</td>
<td>0/9</td>
</tr>
</tbody>
</table>

⁴ Number in parentheses indicates mean survival time after inoculation; d, days.
⁵ ND, Not done.

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Fig. 1. Appearance of liver 6 days after inoculation with $1.3 \times 10^4$ PFU i.p.; (a) Nu/+: parenchymal cells show vacuolization, ballooning, and numerous intranuclear inclusions. (b) Nude: a typical focus of infection. ×500.

noted when as few as $1 \times 10^7$ immune spleen cells were given, but no protective effect was noted with administration of $1 \times 10^6$ immune spleen cells (Table 2). Administration of 0.2 ml of immune serum 24 h after virus inoculation had no effect on the survival of mice (Fig. 2).

Effect of cell transfers on virus titers and histopathology. The livers of recipient mice were examined for histopathological changes and viral content, since the extent of this or-
intranuclear inclusions were present. There was little inflammatory response. In contrast, most of the liver parenchymal cells of mice receiving immune spleen cells appeared normal at 4 and 6 days after inoculation. Moderate numbers of small inflammatory foci and a mild mononuclear cell infiltration around portal areas were noted.

Effect of treatment of immune spleen cells with anti-theta serum. BALB/c mice inoculated with $1.2 \times 10^5$ PFU of MCMV 24 h previously were given $7 \times 10^5$ immune spleen cells treated with either anti-theta serum and complement or normal mouse serum and complement. The protective effect of immune spleen cells was significantly abolished by pretreatment with anti-theta serum and complement ($P < 0.05$, exact test) (Fig. 3).

DISCUSSION

In a recent report, the LD$_{50}$ for nude mice infected with MCMV was found to be $10^{3.9}$ PFU, approximately 10-fold lower than that for Nu/+ mice (12). Histopathological findings were similar in the two groups of mice. The greater susceptibility of nude mice in our study might be

The quantities of virus detected in the liver at various times after inoculation with MCMV are shown in Table 3. Two days after inoculation with MCMV and 24 h after administration of spleen cells, the titers of virus were similar, whether the mice received immune or control spleen cells, but, at 4 and 6 days, the livers of mice receiving immune spleen cells contained less virus than those of mice receiving control spleen cells. Quantities of virus $<10^2$ PFU could not be detected because undiluted liver homogenates produced toxic changes in MEF cultures.

Two days after inoculation with MCMV, occasional cells containing intranuclear inclusions were noted in the livers of mice receiving either immune or control spleen cells. By 4 days after inoculation, areas of ballooning degeneration were noted in the livers of mice receiving control spleen cells. By 6 days, severe degenerative changes were seen in most of the cells; the cytoplasm of many cells appeared granular; many contained small vacuoles, and numerous

**TABLE 2. Survival of BALB/c mice inoculated with MCMV and given various numbers of immune spleen cells**

<table>
<thead>
<tr>
<th>Spleen cells (no.)</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1 \times 10^6$</td>
<td>100</td>
</tr>
<tr>
<td>$3.3 \times 10^6$</td>
<td>100</td>
</tr>
<tr>
<td>$1 \times 10^7$</td>
<td>67</td>
</tr>
<tr>
<td>$1 \times 10^6$</td>
<td>17</td>
</tr>
</tbody>
</table>

$^a$ Mice were inoculated with $1.2 \times 10^5$ PFU and were given immune spleen cells i.v. 24 h later. Immune spleen cells were obtained from BALB/c mice inoculated with $4.8 \times 10^5$ PFU i.p. 6 days previously.

$^b$ Groups consisted of six mice.

**TABLE 3. Virus titers in the livers of BALB/c mice at various times after inoculation with MCMV followed by transfer of control or immune spleen cells**

<table>
<thead>
<tr>
<th>No. of days after inoculation with virus</th>
<th>Control spleen cells</th>
<th>Immune spleen cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>$2.8 \times 10^4$</td>
<td>$2.7 \times 10^4$</td>
</tr>
<tr>
<td>4</td>
<td>$3.3 \times 10^4$</td>
<td>$&lt;10^3$</td>
</tr>
<tr>
<td>6</td>
<td>$3.0 \times 10^4$</td>
<td>$&lt;10^3$</td>
</tr>
</tbody>
</table>

$^a$ Mice were inoculated with $1.2 \times 10^5$ PFU i.p. and received $1 \times 10^6$ control or immune spleen cells i.v. 24 h later.

$^b$ Each value represents the mean of two determinations performed on pooled homogenates from two mice.

**FIG. 2. Survival of BALB/c mice inoculated with $1.2 \times 10^5$ PFU of MCMV (■) and of mice inoculated with the same dose of virus and given $1 \times 10^6$ immune spleen cells (●), $1 \times 10^6$ control spleen cells (△), or 0.2 ml of immune serum (○) 24 h later.**

**FIG. 3. Survival of BALB/c mice inoculated with $1.2 \times 10^5$ PFU of MCMV and given $7 \times 10^6$ immune spleen cells, treated as shown in the figure, 24 h later. There were eight mice in each group.**
due to the genetic background of the mice used, the environment in which they were maintained, or the virulence of the virus strain. Nude mice of the strain we used also showed greatly increased susceptibility to herpes simplex virus and murine hepatitis virus infections compared with Nu/+ mice (1).

In our study, only minimal pathological changes were noted in the livers of nude mice succumbing after inoculation with MCMV. Even when large doses of virus were inoculated, the changes were much more severe in the livers of Nu/+ mice than in the nude mice. The nude mice did not appear to die of hepatitis, in contrast to observations with immunologically intact mice (9).

The reason for relative sparing of the liver in nude mice is unknown. It is possible that Kupffer cells lining liver sinusoids, which act as a barrier to the entrance of some viruses into liver parenchymal cells (7), are more effective in nude mice. It is of interest that peritoneal macrophages of nude mice have recently been shown to be more efficient in phagocytosing and killing Listeria monocyotegenes than those of Nu/+ mice (4). Other explanations include the possibility that Kupffer cells in nude mice fail to phagocytize MCMV, or that their liver cells are refractory to infection.

Despite sparing of their livers, the nude mice succumbed and, at the time of death, had histopathological evidence of infection with MCMV in several organs. These results suggested that T lymphocytes, which the nude mouse virtually lacks, play an important role in recovery from MCMV infection.

Administration of sufficient numbers of immune spleen cells to infected BALB/c mice resulted in significantly increased survival. The protective effect was abolished by pretreatment with anti-theta serum and complement, suggesting that T lymphocytes play an important role in the recovery process. We have recently shown that administration of purified T lymphocytes, obtained by incubating spleen cells in nylon-wool columns, also results in enhanced survival of infected mice (unpublished observations).

T lymphocytes could be functioning in this model by several mechanisms. Products of activated T lymphocytes—macrophage chemotactic factor, migration inhibitory factor, and macrophage activating factors—might act on macrophages, which were implicated as having an important role in recovery from MCMV infections by the studies of Selgrade and Osborn (13). In an earlier study, Osborn and Medearis found no evidence that interferon, another product of T lymphocytes, or other cells contributed to recovery from infection (8). In addition to providing factors acting on macrophages, T lymphocytes might kill MCMV-infected cells since T lymphocyte killing of a number of virus-infected target cells has been reported (1).

The failure of passively administered immune serum and of B lymphocytes depleted of T lymphocytes to prevent mortality does not exclude a possible role for antibodies in the recovery process. Sensitized T lymphocytes might be required for optimal early antibody production, as has recently been shown in studies of Venezuelan encephalitis virus infection of mice (11). Antibody could act by neutralizing virus released as a result of T lymphocyte killing of infected cells, or antibody could potentiate antiviral effects of activated macrophages.

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LITERATURE CITED