Cytomegalovirus-Specific Lymphocyte Proliferation and In Vitro Cytomegalovirus IgG Synthesis for Diagnosis of Cytomegalovirus Infections After Bone Marrow Transplantation

By Per Ljungman, Berit Lönqvist, Gösta Gahrton, Olle Ringdén, and Britta Wahren

With new techniques 19 bone marrow transplant (BMT) recipients were monitored for lymphocyte proliferation and specific IgG production in vitro by cytomegalovirus (CMV) antigen in solid phase. Twelve patients got a reactivated CMV infection as defined by virus isolation or serum IgG conversion. Lymphocyte proliferation and in vitro IgG production responses were significantly stronger in these 12 patients than in seven without ongoing CMV infection (P = .02). CMV infection was indicated by the lymphocyte responses at a mean of 45 days after BMT as against a mean of 79 days that passed before CMV growth in culture was detected (P < .05). Lymphocyte proliferation and in vitro IgG production may thus be used as tools for diagnosis and for monitoring of CMV infections in BMT recipients.

Cytomegalovirus (CMV) infection is a major problem after allogeneic bone marrow transplantation (BMT). Despite antiviral therapy mortality in CMV, pneumonia exceeds 80%. Treatment with a new antiviral drug, phosphonoformic acid (foscarnet), is reportedly effective in BMT patients with encephalitis and pancytopenia and in renal transplant recipients with pneumonia. In a few cases it has also been effective in BMT recipients with CMV pneumonia. An early diagnosis of CMV infection is therefore important in these patients. CMV isolations frequently give positive results, but it may take several weeks for a cytopathic effect to appear in culture. Demonstration of early antigens after short-term culture is feasible, but in our experience may be less sensitive than the classic isolation procedure. Specific IgG and IgM determinations in serum are of minor value for the diagnosis of CMV infections, since in reactivated disease of immunocompromised patients antibody increases often appear late or not at all.

The question whether immunity to herpesviruses is transferred from the donor or persists in the recipient has been addressed. Wahren et al found that the humoral reactivity to viral antigens may either be transferred with donor B cells at BMT or persist among host cells for varying times after BMT. Although engraftment may occur, usually neither long-term transfer nor persistence of herpesvirus-reactive T lymphocytes is seen. The cells that mediate the lymphocyte proliferation response to CMV antigen are mainly T cells of the helper/inducer (T4) phenotype (ref 13 and our own unpublished results). Lymphocyte proliferation to all types of antigens, including herpesvirus antigens, is strongly depressed early after BMT. Together, these findings indicate that a CMV reactivation should be regarded as a new primary infection by the immune system of the BMT recipient.

New techniques have been developed for measuring specific lymphocyte proliferation to CMV antigen and specific lymphocyte IgG production in vitro. The lymphocytes are exposed to the CMV antigen in solid phase. These techniques were found to be specific and sensitive indicators of reactivity to CMV in healthy individuals. They were found to be useful indicators of CMV infection in BMT recipients.

MATERIALS AND METHODS

Patients. The BMT procedure has been described. Out of 20 patients, there were three instances of aplastic anemia, one T cell defect with hypogammaglobulinemia, five acute nonlymphoblastic leukemias (one in first remission, four in partial remission), six acute lymphoblastic leukemias (two in first and four in second remission), one multiple myeloma, and four chronic granulocytic leukemias (two in first chronic phase, one in accelerated phase, and one in second chronic phase (Table 1). All received grafts from HLA-identical, MLC-nonreactive sibling donors. The study was approved by the ethical committee at Huddinge Hospital, and informed consent was obtained from all patients before grafting. The four patients with nonmalignant disease received cyclosporine as prophylaxis against acute graft-versus-host disease (GVHD). The patients with malignant diseases were randomized between cyclosporine (n = 10) and methotrexate (n = 6). Cyclosporine therapy was started on day 1 with 5.0 mg/kg/d intravenously (IV) followed by 12.5 mg/kg/d orally in two divided doses when oral medication was tolerated. Acute GVHD was graded according to Thomas et al. Chronic GVHD was defined according to Shulman et al and Schubert et al and was verified by biopsies.

Eleven cases in which donor and/or recipient were CMV seropositive before BMT were considered to run a high risk of CMV infection developing. They were included in the randomized study for either prophylactic treatment with CMV-hyperimmune plasma or no such treatment. Lymphocytes from these 16 patients were studied weekly from the time the white cell count reached 1.0 x 10⁹ cells/L after the initial hypoplasia until 12 weeks after BMT. Thereafter, tests were performed monthly. Four recipients who were seronegative and had seronegative donors were regarded as a low-risk group for CMV infection developing. All these patients received CMV antibody-negative blood products.

Thirteen out of the 16 patients in the high-risk group and all four in the low-risk group survived the first 3 months after BMT. One patient died of a leukemic relapse on day 37 after BMT, and two died of a CMV pneumonitis on day 70 and 75 respectively. After 3 months, one patient died on day 108 of septicemia and one on day 120 of a pneumonitis caused by Pneumocystis carinii. Acute GVHD of grade III to IV developed in five patients, grade II in five, and grade I in five. All patients with acute GVHD were treated with prednisone, with an initial dose of 2 mg/kg bodyweight, and seven
Table 1. Clinical Characteristics, CMV Infections, Lymphocyte Proliferation, and IgG Production Responses in BMT Recipients

<table>
<thead>
<tr>
<th>UPN</th>
<th>Sex/ Age</th>
<th>Diagnosis/ Remission</th>
<th>CMV Serologic Status</th>
<th>Acute GVHD (Grade)</th>
<th>Chronic Isolation</th>
<th>CMV Symptoms</th>
<th>CMV Specific Lymphocyte Proliferation</th>
<th>IgG Production (A410)</th>
<th>Death (Day)</th>
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<tbody>
<tr>
<td>L096</td>
<td>M 5</td>
<td>ALL 2nd</td>
<td>pos neg</td>
<td>III* no</td>
<td>U + B fever</td>
<td></td>
<td></td>
<td>1,400</td>
<td>0.2</td>
</tr>
<tr>
<td>L100</td>
<td>F 37</td>
<td>CGL 1st</td>
<td>pos pos</td>
<td>IV* no</td>
<td>B + L + H pneum</td>
<td></td>
<td></td>
<td>600</td>
<td>1.6</td>
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<tr>
<td>L099</td>
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<td>ANLL 1st</td>
<td>pos pos</td>
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<td></td>
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<td>55,000</td>
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<tr>
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<td>AA</td>
<td>neg pos</td>
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<td>U none</td>
<td></td>
<td></td>
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<td>12,400</td>
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<tr>
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<td>pos pos</td>
<td>II† yes</td>
<td>B + U + BM panc, fever</td>
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<td>65,000</td>
<td>0.8</td>
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<tr>
<td>L098</td>
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<td>ALL 2nd</td>
<td>pos pos</td>
<td>II† yes</td>
<td>B panc, fever</td>
<td></td>
<td></td>
<td>0</td>
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</tr>
<tr>
<td>A017</td>
<td>M 11</td>
<td>AA</td>
<td>pos pos</td>
<td>II† yes</td>
<td>B + U panc</td>
<td></td>
<td></td>
<td>0</td>
<td>14,000</td>
</tr>
<tr>
<td>L104</td>
<td>M 34</td>
<td>CGL acc</td>
<td>pos pos</td>
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<td>B pneum</td>
<td></td>
<td></td>
<td>500</td>
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<tr>
<td>L106</td>
<td>M 32</td>
<td>MM</td>
<td>pos pos</td>
<td>II† no</td>
<td>B + U + BM none</td>
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<td></td>
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<td>L107</td>
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<tr>
<td>L108</td>
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<td>ALL 2nd</td>
<td>pos pos</td>
<td>II† no</td>
<td>U panc</td>
<td></td>
<td></td>
<td>700</td>
<td>12,000</td>
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<tr>
<td>L103</td>
<td>F 19</td>
<td>ANLL PR</td>
<td>pos neg</td>
<td>IV* no</td>
<td>0 none</td>
<td></td>
<td></td>
<td>300</td>
<td>400</td>
</tr>
<tr>
<td>L101</td>
<td>F 37</td>
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<td>I† no</td>
<td>0 none</td>
<td></td>
<td></td>
<td>0</td>
<td>200</td>
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</tbody>
</table>

Abbreviations: UPN, unique patient number; ANLL, acute nonlymphocytic leukemia; ALL, acute lymphoblastic leukemia; CGL, chronic granulocytic leukemia; acc, accelerated phase; AA, aplastic anemia; MM, multiple myeloma; T cell def, T cell defect; PR, partial remission; B, blood; U, urine; L, lung tissue at autopsy; BM, bone marrow; BR, bronchioalveolar lavage; H, liver at autopsy; O, CMV not isolated; panc, pancytopenia; pneum, pneumonitis.

| *ATG, prednisone, and methylprednisolone. | †Prednisone. |

Patients received high-dose methylprednisolone and/or antithymocyte globulin in addition (ATG, Table 1).

Diagnosis of CMV disease. The diagnosis of a CMV infection was made by virus isolation on human lung (HL) fibroblasts. Samples for CMV isolation were taken weekly from urine and blood cells during the first 12 weeks after BMT and later at least monthly. CMV isolation attempts were also made on bone marrow and on material obtained from bronchoscopies and liver biopsies and on autopsy material. CMV IgG and IgM antibodies in serum were determined weekly by enzyme-linked immunosorbent assay (ELISA). The following serologic criteria were considered significant for a CMV infection: specific IgM production, CMV IgG seroconversion, or a fivefold specific IgG increase. In patients receiving CMV-hyperimmune plasma transfusions, these were considered in the evaluation of the IgG titer rises.

Viral antigens. CMV nucleocapsid and CMV membrane antigens were prepared from HL cells infected with CMV Ad 169 as described. Control antigens were prepared from uninfected HL cells. Lymphocyte proliferation was studied using CMV nucleocapsid antigen because the lymphocytes of healthy CMV-seropositive persons responded better to CMV nucleocapsid than to CMV membrane antigen. CMV membrane antigen was used for stimulation of in vitro CMV IgG production because IgG synthesis in vitro was better with the membrane than with the nucleocapsid antigen. For control purposes, lymphocyte assays were made with herpes simplex virus (HSV) antigens and varicella zoster virus (VZV) antigens. These tests were sometimes precluded by a lack of cells.

Proliferation technique. Coating of 96-well microplates (Nunc Immunoplate I, Nunc, Roskilde, Denmark) was performed overnight at room temperature with 100-μL volumes (1 μg viral protein) of CMV nucleocapsid antigen. Before use, the plates were washed three times with saline containing 0.05% Tween 20. Patient lymphocytes were prepared from heparinized blood by centrifugation on Lymphoprep (Nyegaard & Co, Oslo). After washing, the lymphocytes were suspended in RPMI 1640 supplemented with 10% CMV antibody-negative AB+ human serum, and 1.5 x 10⁶ cells in 200 μL of suspension were dispersed into two antigen-coated plates. After three and five days, 1 μCi of 3H-thymidine was added. Twenty four hours later the cells were harvested (Titertec, Skatron, Lierbyen, Norway), and the activity was measured in a scintillation counter. Each test was performed in triplicate. A net cpm was calculated as the mean cpm from stimulated cultures minus the mean cpm from control antigen-stimulated cultures. The control antigen was non-stimulatory for the lymphocytes (mean cpm < 800, Fig 1). A net cpm of >1,000 is considered specific for DNA synthesis and indicates that the patient has had a CMV infection. A net cpm rise of <1,000 or a more than tenfold rise in the net cpm from a previous test was considered specific for ongoing reactivated CMV infection.

Lymphocyte-produced CMV IgG and IgM determination by ELISA. Microplates were coated with 100-μL volumes (1 μg of viral protein) of CMV membrane antigen. The lymphocytes were suspended in RPMI 1640 with 20% fetal calf serum, and 2.0 x 10⁴ cells were dispersed in duplicate into the antigen-coated plates. After 48 hours the plates were washed three times with saline containing
antihuman IgM (Orion Diagnostika, Helsinki, Finland) was added conjugated goat antihuman IgG (Sigma Chemical Co, St Louis) or 0.05% Tween 20. After washing, 100 μL of alkaline phosphatase-conjugated CMV isolation and total specific IgG titers. Loss of responses considered to be specific for CMV IgG production. A 410 value of cultures stimulated with control antigen. An A 410 rise of 0.4 (0.4 is twice the background level of each assay) was compared with CMV infection compared with CMV isolation and total specific IgG titers. Loss of responses was followed by a new episode of CMV excretion and a second rise of the proliferation response.

0.05% Tween 20. After washing, 100 μL of alkaline phosphatase-conjugated goat antihuman IgG (Sigma Chemical Co, St Louis) or antihuman IgM (Orion Diagnostika, Helsinki, Finland) was added and the plate incubated for two hours at 37 °C. Then, 100 μL of the substrate p-nitrophenyl phosphate (1 mg/mL, Sigma) was added at room temperature, and 30 minutes later the reaction was terminated with 2.5 mol/L NaOH. The absorbance (A) at 410 nm was measured in a Dynatech MR 600 (Arlington, Va). The tests were performed in duplicate. Net absorbance was calculated as the mean A 410 value from CMV-stimulated lymphocytes minus the mean A 410 value of cultures stimulated with control antigen. An A 410 rise of ≥0.4 (0.4 is twice the background level of each assay) was considered to be specific for CMV IgG production.

RESULTS

CMV infections. CMV infection developed in 12 of the 16 high-risk patients. Fifteen were confirmed by CMV isolations. Three of them also had serum CMV IgG titer rises. One patient had a CMV IgG seroconversion. None had detectable (titer <50) serum CMV IgM. CMV pneumonitis developed in three patients and two died. The third patient was treated with phosphonoformic acid (foscarnet) and survived the pneumonitis but later died of sepsis. Five patients with CMV infection had pancytopenia and/or fever. Three of the high-risk patients had neither clinical signs of CMV disease nor virologic evidence of a CMV infection. One patient was not evaluable because virus isolations were not adequately sampled. No CMV infection developed in any of the low-risk patients.

Lymphocyte proliferation responses. CMV-specific lymphocyte proliferation responses developed in ten of 12 patients with CMV infections (minimum values for previous tests and maximum cpm values for each patient are shown in Table 1). Eight of these patients had a net cpm of more than 10,000 and all had a more than tenfold rise in the net cpm compared with a previous test (Table 1, Fig 1). None of the seven patients without CMV infection had a net cpm of more than 3,400 (Table 1, Fig 2). Table 2 shows a significant difference in the mean responses between the two patient groups (P <0.02 by the two-tailed Mann-Whitney U test).

The proliferation responses rapidly rose to a peak value, and during follow-up the responses usually diminished (Fig 1). In three patients, lymphocyte proliferation responses before treatment with ATG (net cpm of 148,000, 48,000, and 8,000, respectively) became deeply depressed after this treatment (net cpm <1,000). In three out of four patients in whom chronic GVHD developed, a strong CMV-specific proliferation response at the diagnosis of CMV infection was lost during follow-up.

The strong proliferation responses occurred specifically in response to infection. In every case where we found a net cpm of >10,000 after BMT against any herpesvirus antigen (CMV, HSV, VZV), a virus reactivation was confirmed by other means.

Two patients had a herpesvirus antigen-stimulated net cpm of between 5,000 and 10,000 the first weeks after marrow take. This was followed by a rapid decrease. One of these patients (L100) is presented in Fig 3. CMV was later isolated, and a second rise to a net cpm of 12,000 was seen before the patient died of CMV pneumonitis. This patient was CMV-seropositive before BMT and had a seropositive donor. Either the transfer of CMV-reactive lymphocytes from the donor or the persistence of the patient's own lymphocytes would explain the stimulation response directly after BMT. The second patient (L97) was seronegative for HSV before BMT and had an HSV-seropositive donor. This patient had a positive lymphocyte response with HSV antigen without signs of HSV infection during the first 2 months.

Table 2. Mean Maximum CMV-Specific Lymphocyte Proliferation and Mean Specific Antibody Production In Vitro for BMT Recipients With and Without CMV Infection

<table>
<thead>
<tr>
<th>Patients</th>
<th>Mean Net cpm (Range)*</th>
<th>Mean Net A 410 (Range)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>With CMV infection (n = 12)</td>
<td>31,400 (400-148,000)</td>
<td>0.9 (0.2-2.0)</td>
</tr>
<tr>
<td>Without CMV infection (n = 7)</td>
<td>900 (200-1,400)</td>
<td>0.2 (0.0-1.0)†</td>
</tr>
</tbody>
</table>

*P = .02, Mann-Whitney U test, two tailed.
†One patient died early in relapse.
LYMPHOCYTE PROLIFERATION IN CMV INFECTION

Fig 3. Lymphocyte proliferation (■, patient; ○, donor) and IgG production (□, patient; ▲, donor) responses in patient (L100) who died of CMV pneumonitis. In this patient transfer or persistence of CMV-reactive lymphocytes may have occurred (first peak of specific proliferation and IgG responses).

after BMT. The response was lost later. This indicates a transfer of reactive cells from donor to recipient.

CMV-specific antibody production in vitro. Specific IgG production by lymphocytes could be detected (net A 410 > 0.4) in nine of 12 patients with CMV infection. Eight had at least a fivefold rise in the amount of CMV IgG produced as judged by the absorbance levels. In eight of these patients, at least two successive positive tests were found. In six of the seven patients without CMV infection, no IgG production could be detected (A ≤ 0.4; P < .05 by the two-tailed Mann-Whitney U test). One patient, who died early in relapse, had an A 410 of 1.0 that later decreased rapidly. No CMV IgG rises were seen in patients without a CMV reactivation. No lymphocyte-produced CMV-specific IgM was detected in any of the patients.

In six patients an initial IgG response to CMV antigen was seen, followed by a decrease (Fig 3). Five of these recipients were seropositive before BMT with seropositive donors. CMV infection developed in these patients, and in three of them, later, strong CMV IgG production was seen. Both transfer and persistence of IgG-producing lymphocytes are feasible in these cases.

CMV-specific lymphocyte proliferation and CMV IgG production in vitro correlated with the diagnosis of CMV infection. Lymphocyte proliferation responses and in vitro IgG production both quickly indicated a CMV infection after BMT (Table 3). When the two methods were combined, 11 of 12 CMV infections were detected. The 12th patient had severe acute GVHD and was treated with methylprednisolone and ATG. He later died of a CMV pneumonitis. A positive CMV-specific lymphocyte proliferation and/or an in vitro IgG production response developed significantly sooner than growth of CMV in culture or a CMV IgG titer rise in serum (P < .05 by Wilcoxon’s rank sum test). In six of seven patients, either positive CMV-specific lymphocyte proliferation or IgG production was found before either CMV isolation or a serum antibody increase. In the seventh patient, a CMV IgG increase in serum was the first sign of CMV infection.

DISCUSSION

The magnitude of CMV-specific lymphocyte proliferation responses was found to differ significantly between patients with and without CMV infection. There was also a difference in the in vitro CMV IgG production response. Responses seen early after BMT may reflect transfer or persistence of reactive lymphocytes, but such early responses usually diminished during the first weeks. Patients with proven reactivated CMV infections had secondary strong rises of in vitro lymphocyte responses to CMV. A strong rise in the lymphocyte proliferation response and/or a rise in the production of CMV-specific IgG in vitro may therefore be used as adequate diagnostic signs of a CMV infection.

We used unfractionated lymphocyte populations to minimize the loss of cells with specific functions, especially those of antigen presentation. This also has the advantage that the T cell and B cell responses can be analyzed simultaneously.

It is difficult to define exactly the time after BMT at which a CMV reactivation starts. We therefore calculated the number of days from BMT until the first positive assay for CMV was obtained with each of the methods. With the criteria used (a net cpm rise to >10,000, a tenfold rise in net cpm, or a net A 410 rise of >0.4), the time to diagnosis of a CMV infection was determined by the time at which first positive CMV isolation was obtained. Considering the time required for CMV replication in culture and the in vitro lymphocyte tests, the detection of CMV reactivation was achieved significantly sooner with the lymphocyte proliferation/IgG production tests.

We noted that CMV-specific lymphocyte proliferation responses reached an early peak and often diminished later. The reason for this is unknown. It may have to do with a decreased activity of T4 cells. CMV infection has been associated with a depressed T4/T8 ratio, and in four of seven patients with CMV infection, a low T4/T8 ratio was
found (data not shown). Patients who received ATG treatment rapidly acquired a depressed lymphocyte proliferation response to CMV. A decreased activity of T4 cells may be mediated by suppression, for instance, induced by CMV. In two of three patients in this study in whom chronic GVHD has thus far developed, the lymphocyte proliferation responses were lost while CMV excretion has continued. This is in agreement with our earlier results showing that lost CMV-specific lymphocyte proliferation responses were more common in patients with chronic GVHD. Chronic GVHD is associated with immunoincompeience, and nonspecific suppressor cells have been found in patients with chronic GVHD (for a review see Tsoi et al, ref 27).

In summary, we have found that increased CMV-specific lymphocyte proliferation and in vitro IgG production to CMV antigen may be used as tools for the early diagnosis of CMV infection after BMT.

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REFERENCES

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