Rat Cytomegalovirus-Induced Pneumonitis after Allogeneic Bone Marrow Transplantation: Effective Treatment with (S)-1-(3-Hydroxy-2-Phosphonyl-Methoxypropyl)Cytosine

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Two antiviral compounds, (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC) and 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG), were evaluated for their effects on rat cytomegalovirus (RCMV)-induced interstitial pneumonitis after allogeneic bone marrow transplantation (BMTx). Eight-week-old Brown Norway rats immunosuppressed by a lethal dose of total body irradiation were inoculated with RCMV and received allogeneic bone marrow cells from Lewis rats. Animals were treated with either HPMPC (20 mg/kg of body weight as a single dose) or DHPG (20 mg/kg as two daily doses for 5 days). The effect of antiviral therapy was monitored by measuring RCMV titers in different organs and the histopathologic changes in lungs at 8 to 10 days postinfection. In RCMV-infected allogeneic BMTx recipients, severe diffuse thickening of alveolar septa (6.02 μm) with a diffuse infiltration of mononuclear cells occurred, whereas in the noninfected allogeneic BMTx recipients, the septal width was on the order of 2 μm (P < 0.01). Treatment with DHPG (20 mg/kg in two daily doses for 5 days) resulted in a decrease in virus titers (log10 PFU per gram of tissue) in lungs and spleens from 3.81 ± 0.34 and 4.29 ± 1.07 (untreated animals) to 1.26 ± 0.53 and 3.22 ± 0.27 (treated animals), respectively. Treatment with HPMPC (20 mg/kg as a single dose) resulted in a complete reduction of virus titers in all organs to below the detection level (P < 0.01). Furthermore, antiviral treatment resulted in a reduction of the alveolar septal width from 6.02 ± 1.59 μm (untreated animals) to 4.67 ± 1.70 and 3.32 ± 0.63 μm after DHPG and HPMPC treatment, respectively. Furthermore, the influx of mononuclear cells in the alveolar septa was significantly impaired after treatment with HPMPC (P < 0.01). We conclude that in the described rat model, HPMPC is highly effective in suppressing RCMV-induced interstitial pneumonitis after allogeneic BMTx.

Cytomegalovirus (CMV) occurs in about 50 to 60% of patients after organ and bone marrow transplantation (BMTx) (16, 21). After allogeneic BMTx, CMV-induced interstitial pneumonitis (IP) is observed in 15 to 18% of the cases and represents a major cause of death, with a mortality rate of up to 83% (16, 35).

Ganciclovir combined with immunoglobulin reduces mortality in human CMV pneumonitis in BMTx recipients (12, 19, 23), and administration at the time that viral shedding is identified reduces the subsequent risk of CMV disease (14, 18). However, the clinical use of this agent is limited by adverse reactions (9). Moreover, treatment of IP after allogeneic BMTx remains a major clinical problem, partly because of the poor understanding of the pathogenesis of CMV-induced IP (25, 27) and partly because of the unavailability of potent and selective inhibitors of human CMV replication in vivo (2). Recently, a new class of acyclic nucleoside phosphonate analogues has been described (10, 11, 34). Of these compounds, (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC) seems to be the most potent and most selective inhibitor of CMV replication in vitro (29). In the treatment of generalized rat CMV (RCMV) infection in immunocompromised rats, HPMPC appears to be much more effective (31) than 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG).

To study new therapeutic approaches for the treatment of CMV-induced IP, an animal model resembling the human situation is required. Using RCMV isolated from wild rats, we developed an animal model for generalized CMV infections in immunocompromised rats (6, 30). In immunocompromised rats, however, diseases including hepatitis, splenitis, and thrombocytopenia occur. We have studied the pathologic and virologic course of CMV-induced IP after allogeneic BMTx in rats and determined the effects of antiviral strategies with HPMPC and DHPG on these parameters.

MATERIALS AND METHODS

Animals. Eight-week-old inbred specific-pathogen-free male Brown Norway (BN) rats with total body weights of between 140 to 180 g were used as recipients of BMTx. As donors for allogeneic bone marrow cells, adult specific-pathogen-free Lewis rats were used and syngeneic bone marrow cells were harvested from adult BN rats. By using an enzyme-linked immunosorbent assay by the method of Engvall and Perlmann (13), neither donor animals nor recipients were found to have antibodies to CMV at the start of the experiment. All rats were bred and housed at the Department of Experimental Animal Services at the Biomedical Center, University of Limburg, Maastricht, The Netherlands. All animal experiments were approved by the University Ethical Committee for animal studies.

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RCMV infection and total body irradiation. The RCMV stock consisted of a pool of salivary glands harvested from acutely infected laboratory rats (5). The salivary glands were homogenized, and the supernatant was collected after centrifugation and stored at −70°C. Each rat received from the RCMV stock 10^7 PFU by the intraperitoneal (i.p.) route as described before (6). For immunossuppression, animals received 9.6 Gy of total body X-irradiation (TBI) one day before i.p. RCMV inoculation (7).

BMTx. Syngeneic and allogeneic bone marrow cells from donors were obtained as described earlier (3). One day after TBI and 6 h postinfection (p.i.), each rat received 5 × 10^7 viable bone marrow cells intravenously.

To prevent bacterial infections with potential pathogenic bacteria after TBI, the aerobically growing gram-negative bacteria were eliminated from the intestines by selective bowel decontamination (36). Therefore, BMTx recipient rats received 1 week before transplantation 0.2 ml of a 10% solution enrofloxacin (a fluorquinolone; Bayer, Mijdrecht, The Netherlands) per liter of drinking water, resulting in a final dose of approximately 2 mg/kg of body weight per day (32). From the start of selective bowel decontamination, all the recipient rats were kept in sterile cages and received sterile food to avoid bacterial infections. Control bacterial cultures of rat feces proved that the gram-negative aerobic bowel flora was completely eliminated at the time of BMTx.

Experimental design. The study consisted of two parts, the first being an evaluation of the description of the pathologic events in RCMV-infected rats after allogeneic BMTx compared with that in syngeneic BMTx recipients. The second part consisted of the antiviral treatment of CMV infection after allogeneic BMTx. For allogeneic BMTx, bone marrow cells from Lewis rats were administered to BN recipients (group 1); for syngeneic BMTx, bone marrow cells from BN rats were administered to BN recipients (group 2). Noninfected allogeneic control rats were also included in the study (group 3).

Antiviral treatment was performed with optimal dosage schedules, as determined earlier (31).

DHPG was obtained from Syntex Inc., Palo Alto, Calif., and HPMPC was synthesized by I. Rosenberg and A. Hóly (Czechoslovak Academy of Sciences, Prague, Czechoslovakia) (17). The antiviral agents were dissolved in sterile phosphate-buffered salt solution (PBS; pH 7.40) and were administered to the animals as described below.

DHPG (20 mg/kg/day) was administered to CMV-infected allogeneic BMTx recipients by the i.p. route as two daily doses for 5 days, starting at 6 h p.i. (group 4), and HPMPC was administered as one single dose at 6 h p.i. (group 5).

Animals were checked every day for physical signs of symptomatic disease, such as tachypnea, dyspnea as signs for lung involvement, ocular discharges, brushy hairs, and impaired reactivity to exogenous stimuli as signs of generalized infection and hunchback as a sign of peritonitis (30).

From about 8 days p.i. and on the basis of the criteria mentioned above, rats were examined, and just before the untreated rats died (groups 1 and 2), all groups were sacrificed on the same day (between days 8 and 10 p.i.). Then, lungs, spleens, livers, kidneys, and salivary glands were resected and processed for histological examination and virus plaque assays as described earlier (5, 30). Briefly, samples of salivary glands, spleens, livers, and lungs were homogenized within 3 h after resection, serial 10-fold dilutions of a 1:10 (wt/vol) organ suspension in Eagle’s minimal essential medium were cultured on rat embryonal fibroblasts, and the amount of virus was expressed as the logarithm of PFU per gram of tissue.

Histological examination. Samples of the organs mentioned above were fixed in paraformaldehyde-lysine-periodate and were embedded in paraffin. Serial 3.5-μm-thick sections were prepared for hematoxylin-eosin staining, Jones silver staining, and immunoperoxidase staining by using monoclonal antibodies to RCMV antigens, as described below. For morphometric analyses, the thickness of the alveolar wall was measured 10 times for each lung by two different observers. There was a high level of agreement between the two series of measurements (P > 0.95). For the final analysis, the mean of 20 measurements from one rat was used. The amount of inflammatory infiltrate in alveolar septa and alveolar spaces was assessed semiquantitatively in each section. The absence of inflammatory cells was scored as 0, slight inflammatory infiltrate was scored as 1, moderate dense infiltrate was scored as 2, and dense inflammatory infiltrate was scored as 3 (see Table 2).

Immunohistochemistry. After deparaffinization, sections were blocked for their endogenous peroxidase activity by incubation in 100% methanol containing 0.3% H2O2 for 30 min. They were preincubated for 30 min at room temperature with 10% normal rabbit serum, washed with PBS-0.05% Tween 20, and then incubated for 1 h in moist air (30) with a mouse monoclonal antibody, termed monoclonal antibody 35, directed against the 29-kDa early structural antigen of RCMV. This gave a specific diffuse nongranular staining pattern in the cytoplasm of CMV-infected cells (8). The sections were washed and incubated with biotinylated, affinity-purified sheep anti-mouse immunoglobulin (Amersham Nederland B.V., Houten, The Netherlands) in PBS (1:200) containing 1% bovine serum albumin for 60 min; this was followed by washing and incubation for 45 min with biotin-streptavidin-horseradish peroxidase complex in PBS (1:400; Amersham) containing 1% bovine serum albumin. The sections were then washed again and incubated for 10 min in diaminobenzidine in PBS with H2O2. After washing, the sections were counterstained with hematoxylin, dried, and embedded in Entellan (30).

Statistical analysis. For statistical analysis, the chi-square test and the Mann-Whitney test were used. P values of <0.01 were considered significant. For interobserver analyses, the paired Student’s t test was used.

RESULTS

Characterization of RCMV pneumonia model. At about 8 days after allogeneic BMTx and RCMV inoculation, the virus reached maximum titers (4.29 ± 1.07 log10 PFU/g of tissue) in the spleens of rats; all rats then suffered from symptomatic RCMV disease (group 1). However, in lungs, livers, and kidneys, RCMV titers were 0.5 to 2.0 log10 PFU/g lower. After syngeneic BMTx and subsequent RCMV inoculation (group 2), virus titers in spleens, lungs, livers, and kidneys were comparable (Table 1). RCMV titers in the salivary glands of both groups of rats were near the detection level (log10 1.25 PFU/g).

All untreated RCMV-infected rats (group 1) showed severe symptoms of infection, such as ocular discharge, hunchback, and impaired reactivity to exogenous stimuli. In contrast to syngeneic RCMV-infected recipients (group 2), animals which received allogeneic BMTx (group 1) showed severe tachypnea and dyspnea shortly before dying. Furthermore, group 1 animals had hemorrhagic fluid in their peritoneums and petechiae and ecchymoses on their livers.
and spleens, findings that have already been described for immunocompromised RCMV-infected rats without BMTx (30). In addition, many of the group 1 animals showed hemorrhagic lesions in the lungs. Lungs of RCMV-infected allogeneic BMTx recipients (group 1) showed dilatation of alveolar vessels, alveolar edema, and infiltration of alveolar stroma with mononuclear cells (Fig. 1). As shown in Table 2, severe thickening of the alveolar septa was noted in group 1 compared with that in noninfected allogeneic controls (group 3). In the untreated, RCMV-infected syngeneic BMTx recipients (group 2), thickening of the alveolar septa occurred to a far lesser extent compared with that in the allogeneic BMTx recipients (group 1) (P < 0.01), the inflammatory infiltrate was more focally located, and edema was less pronounced or even absent compared with that in the allogeneic BMTx group.

In the untreated RCMV-infected allogeneic BMTx recipients (group 1), numerous alveolar lining cells and alveolar macrophages showed cytoplasmic immunoreactivity to CMV antigens. RCMV antigen was also found in large amounts in the spleens and livers and, to a lesser extent, in the kidneys and some of the salivary glands. Similar findings were obtained for RCMV-infected syngeneic BMTx recipients (group 2).

Effect of antiviral treatment. As shown in Table 1, antiviral treatment resulted in a significant decrease in RCMV titers in lungs from DHPG-treated (group 4) and HPMPC-treated (group 5) animals compared with the titers in untreated rats (P < 0.01). However, following DHPG treatment virus titers in the spleens and livers decreased only slightly and not significantly. After HPMPC treatment, no virus was detected in the spleens, lungs, or any of the other organs (P < 0.01).

Compared with the thickness of the alveolar septa (1.85 ± 0.23 μm) in untreated rats after allogeneic BMTx without RCMV infection (group 3) and after syngeneic BMTx with RCMV infection (group 2), the thicknesses of the septa were almost identical (2.45 ± 0.36 μm), but the width was increased compared with that of the alveolar septa of normal healthy rats (±1.0 μm) (data not shown). However, in rats which received both allogeneic BMTx and RCMV (group 1), the alveolar septa were extremely thickened (6.02 ± 1.59 μm). Treatment with DHPG reduced the thickness of alveolar septa only slightly, but HPMPC treatment led to complete reduction of the alveolar wall. As shown in Table 2, DHPG and HPMPC treatments also reduced the amount of inflammatory infiltration; in particular, HPMPC suppressed mononuclear cell infiltration in lung tissue.

### DISCUSSION

In the rat model, CMV infection led to IP in allogeneic BMTx recipients. A significant difference in lung pathology was observed between allogeneic and syngeneic BMTx recipients, despite comparable infectious virus titers in both host systems. After acute CMV infection, the lungs of allogeneic BMTx recipient rats showed a prominent influx of mononuclear cells. Perivascular infiltrates with mononuclear cells were frequent in both allogeneic and syngeneic BMTx recipients. Compared with noninfected allogeneic BMTx recipients, RCMV-infected recipients showed severe thickening of the alveolar septa. However, alveolar septa were not thickened in rats after syngeneic BMTx.

These findings are in accord with the findings of Grundy et al. (15), who reported that in a model for pneumonia in immunocompetent F1 mice, severe diffuse-type pneumonia occurred only when acute graft (allogeneic spleen cells)-versus-host reaction, in addition to CMV infection, was present. Syngeneic spleen cells, however, did not induce diffuse-type pneumonia. Despite the high incidence of CMV infection after syngeneic BMTx in humans, IP occurs very infrequently (1). Two factors appear to be necessary for the occurrence of CMV-induced IP: active CMV infection and allogeneic cells. However, the mechanism by which IP is induced remains unclear.

As pointed out in previous reports, the control of CMV infection is mainly based on T-cell immunity (22), and this immunity is restricted by major histocompatibility complex class I-restricted control of CMV infection seems to be impaired. Treatment of CMV-induced IP after allogeneic BMTx remains a major clinical problem (9). DHPG, at present the most potent therapeutic agent available for treatment of CMV infections in humans, gives poor results for the treatment of CMV-induced IP after allogeneic BMTx (2). In the present study, the effect of DHPG treatment on the thickness of the alveolar septa and the amount of perivascular infiltrates was insignificant, despite a significant reduction in RCMV titers in the lungs and other tissues. Similar results were described for mice (26) and humans who received DHPG treatment for IP (28). An explanation of the finding could be that CMV-induced IP is an immunopathological process, and therefore, antiviral treatment may not be expected to be (very) effective (28); or the reduction in virus titers is only partial, and therefore, protection from CMV-induced IP was incomplete.

In an attempt to answer these questions, we treated the rats with HPMPC, which has previously been proven to be a

### TABLE 1. Virus titers in organs of rats after BMTx with or without antiviral treatmenta

<table>
<thead>
<tr>
<th>Group</th>
<th>BMTx</th>
<th>Treatment</th>
<th>No. of animals</th>
<th>Log_{10} PFU/g of tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lung</td>
</tr>
<tr>
<td>1</td>
<td>Allogeneic</td>
<td>None</td>
<td>12</td>
<td>3.81 ± 0.34</td>
</tr>
<tr>
<td>2</td>
<td>Syngeneic</td>
<td>None</td>
<td>8</td>
<td>4.08 ± 0.22</td>
</tr>
<tr>
<td>4</td>
<td>Allogeneic</td>
<td>DHPGb</td>
<td>11</td>
<td>1.26 ± 0.53</td>
</tr>
<tr>
<td>5</td>
<td>Allogeneic</td>
<td>HPMPCc</td>
<td>11</td>
<td>&lt;1.25</td>
</tr>
</tbody>
</table>

* Groups of at least five BN rats each received 9.6 GY of TBI and 2 × 10⁷ viable allogeneic (Lewis) or syngeneic (BN) bone marrow cells at 2 h before inoculation with 10⁷ PFU of RCMV i.p. Organs were harvested at 8 days p.i. Data represent two separate experiments.

a DHPG at 20 mg/kg/day twice daily for 5 days starting 6 h p.i.

b P < 0.01, compared with group 1.

c HPMPC at 20 mg/kg as a single dose at 6 h p.i.
FIG. 1. Hematoxylin-eosin staining of lung sections from rats 8 days after allogeneic BMTx and subsequent RCMV infection. (A) Lung section from an untreated animal. The alveolar septa were severely thickened, and the alveolar stroma was infiltrated with mononuclear cells. (B) Lung section of HPMPC-treated rat. The thickness of the alveolar septal wall is reduced to normal size. The alveolar stroma is infiltrated with only a few mononuclear cells. Magnification, x395.
very potent and selective inhibitor of RCMV infections in vitro and in vivo (31). In contrast to DHPG, HPMPC, which eliminated RCMV completely from the lungs and other internal organs, reduced the thickness of the alveolar septa almost completely ($P < 0.01$). Taken together, these observations suggest that, when virus is absent (e.g., below the detection level) in any internal organ, immunopathologic findings in the lungs do not ensue. Therefore, it is postulated that some degree of active CMV replication and presentation of viral antigens (not necessarily originating in the lungs) is obligatory for producing CMV-induced IP.

A single dose of HPMPC administered early after infection completely prevented CMV-induced IP. This drug has the advantage over DHPG in that it has a 10-fold higher selectivity and potent activity against CMV (whether rat, mouse, or human CMV) in vitro and it is also far more potent than DHPG against both RCMV (31) and murine CMV (20) in vivo. Toxicity studies for HPMPC in mice failed to detect toxicity when it was used at 200 and 400 mg/kg, respectively (4, 10), which is of the same order as the 50% lethal dose for DHPG in these animals. In rats, dosages of 300 mg/kg for 3 days were not toxic (31). Phase I studies in humans will be performed soon.

In conclusion, HPMPC is far more active than DHPG against RCMV-induced IP, and HPMPC can be considered a promising candidate drug for the treatment of CMV-induced IP in allogeneic BMTx recipients.

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