Recovery of HLA-Restricted Cytomegalovirus (CMV)-Specific T-Cell Responses After Allogeneic Bone Marrow Transplant: Correlation With CMV Disease and Effect of Ganciclovir Prophylaxis

By Cheng-Rong Li, Philip D. Greenberg, Mark J. Gilbert, James M. Goodrich, and Stanley R. Riddell

Protection from cytomegalovirus (CMV) disease in immunocompromised hosts has been shown to correlate with recovery of the host virus-specific CD8+ T-cell response. The administration of ganciclovir to immunosuppressed transplant recipients as antiviral prophylaxis has reduced the early risk of CMV disease, but late disease is observed with increased frequency, suggesting that recovery of the CMV-specific T-cell responses necessary for protective immunity may be delayed in these patients. Therefore, we evaluated reconstitution of CMV-specific T-cell responses in 47 bone marrow transplant (BMT) recipients entered on a randomized placebo-controlled study of ganciclovir. The study drug was initiated at a mean of 24 days after BMT. At day 30 to 40, a minority of patients had recovery of T-cell immunity to CMV and the frequency of reconstitution was equivalent in patients randomized to ganciclovir or placebo. The failure of ganciclovir to effect early reconstitution may reflect the short duration of treatment. Early recovery was associated with the infusion of BM from a CMV seropositive donor \( r^P = .07 \) for CD8+ cytotoxic T cell (CTL). \( P = .04 \) for CD4+ Th. Between day 40 and day 90, recovery of deficient CD8+ and CD4+ CMV-specific T-cell responses occurred in the majority of individuals that received placebo, but in a minority of ganciclovir recipients. Two cases of late-onset CMV disease occurred in ganciclovir recipients. In all patients, the presence of a CTL response to CMV conferred protection from subsequent CMV disease \( (P = .005) \), and these protective CTL responses are shown to be specific for structural virion proteins similar to the responses in immunocompetent CMV seropositive individuals. These data confirm the importance of CMV-specific T-cell responses and suggest that a delay in recovery of these responses as a result of ganciclovir prophylaxis may contribute to the occurrence of late CMV disease.

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stitution of CD4+ and CD8+ HLA-restricted CMV-specific T-cell responses were evaluated in 47 allogeneic BMT recipients entered onto a double-blind randomized trial in which individuals received ganciclovir or placebo prophylaxis after recovery of peripheral neutrophil counts. The data show that the recovery of CD4+ and CD8+ CMV-specific T-cell responses early after BMT is more frequently observed in recipients of BM from CMV seropositive donors consistent with the transfer of primed T cells in the marrow inoculum contributing to CMV-specific immune recovery in these recipients. The results confirm previous studies showing that recovery of CD8+ CMV-specific CTL responses confers protection from CMV disease in BMT recipients not receiving ganciclovir prophylaxis. This observation is extended to show that the protective CD8+ CTL responses that develop after BMT are specific for structural virion proteins, similar to the immunodominant response maintained in healthy CMV-seropositive individuals.4 The data also show that patients receiving ganciclovir prophylaxis have delayed recovery of HLA-restricted CMV-specific T-cell responses including CD8+ CTL, a finding that may explain the cases of late-onset CMV disease observed in BMT and lung transplant recipients receiving ganciclovir prophylaxis.11,13,16

MATERIALS AND METHODS

Patient population. Forty-seven CMV seropositive patients undergoing allogeneic BMT at the Fred Hutchinson Cancer Research Center were randomly assigned to receive ganciclovir or placebo as prophylaxis for CMV infection. These patients represent a random sample of a larger number of patients entered onto a study evaluating the antiviral effects of ganciclovir prophylaxis. The results of the efficacy and toxicity of ganciclovir prophylaxis in the entire study group has been published.13 The study was approved by the Institutional Review Board and all patients gave informed consent. Patients were evaluated for recovery of CMV-specific T-cell responses between days 30 and 40, and between days 80 and 90 after transplant. In one patient in the ganciclovir group, the second evaluation was not done until day 125. Eight of the patients died or relapsed and one patient withdrew from the study before the first evaluation of CMV-specific T-cell responses. Four patients were studied for CMV-specific T-cell reconstitution, but at the time of randomization, were excluded from receiving the study drug because of renal failure (N = 2), or a positive pretransplant CMV culture (N = 2). The nonrandomized patients did not receive ganciclovir unless CMV disease developed. Thirty-four patients received the study drug with 15 receiving ganciclovir and 19 receiving placebo. The clinical characteristics of these patients are shown in Table 1. Prophylaxis for graft-versus-host disease (GVHD) was similar in both groups with the majority of patients receiving methotrexate and cyclosporine. The severity of acute GVHD was graded according to published criteria.21 The randomization code was not broken until the study was completed.

CMV-specific CTL generation. Short-term CMV-specific CTL lines were generated as previously described.4,22 Briefly, skin biopsies were obtained from each marrow donor to establish a fibroblast line for use as stimulator and target cells. Fibroblasts were propagated in Waymouth's media supplemented with 15% fetal calf serum, penicillin (50 U/mL), and streptomycin (50 μg/mL). Peripheral blood mononuclear cells (PBMC) were obtained from the BMT recipient and cultured at a responder to stimulator ratio of 20:1 with HLA-identical fibroblasts derived from the marrow donor and infected for 6 hours with AD169 strain CMV at a multiplicity of infection (MOI) of 5. Although the lymphocyte yield varied amongst patients, there was no significant difference in the mean number of lymphocytes between the placebo and ganciclovir groups. The T-cell culture media was RPM1 1640 supplemented with 10% human AB positive, CMV seronegative serum, 2 mmoL L glutamine, penicillin (50 U/mL), streptomycin (50 μg/mL), and 2.5 × 10−3 mol/L 2-mercaptoethanol. The T-cell cultures were stimulated again 7 days later with fresh CMV-infected fibroblasts and supplemented with autologous irradiated (3,300 rads) PBMC as feeder cells, and fed with recombinant interleukin-2 (IL-2) (2 U/mL), 2 and 10% human AB positive, CMV seronegative serum, 2 mmoL L glutamine, penicillin (50 U/mL), streptomycin (50 μg/mL), and 2.5 × 10−3 mol/L 2-mercaptoethanol. The T-cell cultures were stimulated again 7 days later with fresh CMV-infected fibroblasts and supplemented with autologous irradiated (3,300 rads) PBMC as feeder cells, and fed with recombinant interleukin-2 (IL-2) (2 U/mL), 2 and 4 days after restimulation. Fibroblasts express abundant class I HLA molecules, but little or no class II HLA molecules; thus, when used as stimulators, CD3+, CD8+, CD4+ class I-restricted CTL precursors present in PBMC are preferentially activated and expanded.14,15,23,24 Cytotoxicity assay. T-cell cultures were assayed for cytolytic activity in a 5-hour chromium release assay 6 to 7 days after restimulation as previously described.4,25 The panel of target cells used for each assay included HLA-identical and HLA class I-mismatched CMV-infected and mock-infected fibroblasts. Fibroblast targets were preincubated for 48 hours with 100 U/mL recombinant interferon γ (IFNγ) (Boehringer Mannheim, Indianapolis, IN), before infection with CMV at an MOI of 5, then labeled with Cr37 for 16 to 20 hours. The use of IFNγ pretreatment of target cells has been shown to increase the sensitivity of the assay for the detection of CD8+ CMV-specific CTL.25 CMV-infected fibroblasts are lysable by CD8+ CTL lines and clones, but do not express sufficient class II HLA molecules to elicit or be lysed by CD4+ class II HLA-restricted CMV-specific T cells,4 and unpublished data, November 1992). Spontaneous chromium release was determined from target cells incubated with media and maximum release from target cells incubated with 1% NP-40. In all experiments, spontaneous release was less than 25% of maximal release. Specific lysis was calculated by the standard formula. A CTL response in the patients was defined as positive if the cultures showed lysis of HLA identical CMV-infected targets at least 10%
greater than the level of lysis observed with HLA-identical mock-infected and HLA class I-mismatched CMV-infected and mock-infected target cells. This definition of a positive response is based on the results of cultures from six consecutive CMV seronegative normal individuals. These cultures from seronegative individuals all showed less than 2% greater lysis of autologous CMV-infected target cells than the lysis observed against either mock-infected or HLA-mismatched CMV-infected target cells (unpublished data, August 1993).

In a subset of recipients with demonstrable CD8+ CMV-specific CTL activity, the specificity of CTL responses for structural virion proteins was examined by assaysing for lysis of HLA-identical and HLA-mismatched target cells infected with CMV in the presence of actinomycin D as previously described. In selected patients where sufficient T cells were available after culture, the phenotype of the cytolytic effector cell was confirmed by depletion of CD4+ T cells or CD8+ T cells using tissue-culture flasks coated with either anti-CD4 or anti-CD8 monoclonal antibodies (MoAbs) (Applied Immune Sciences, Menlo Park, CA). This method results in 90% to 95% depletion of the selected T-cell subset (data not shown).

Lymphoproliferation assay. The CD4+ Th response of PBMC to soluble CMV antigen, a control antigen, and to phytohemagglutinin (PHA) (Difco Laboratories, Detroit, MI) was determined at the time CTL cultures were initiated. The coculture of PBMC with CMV antigen has been shown to selectively expand and expand CD4+ class II HLA-restricted T cells presumably because there is no cell in PBMC sufficiently permissive for CMV infection to present viral antigen via the class I antigen-processing pathway, but there is uptake and presentation of viral antigen by antigen-presenting cell via the class II pathway to elicit CD4+ Th proliferative responses.

Thus, depletion of CD4+ T cells before initiating the cultures or blocking with anti-class II HLA MoAbs abrogates or inhibits the proliferative response of PBMC from normal CMV seropositive individuals, (and unpublished data, November 1992). One hundred microliters of PBMC (1 × 10^6 cells) in T-cell culture media was dispensed in triplicate wells of 96-well round-bottom plates. Mock antigen, CMV and PHA were added at final concentrations of 1:200, 1:200, and 10 μg/mL respectively, whereas control triplicates received media alone. The cultures were pulsed with 1 μCi per well of H thymidine for the final 16 hours of a 96-hour incubation. Wells were harvested and incorporation of H thymidine determined by beta scintillation counting. Results are expressed as a stimulation index (SI) by dividing the mean counts per minute (CPM) incorporated by cells exposed to CMV or PHA by the mean CPM of cells exposed to control antigen. A stimulation index of greater than 1.5 was considered to indicate a positive lymphoproliferative response. This was determined by prior analysis of ten CMV seronegative individuals in whom the mean SI was 0.9 (range 0.5 to 1.2) with this CMV antigen preparation and identical culture conditions (unpublished data, November 1992 and August 1993).

Study drug administration. The study drug (ganciclovir or placebo) was initiated after recovery of the absolute neutrophil count to greater than 750/mm³ for 2 consecutive days. Ganciclovir was administered at a dose of 5 mg per kg twice daily for the first 7 days and then once daily until 100 days after transplant. The study drug was discontinued if the neutrophil count fell below 750/mm³ for 2 consecutive days.

Virologic surveillance and definition of CMV disease. Specimens were obtained weekly from the throat, urine, and blood and were examined for CMV both by conventional culture in which specimens were inoculated onto monolayers of human fibroblasts and by centrifugation culture. CMV pneumonia was defined as evidence of CMV in bronchoalveolar lavage associated with clinical signs and symptoms; CMV disease at other sites was defined by evidence of CMV in tissue sections associated with clinical signs and symptoms.

Statistical analysis. Comparisons of dichotomous variables were analyzed using Fisher's exact test.

RESULTS
Recovery of CD8+ and CD4+ CMV-specific T-cell responses at days 30 to 40 after BMT. To determine the kinetics of recovery of CMV-specific T-cell responses in patients entered onto this placebo-controlled study of ganciclovir prophylaxis, patients were analyzed on two occasions for the presence of CD8+ class I HLA-restricted and CD4+ class II HLA-restricted CMV-specific T-cell responses. Blood samples were first obtained and assayed between days 30 and 40 after the BMT. At this time all patients had evidence of marrow engraftment as defined by an absolute neutrophil count of greater than 750/mm³ and had been randomized to study drug or excluded from randomization according to the protocol. The mean day of initiating study drug was 24 (range 14 to 36). CD8+ class I HLA-restricted CMV-specific CTL responses were evaluated by stimulating PBMC with HLA-identical CMV-infected fibroblasts that express class I HLA, but not class II HLA molecules and selectively activate CD8+ CTL precursors. CD8+ CMV-specific CTL were detected in 4 of 19 (21%) and 4 of 15 (26%) patients randomized to placebo and ganciclovir respectively (Table 2). CTL responses were detected in 1 of 4 (25%) patients who were initially entered on study, but were not randomized at the time of engraftment because of renal failure or a positive pretransplant CMV culture.

In the eight patients randomized to ganciclovir or placebo and with detectable CTL responses, the cytolytic reactivity observed was class I HLA restricted as shown by lysis of HLA-identical CMV-infected cells, but not class I HLA-mismatched target cells (Fig 1). In a subset of these patients, selective depletion of either CD4+ T cells or CD8+ T cells confirmed that the effector cell responsible for lysis was CD8+ and not CD4+ (Fig 2). The mean lytic activity at an effector to target ratio of 15:1 was 32% ± 10.3% (range 25% to 49%) for ganciclovir recipients and 22% ± 5.8% for placebo recipients.
A. 40
30
20
10
0
Lysis

30
20
10
0
Lysis

Fig 1. Class I HLA-restricted cytotoxic T-cell responses in BMT recipients at day 30 to 40 after transplant. Cytotoxic T-cell responses were generated from peripheral blood lymphocytes isolated from recipients by stimulation with donor-derived HLA-identical fibroblasts as described in Materials and Methods. These cultures were assayed for recognition of HLA-matched CMV-infected (●) and mock-infected (□), and class I HLA-mismatched CMV-infected (□) and mock-infected (□) targets. Each recipient is identified by a unique patient number and the data are shown for each recipient at an effector to target ratio of 15:1. The data in (A) shows the responses observed in individuals randomized to placebo and the data in (B) show the responses in individuals randomized to ganciclovir.

(range 14% to 29%) for patients randomized to placebo (Fig 1).

At days 30 to 40, CD4+ CMV-specific Th responses were evaluated by stimulating PBMC with CMV antigen, which elicits CD4+ class II HLA-restricted Th responses.9,22 Responses were detected in 8 of 19 (42%) and 8 of 15 (53%) patients randomized to placebo and ganciclovir, respectively, and in 2 of the 4 (50%) nonrandomized patients (Table 2).

Positive Th responses were detected in all patients that had CD8+ CTL responses. The mean stimulation index for CMV-specific CD4+ Th responses in the placebo group was 10.1 ± 13.8 (range 1.6 to 45.1) and in the ganciclovir group was 13.2 ± 14.4 (range 1.8 to 46.8) (Table 2). Thirty-three of the 34 patients randomized to ganciclovir or placebo and 4 of 4 nonrandomized patients had a positive proliferative response to PHA with a mean stimulation index of 88.1 ± 64 (range 3.7 to 378) for placebo recipients, 99.1 ± 82.4 (range 2.1 to 294) for ganciclovir recipients, and 70.8 for nonrandomized patients. Thus, the similar numbers of patients recovering detectable CMV-specific immune responses in the ganciclovir and placebo groups shows that administration of ganciclovir after engraftment has no discernible impact on CMV-specific T-cell recovery when analyzed within 7 to 14 days after initiation of the drug.

Influence of marrow donor serology on early reconstitution of CMV-specific T-cell responses. The analysis at day 30 to 40 showed that early recovery of CMV-specific T-cell responses is observed in only a minor subset of patients. This may reflect the transfer of primed antigen-specific T cells in the marrow inoculum as has been shown for CD4+ Th responses to nonviral antigens.27 The recovery of CMV-specific CD8+ CTL and CD4+ Th responses at days 30 to 40 after BMT was analyzed in relationship to donor CMV serology for the 34 patients randomized to ganciclovir or
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Table 3. Influence of Donor CMV Serology on HLA-Restricted CMV-specific T-Cell Reconstitution at Day 30 to 40 After BMT

<table>
<thead>
<tr>
<th>Donor Serostatus</th>
<th>CMV Positive</th>
<th>CMV Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8+ CTL recovery</td>
<td>8/22 (36%)</td>
<td>1/16 (6%)</td>
</tr>
<tr>
<td>CD4+ Th recovery</td>
<td>14/22 (64%)</td>
<td>4/16 (25%)</td>
</tr>
</tbody>
</table>

Patients were evaluated between day 30 and 40 after BMT for the presence of CMV-specific CD8+ CTL and CD4+ Th responses as described in Materials and Methods. Donor CMV serologies were determined by enzyme immunoassay (Whittaker Bioproducts, Walkersville, MD). Statistical analysis comparing the proportions of patients with recovery of CD8+ CTL and CD4+ Th responses in relationship to donor CMV serology was done using Fisher's exact test.

placebo and the 4 patients who were not eligible for randomization. At this early time posttransplant, CD8+ CTL responses had recovered in 8/22 patients receiving marrow from a CMV seropositive donor compared with 1/16 patients receiving marrow from a CMV seronegative donor (P = .07) (Table 3). CD4+ Th responses had recovered in 14/22 recipients of marrow from a CMV seropositive donor compared with 4/16 recipients of marrow from a CMV seronegative donor (P = .04) (Table 3). Thus, donor CMV serology appears to be a major factor influencing the early detection of CMV-specific immune responses after BMT.

Influence of ganciclovir prophylaxis on CMV-specific T-cell recovery between days 40 and 90 after BMT. An effect of ganciclovir prophylaxis on CMV-specific T-cell reconstitution should be most discernible in patients without detectable responses at day 30 to 40. Thus, using identical culture conditions and target cells, CMV-specific T-cell responses were reevaluated between day 80 and 90 after BMT in the 29 survivors. Overall, at this later time, 10/16 (63%) placebo patients and 5/13 (39%) ganciclovir patients had measurable CD8+ CMV-specific CTL responses with 12/16 (75%) placebo recipients and 9/13 (69%) ganciclovir recipients having CD4+ CMV-specific Th responses. All patients that were positive for CTL and Th at the day 30 through 40 evaluation retained these responses at the day 90 evaluation and patients with CD8+ CTL responses had preexisting or concurrent recovery of CD4+ Th.

Twelve of the 15 placebo patients and 9 of the 11 ganciclovir recipients that were deficient in CD8+ CTL at day 40 were alive and reevaluated at day 90. Six of the 12 placebo recipients, but only 1 of the 9 ganciclovir recipients had reconstituted CD8+ CMV-specific CTL at reevaluation (Table 4). The patient in the ganciclovir group that showed recovery of CD8+ CTL did not have ganciclovir therapy interrupted and did not excrete CMV on surveillance cultures. This patient had received marrow from a CMV seropositive donor and showed a positive CD4+ CMV-specific Th response at the initial evaluation. Eight of the 11 placebo and 5 of the 7 ganciclovir recipients that were deficient in CD4+ Th responses were alive and reevaluated at day 90. Five of the 8 placebo recipients and 1 of the 5 ganciclovir recipients reconstituted CMV-specific Th responses between the first and second evaluation (Table 4). The one patient with recovery of CD4+ Th had ganciclovir discontinued at day 75 and was not evaluated until day 125, and thus, this recovery may have reflected a T-cell response to reactivation of CMV occurring in the interval off ganciclovir.

These results show a strong correlation between ganciclovir prophylaxis and failure to recover both CD8+ CTL and CD4+ Th CMV-specific T-cell responses between day 40 and 90 after BMT. Perhaps because of the small numbers of patients on this study, the difference between ganciclovir and placebo recipients in recovery of CD4+ Th or CD8+ CTL responses during the day 40 to 90 interval did not achieve statistical significance when each response was analyzed as an isolated variable (Table 4). However, if the total number of CMV-specific T-cell responses (ie, CD4+ Th plus CD8+ CTL) that recovered between day 40 and 90 in placebo patients is compared with the total number of T-cell responses recovering in ganciclovir recipients the differences between the two groups is significant. Eleven of 20 deficient CMV-specific T-cell responses recovered between the first and second evaluation in individuals receiving placebo compared with 2 of 14 deficient responses in those receiving ganciclovir (P = .04) (Table 4).

Influence of graft-versus-host disease on endogenous CMV-specific T-cell reconstitution. GVHD has previously been shown to be a risk factor for CMV disease, and by inference would be associated with delayed reconstitution of CMV-specific T-cell responses. We evaluated the effect of GVHD on reconstitution of CMV-specific immune re-

Table 4. Recovery of CMV-Specific CD8+ CTL and CD4+ Th Responses at Day 80 to 90 After BMT in Patients Deficient in These Responses at Day 40

<table>
<thead>
<tr>
<th>Recovery of a CMV-specific T-cell response</th>
<th>Placebo</th>
<th>Ganciclovir</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8+ CTL response*</td>
<td>6/12(50%)</td>
<td>1/9 (11%)</td>
</tr>
<tr>
<td>CD4+ Th response*</td>
<td>5/8 (63%)</td>
<td>1/5 (20%)</td>
</tr>
<tr>
<td>No. of deficient CMV-specific Th responses recovering#</td>
<td>11/20 (55%)</td>
<td>2/14 (14%)</td>
</tr>
</tbody>
</table>

* The presence of a CD8+ CMV-specific CTL response in PBMC at day 80 to 90 was evaluated in patients randomized to ganciclovir or placebo using the CTL generation assay described in Materials and Methods.

† The presence of a CD4+ CMV-specific Th response in PBMC at day 80 to 90 was evaluated in patients randomized to placebo or ganciclovir using the lymphoproliferation assay described in Materials and Methods.

In (* and †), the numerator represents the number of patients that recovered the response, and the denominator represents the total number of patients who were deficient in the response at the day 30 to 40 evaluation and were reevaluated after day 80.

‡ The total number of deficient CMV-specific immune responses that recovered in placebo patients and the total number of deficient CMV-specific immune responses that recovered in ganciclovir recipients. The numerator is the number of CMV-specific T-cell responses (CD4+ Th plus CD8+ CTL) that recovered between day 40 and 90 and the denominator is the total number of T-cell responses that were deficient at day 40.
Table 5. Effect of GVHD Grade on Reconstitution of CMV-Specific CD8+ CTL and CD4+ Th Responses at Day 90

<table>
<thead>
<tr>
<th>Recovery of CD8+ CTL</th>
<th>Grade 0 to 1 GVHD</th>
<th>Grade 2 to 4 GVHD</th>
<th>Grade 0 to 1 GVHD</th>
<th>Grade 2 to 4 GVHD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery of CD4+ Th</td>
<td>10/14</td>
<td>5/15</td>
<td>P = .10</td>
<td>P = .01</td>
</tr>
<tr>
<td>Recovery of both CD8+</td>
<td>14/14</td>
<td>7/15</td>
<td>P = .01</td>
<td>P = .01</td>
</tr>
</tbody>
</table>

CMV-specific T-cell responses were analyzed as described in Materials and Methods. GVHD was graded by standard criteria,21 and the maximum grade of GVHD developing during the first 90 days post-transplant was used for data analysis.

of the 7 placebo recipients tistically significant by day 40 and grade 0 to 1 GVHD reconstituted both CD8+ and CD4+ CTL and CD4+ Th responses in the day 40 to 90 interval in the patients with grade 0 to 1 GVHD (10/14), than in patients with grade 2 to 4 GVHD (5/15), although this was not statistically significant (P = .10).

Sixteen of the 29 patients evaluated through day 90 received placebo and 13 received ganciclovir. GVHD occurred with similar frequency and severity in both arms of the study with 8 of 16 placebo recipients and 6 of 13 ganciclovir recipients developing grade 0 to 1 GVHD. Seven of the 8 placebo recipients and 3 of the 6 ganciclovir recipients with grade 0 to 1 GVHD reconstituted both CD8+ and CD4+ CMV-specific T-cell responses. Of the 7 placebo recipients with grade 0 to 1 GVHD and complete reconstitution of CMV-specific T-cell responses, 3 recovered these responses by day 40 and 4 recovered the responses in the interval between day 40 and 90. All 3 of the ganciclovir recipients with grade 0 to 1 GVHD and complete recovery of CMV-specific T-cell responses showed recovery by day 40, and no recovery was observed in the day 40 to 90 interval in the other 3 patients. Of the 8 remaining placebo recipients and 7 ganciclovir recipients with grade 2 to 4 GVHD, complete recovery of CMV-specific T-cell responses was observed in 3 placebo and 2 ganciclovir recipients, respectively. Although these numbers are clearly too small for a meaningful statistical analysis, the failure of 3 ganciclovir patients with grade 0 to 1 GVHD to reconstitute CMV-specific T-cell immunity suggests that GVHD is not the sole factor explaining the failure of CMV-specific immune recovery in this group of patients.

Virologic monitoring of ganciclovir and placebo patients. The development of antigen-specific T-cell responses after BMT can reflect the transfer of memory T cells from the marrow donor, but in circumstances where these cells are transferred in small numbers or where the donor has not been primed to the respective antigen, in vivo stimulation is likely to be required for expansion of specific T-cell precursors. Thus, recovery of T-cell responses to CMV may require reactivation of virus in vivo and stimulation of CMV-specific T-cell precursors. The results of weekly cultures for CMV provide support for the hypothesis that the delay in reconstitution of CMV-specific T-cell responses in patients receiving ganciclovir may reflect a lack of viral antigen stimulation. Five of the six placebo patients with recovery of CD8+ CMV-specific CTL responses after day 40 had culture evidence from throat, blood, or urine of CMV reactivation, but none of the ganciclovir recipients had positive cultures. Thus, these results suggest that the delayed recovery of CMV-specific T-cell immunity in patients receiving ganciclovir prophylaxis is caused by effective suppression of virus replication during the period of drug administration.

Correlation of CMV-specific T-cell responses and CMV disease occurrence. CMV disease occurred in 8 of the 34 patients randomized to either ganciclovir or placebo and 3 of 4 nonrandomized patients. Seven of these 11 patients developed CMV interstitial pneumonia and 4 patients developed CMV enteritis as the initial manifestation of CMV disease. Two of the four patients with CMV enteritis subsequently developed CMV pneumonia. Of the 11 cases of CMV disease, 2 occurred in patients in the ganciclovir arm and both were pneumonia. These cases occurred at days 125 and 153 after BMT and in both patients ganciclovir had been discontinued before or on day 100. The median day of onset of CMV disease in the patients not receiving ganciclovir prophylaxis was day 48 (range 43 to 74), which is consistent with the median of day 56 observed in prior studies. None of the 11 patients developing CMV disease had CD8+ CMV-specific CTL detected in the peripheral blood in the assay before the development of CMV disease or when reevaluated with 2 days of the establishment of the diagnosis of CMV disease. However, 3 of these 11 patients had detectable CD4+ CMV-specific Th responses although the stimulation indices were low (mean 2.6, range 1.6 to 4.7). Sixteen of the 38 patients reconstituted CD8+ CMV-specific CTL responses during the course of this study, and none of these patients developed CMV disease subsequent to the endogenous recovery of CD8+ CTL. The association of a positive CD8+ CTL response and protection from CMV disease was highly significant (P = .005) and substantiates previous findings in a smaller study showing the correlation of deficient class I HLA-restricted CMV-specific CD8+ CTL responses with the occurrence of CMV disease. Despite the occurrence of CMV disease in a small number of patients with CD4+ Th responses, overall the reconstitution of lymphoproliferative responses to CMV was associated with protection from CMV disease (P = .01), consistent with the requirement for CD4+ Th for the in vivo generation of CD8+ CTL.

Specificity of CD8+ CMV-specific CTL responses developing in BMT recipients. Although CMV encodes a large number of proteins that are potential target antigens, the host CTL response effective in controlling persistent infection in seropositive individuals is specific for a small number of viral proteins. The dominant target antigens recognized are structural viral proteins and the majority of CTL clones iso-
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Many patients reactivated HSV when acyclovir was discontinue in studies evaluating acyclovir for HSV infection.29 A similar approach using the nucleoside analog ganciclovir as prophylaxis for CMV infection in immunocompromised hosts has reduced the incidence of CMV disease, but late-onset disease is observed with increased frequency.11,12 Moreover, the mortality rate for patients with late-onset CMV pneumonia at our center is 70% (M. Boechk, personal communication, December 1993).

In immunocompetent hosts, strong HLA-restricted CMV-specific T-cell responses are maintained to control reactivation of CMV infection.13,14 In BMT recipients CD8+ CTL are required for complete protection from CMV disease and the recovery of CD4+ CMV-specific lymphoproliferative responses is obligatory for the endogenous reconstitution of CD8+ CTL.5,9 The occurrence of late CMV disease in patients receiving ganciclovir as early treatment for CMV infection suggests that the recovery of protective CMV-specific T-cell responses may be delayed in patients receiving this antiviral agent. The analysis of the kinetics of endogenous reconstitution of CD4+ and CD8+ CMV-specific T-cell responses in patients entered onto a prospective randomized placebo-controlled study of ganciclovir prophylaxis has identified that marrow donor serology has an important influence on the early detection of virus-specific T-cell responses. The results evaluating later reconstitution at day 90 also support the hypothesis that effective antiviral prophylaxis may impact CMV-specific T-cell recovery in those individuals who were deficient in these responses at day 30 to 40. Between days 40 and 90, a higher proportion of patients receiving placebo recovered CD8+ CTL and/or CD4+ Th responses and this was not explained by more severe GVHD in the ganciclovir patients. Thus, a result of using ganciclovir prophylaxis is that a larger fraction of patients are deficient in CMV-specific CD4+ proliferative and CD8+ cytotoxic responses at day 100 when ganciclovir is discontinued. Consistent with this, two cases of CMV pneumonia occurred after day 100 in patients who had received ganciclovir prophylaxis and had not recovered protective CD8+ CMV-specific CTL.

Ganciclovir may contribute to the delay in recovery of CMV-specific T-cell responses by several mechanisms. Ganciclovir inhibits mitogen- and antigen-induced T-cell proliferation in vitro because of effects on cellular DNA synthesis.18 These effects are most significant at high in vitro concentrations of the drug and may be of limited relevance in vivo. This effect would be nonselective and should result in a general depression of T-cell immunity in patients receiving the drug. The equivalent responses to PHA observed in ganciclovir and placebo recipients in this study are against such a general effect on T-cell function. The results do support an alternative mechanism for the delay in recovery of CMV-specific T-cell responses in ganciclovir recipients—sufficient suppression of CMV replication to preclude in vivo priming and expansion of CMV-specific T-cell precursors.

A focus of current clinical trials is to determine parameters that predict which immunosuppressed patients are at highest risk of CMV reactivation, and to develop strategies to correct parameters that predict which immunosuppressed patients are at highest risk of CMV reactivation, and to develop strategies to correct these parameters at an earlier time point.

Discussion

The strategy of using an antiviral drug as prophylaxis for infection caused by reactivation of latent or persistent viruses in immunosuppressed hosts was initially shown to be effective in studies evaluating acyclovir for HSV infection.29 Many patients reactivated HSV when acyclovir was discontinue, and analysis of CD4+ HSV-specific lymphoproliferative responses showed that treatment with acyclovir delayed the recovery of these responses.30 A similar approach using the nucleoside analog ganciclovir as prophylaxis for CMV infection in immunocompromised hosts has reduced the incidence of CMV disease, but late-onset disease is observed with increased frequency.11,12 Moreover, the mortality rate for patients with late-onset CMV pneumonia at our center is 70% (M. Boechk, personal communication, December 1993).

In immunocompetent hosts, strong HLA-restricted CMV-specific T-cell responses are maintained to control reactivation of CMV infection.13,14 In BMT recipients CD8+ CTL are required for complete protection from CMV disease and the recovery of CD4+ CMV-specific lymphoproliferative responses is obligatory for the endogenous reconstitution of CD8+ CTL.5,9 The occurrence of late CMV disease in patients receiving ganciclovir as early treatment for CMV infection suggests that the recovery of protective CMV-specific T-cell responses may be delayed in patients receiving this antiviral agent. The analysis of the kinetics of endogenous reconstitution of CD4+ and CD8+ CMV-specific T-cell responses in patients entered onto a prospective randomized placebo-controlled study of ganciclovir prophylaxis has identified that marrow donor serology has an important influence on the early detection of virus-specific T-cell responses. The results evaluating later reconstitution at day 90 also support the hypothesis that effective antiviral prophylaxis may impact CMV-specific T-cell recovery in those individuals who were deficient in these responses at day 30 to 40. Between days 40 and 90, a higher proportion of patients receiving placebo recovered CD8+ CTL and/or CD4+ Th responses and this was not explained by more severe GVHD in the ganciclovir patients. Thus, a result of using ganciclovir prophylaxis is that a larger fraction of patients are deficient in CMV-specific CD4+ proliferative and CD8+ cytotoxic responses at day 100 when ganciclovir is discontinued. Consistent with this, two cases of CMV pneumonia occurred after day 100 in patients who had received ganciclovir prophylaxis and had not recovered protective CD8+ CMV-specific CTL.

Ganciclovir may contribute to the delay in recovery of CMV-specific T-cell responses by several mechanisms. Ganciclovir inhibits mitogen- and antigen-induced T-cell proliferation in vitro because of effects on cellular DNA synthesis.18 These effects are most significant at high in vitro concentrations of the drug and may be of limited relevance in vivo. This effect would be nonselective and should result in a general depression of T-cell immunity in patients receiving the drug. The equivalent responses to PHA observed in ganciclovir and placebo recipients in this study are against such a general effect on T-cell function. The results do support an alternative mechanism for the delay in recovery of CMV-specific T-cell responses in ganciclovir recipients—sufficient suppression of CMV replication to preclude in vivo priming and expansion of CMV-specific T-cell precursors.

A focus of current clinical trials is to determine parameters that predict which immunosuppressed patients are at highest risk of CMV reactivation, and to develop strategies to correct these parameters at an earlier time point.

Table 6. Endogenously Recovering CD8+ CMV-Specific CTL in BMT Recipients Recognize CMV-Infected Cells in the Absence of Viral Gene Expression

<table>
<thead>
<tr>
<th>Target Cell (% Lysis)</th>
<th>HLA Identical</th>
<th>MHC Mismatched</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPN</td>
<td>E/T</td>
<td>CMV</td>
</tr>
<tr>
<td>5937</td>
<td>5:1</td>
<td>42</td>
</tr>
<tr>
<td>20:1</td>
<td>61</td>
<td>62</td>
</tr>
<tr>
<td>6213</td>
<td>5:1</td>
<td>35</td>
</tr>
<tr>
<td>20:1</td>
<td>62</td>
<td>60</td>
</tr>
<tr>
<td>5950</td>
<td>5:1</td>
<td>22</td>
</tr>
<tr>
<td>28:1</td>
<td>29</td>
<td>32</td>
</tr>
<tr>
<td>6204</td>
<td>5:1</td>
<td>22</td>
</tr>
<tr>
<td>20:1</td>
<td>28</td>
<td>30</td>
</tr>
</tbody>
</table>

Polyclonal CD8+ CTL lines were generated from PBL obtained from BMT recipients. UPN 5937 and 6213 had received placebo whereas UPN 5937 and 6204 had received ganciclovir. Three patients, UPN 5937, 6213, and 6204, first showed CD8+ CTL recovery on the day 30 to 40 evaluation, whereas UPN 8204 did not show CD8+ CTL recovery until the evaluation at day 80 to 90. The CTL cultures were assayed 12 to 14 days after initiation for cytolytic reactivity against Cr+ labeled HLA-identical and -mismatched fibroblasts either mock infected (Mock), infected with CMV for 16 hours (CMV), or infected with CMV for 4 hours in the presence of the transcriptional inhibitor actinomycin D (CMV/ActD).
risk for CMV disease. This would permit ganciclovir therapy to be selectively utilized and minimize ganciclovir-related hematopoietic toxicity, which is observed in 30% of patients receiving ganciclovir prophylaxis and is associated with an increased risk of bacterial infections.\textsuperscript{13,31} The results of our study suggest that evaluating patients for recovery of CD8\textsuperscript{+} CMV-specific CTL responses would identify those patients that have reconstituted these responses and do not need ganciclovir prophylaxis. This approach would eliminate 25% of patients from consideration for prophylactic ganciclovir by day 30 to 40 after BMT. A similar evaluation of patients beyond day 100 may help guide the clinical use of ganciclovir late after BMT. Such an approach should reduce the toxicities incurred with long-term ganciclovir therapy while ensuring that immunocompromised patients still at risk of CMV disease are identified.

An alternative strategy to prevent CMV infection after allogeneic BMT is to reconstitute protective immune responses by the adoptive transfer to the BMT recipient of CD8\textsuperscript{+} CMV-specific T-cell clones derived from the BM donor and propagated by in vitro culture.\textsuperscript{32} In a previous study, we reported that this therapy was not associated with any toxicity and resulted in the selective and persistent reconstitution of CMV-specific CTL responses in all treated patients.\textsuperscript{10} The CD8\textsuperscript{+} CMV-specific CTL isolated from healthy CMV seropositive BM donors are predominantly specific for structural viral proteins processed and presented by infected cells without the requirement for viral gene expression.\textsuperscript{4} The epitopes recognized by CTL are conserved in genetically distinct clinical isolates of CMV including ganciclovir-resistant strains (S.R. Riddell, unpublished data, August 1992). Thus, adoptively transferred CTL may provide protection against the ganciclovir-resistant CMV strains that are increasingly identified in immunosuppressed hosts receiving ganciclovir.\textsuperscript{32-35} In this report, we have extended the observation that recovery of CD8\textsuperscript{+} CTL after BMT correlates with protection from CMV disease to show that the specificity of endogenously recovering CTL is predominantly directed at structural virion proteins. Thus, the nature of the protective responses that develop after BMT appear to be the same as those maintained in healthy seropositive individuals. This further affirms that adoptive immunotherapy with T-cell clones derived from the donor to reconstitute immunity in the recipient may be particularly useful in selected clinical circumstances where prolonged immunodeficiency is predicted and ganciclovir therapy is poorly tolerated.

REFERENCES

Recovery of HLA-restricted cytomegalovirus (CMV)-specific T-cell responses after allogeneic bone marrow transplant: correlation with CMV disease and effect of ganciclovir prophylaxis

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