Cytomegalovirus Antigen Detection in Peripheral Blood Leukocytes After Allogeneic Marrow Transplantation

By Michael Boeckh, Raleigh A. Bowden, James M. Goodrich, Mary Pettinger, and Joel D. Meyers†

Detection of cytomegalovirus (CMV) antigenemia was compared with shell vial centrifugation cultures for rapid detection of CMV infection. In a prospective study, 59 CMV seropositive patients were monitored weekly during the first 100 days after allogeneic marrow transplantation for virus excretion from urine, throat, and blood and for antigenemia by direct staining of peripheral leukocytes using an antibody pool directed against pp 65. Antigenemia was present in 21 of 22 patients with culture-proven CMV infection and in 3 of 37 without culture-proven CMV infection (sensitivity 95%, specificity 91%). The median time of onset of antigenemia and shell vials was day 47 and 55 after transplant, respectively (P = .0006). Among patients who developed CMV disease without preceding cultures, antigenemia was detected in all patients with CMV pneumonia (N = 6) and in two of three patients with gastrointestinal disease by a median of 10 and 7 days, respectively, before the onset of disease (P = .0002). Levels of antigenemia were significantly higher in patients with disease or viremia than in patients with excretion from urine or throat (P < .05). Whether the antigenemia assay is more sensitive than rapid culture methods to focus antiviral prophylaxis in marrow transplant patients must be determined in controlled studies.

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Patients and Methods

Patients and specimens. Between December 1990 and September 1991 consecutive CMV seropositive patients undergoing allogeneic marrow transplantation were monitored weekly between days 15 and 100 posttransplant for CMV antigenemia. CMV excretion in urine, throat, and blood was monitored weekly by both shell vial centrifugation cultures and conventional cultures between start of conditioning and day 100 posttransplant. All patients received acyclovir prophylaxis (500 mg/m² intravenously [IV] every 8 hours) from conditioning until day 30 after transplant.14 In addition, patients received ganciclovir after CMV excretion was detected (5 mg/kg twice daily for 7 days followed by 5 mg/kg through day 100 posttransplant). Patients were also eligible to participate in a double-blind placebo-controlled study of prophylactic ganciclovir. In this study, 26 patients received ganciclovir (5 mg/kg twice daily for 5 days followed by 5 mg/kg once daily) after engraftment (defined as > 750 neutrophil cells per microliter for more than 2 days) until day 100 posttransplant unless ganciclovir had to be discontinued because of toxicity. Antigenemia was determined for two additional nonconsecutive patients with CMV pneumonia for correlation of antigenemia with disease activity.

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Definitions. CMV infection was defined as identification of virus from urine, throat, or blood determined by shell vial or conventional cultures. CMV disease was defined as recovery of virus from tissue (eg, lung, gastrointestinal [GI] tissue) or by BAL in presence of new or changing pulmonary infiltrates.2 Serologic changes were not used to define CMV infection.

Viral cultures. Processing and inoculation of specimens of urine, polymorphonuclear leukocytes (PMNL), throat, BAL, and tissue for shell vial centrifugation and conventional cultures were performed as previously described.1516 Coverslips of shell vial centrifugation cultures were stained after 20 and 40 hours. Conventional cultures were examined for inoculation and for the antigenemia assay. Cytospin preparations were made with buffered saline (PBS) and divided in duplicate for calculations of sensitivity and specificity but not for the antigenemia assay. The pellet containing PMNL was suspended in 0.2 mL phosphate-buffered saline (PBS) and divided in duplicate for culture inoculation and for the antigenemia assay. Cytospin preparations were made with 100 µL of a suspension of 1.5 × 10⁶ cells/mL by centrifugation for 4 minutes at 800 rpm. Detection of CMV antigenemia by immunoperoxidase staining was performed according to van der Bij et al.6 Cytospin preparations were fixed in acetone, air dried, and stained within 24 hours after storage at 4°C for specimens held more than 2 hours. Before staining, slides were placed in PBS and then incubated in duplicate with 35 µL of an MoAb mixture (C 10 and C 11, diluted 1:10 in PBS and 1% human albumin; Biotest Diagnostics Corp, Denville, NJ and Biotest AG, Dreieich, Germany) for 45 minutes at room temperature in a humid chamber. Slides were washed in PBS and incubated with 35 µL of a peroxidase-labeled rabbit antimouse Ig (DAKO, Copenhagen, Denmark) diluted 1:20 in PBS with 1% human albumin at room temperature for 45 minutes in a humid chamber. After rinsing in PBS, freshly prepared 3-amino-9-ethylcarbazole solution (Sigma chemicals Co, St Louis, MO; No. 5754) was applied for 10 minutes at room temperature as described by van der Bij et al.6 Slides were then washed with acetate buffer (pH 4.9, 0.05 mol/L), rinsed, counterstained with hematoxylin for 30 seconds to 1 minute, rinsed again, and mounted. For detection of CMV antigenemia by immunofluorescence, slides were processed as described above but incubated with 35 µL fluorescein-labeled goat antimouse IgG (DAKO) with 0.005% Evans Blue for 45 minutes instead of the peroxidase-labeled Ig according to Gerna et al8 and Revello et al.13 Infected cells presented with dark-brown (immunoperoxidase) or yellow-green (immunofluorescence) nuclear staining. Positive controls consisted of CMV infected fibroblasts and leukocytes from known antigenemia-positive patients. Results were reported as number of antigen-positive cells per 50,000 cells (average number of cells per slide).

Statistical evaluation. A positive antigenemia assay was defined as any positive results within the first 100 days after transplant. For calculation of predictive values only patients monitored until day 100 were considered. Maximum levels of antigenemia were compared with the infection status groups by nonparametric Kruskal-Wallis test statistics using rank sums.18 Time to occurrence of antigenemia and cultures in patients monitored until day 100 after transplant were calculated according to Kaplan and Meier and compared using a paired log-rank statistic.

RESULTS

Patients. In 9 of 59 patients enrolled in this study monitoring was stopped between day 60 and 70 because of relapse or death. All nine patients remained both antigenemia- and culture-negative. These patients were considered for calculations of sensitivity and specificity but not for the onset of antigenemia relative to cultures. Of 50 patients monitored until day 100 after transplant, 20 (40%) received ganciclovir after engraftment for a median time of 62 (range 6 to 76) days.

CMV excretion occurred in 13 (26%) patients. Two of these patients subsequently developed CMV disease: one patient developed both GI disease and pneumonia 2 and 9 days, respectively, after institution of ganciclovir treatment for viremia; the other patient developed GI disease after 15 days of ganciclovir for CMV excretion from urine. Eleven of the 13 patients did not receive prophylactic ganciclovir after engraftment. Two ganciclovir recipients developed viremia at day 80 and 104 after marrow transplant, respectively. Ganciclovir was stopped in these patients because of neutropenia 36 and 40 days before excretion occurred, respectively.

CMV disease developed in a total of 11 patients at a median time of 55 days after transplant. In 9 of the 11 patients CMV disease was diagnosed before or coincident with CMV excretion. None of the 11 patients with disease received prophylactic ganciclovir from the time of engraftment. Five of seven patients with CMV pneumonia and one of four patients with CMV GI disease died.

Sensitivity, specificity, and predictive value of antigenemia. CMV antigenemia occurred in 21 of 22 patients with culture-proven CMV infection and in all with viremia (N = 10), indicating a sensitivity of 95% and 100%, respectively (Table 1). One patient with excretion in urine remained antigenemia-negative throughout the transplant course. Antigenemia occurred in three patients without documentation of CMV infection by culture. Two of these three patients received prophylactic ganciclovir after engraftment and had antigenemia detected at one and two occasions, respectively; the third patient did not receive ganciclovir and had antigenemia detected at one single occasion. If all three of these patients were considered false positive the specificity was 91.2%. Sensitivity and specificity were not changed when only patients not receiving prophylactic ganciclovir were considered (Table 1). A direct comparison of antigenemia and blood cultures is shown in Table 2. Of the 15 positive blood cultures, six were positive

| Table 1. Antigenemia in Patients With Culture-Proven CMV Infections and With Viremia Only |
|---------------------------------|---------------|-------------|---------------|
|                                | All Patients  | Patients Not Receiving Prophylactic Ganciclovir (N = 33) |
|                                | (N = 59)      | (N = 33)    |
| Positive                        | 21†           | 19          | 1             |
| Negative                        | 3             | 1           | 12            |
| Viremia*                        | 10            | 8           | 0             |
| Negative                        | 14            | 35          | 12            |

*Includes recovery from blood, urine, throat, tissue, BAL. †Entries indicate number of patients: a positive test was defined as any positive result during the first 100 days after marrow transplant. Includes all patients with viremia, including patients coincident with disease (N = 2) or after excretion from other sites (N = 1) and patients with viremia detected by conventional cultures only (N = 2).
by shell vial and conventional cultures and nine were positive by conventional cultures only. In five of these specimens, shell vial cultures were either negative or toxic and in four specimens shell vial cultures were not done because the patient had already started ganciclovir.

Because all patients with viremia were treated with ganciclovir\(^2\) in this study and viremia is the most significant risk factor for subsequent disease,\(^1,2\) we calculated the predictive values for both disease and disease or viremia. Positive and negative predictive values for subsequent CMV disease in patients not receiving prophylactic ganciclovir were 52.6% and 90.9%, respectively, and for CMV disease or viremia, 68.4% and 90.9%, respectively. When all patients were considered, positive predictive values were 43.5% and 65.2% for disease and disease or viremia, respectively. The negative predictive values was 96.2% for both disease and disease or viremia.

**Technical aspects.** All 570 specimens tested for antigenemia were stained by the peroxidase method and 270 consecutive specimens were also stained by the immunofluorescence technique in parallel. Discordant results occurred in 11 of 270 specimens (4%) in specimens with low-grade antigenemia (one positive cell per slide). Eight of the 11 specimens were positive by immunofluorescence only while three specimens were positive by immunoperoxidase only. Staining of additional slides in these cases usually yielded in concordant results. Because of endogenous peroxidase, 5.7% of slides were not interpretable. Using the immunofluorescence technique, artifacts (ie, green background staining) were less than 1%. Sixteen of 570 (2.8%) specimens did not yield sufficient number of cells for antigenemia testing. Thirteen of these were obtained before day 25 after transplant when the absolute neutrophil count was less than 200/mm\(^3\) and three specimens were from patients with secondary graft failure. The average processing time was 5 hours (immunoperoxidase) and 4.5 hours (immunofluorescence), respectively.

**Onset of antigenemia versus first positive culture.** The onset of antigenemia versus the first positive shell vial culture is shown in Fig 1 and Table 3. For shell vial centrifugation cultures the time of onset indicates when cultures where taken, not when result was available (processing time not considered). The median time of onset of antigenemia and shell vial cultures was at day 47 and 55, respectively (\(P = .0006,\) log-rank test), and antigenemia could be detected in 17 of 22 patients before the first positive culture. All patients presenting with pneumonia as first evidence of CMV infection had preceding antigenemia even though they had negative surveillance cultures before the onset of pneumonia. The median time of onset of antigenemia before the diagnosis of pneumonia was 10 days (range 2 to 22 days). In an additional patient with CMV pneumonia and GI disease who had preceding viremia for 2 days, antigenemia was detected 10 days before the onset of disease.

Among patients with GI disease without preceding cultures, two of three had antigenemia detected before the diagnosis (both 7 days) while the third patient had antigenemia coincident with the diagnosis. This patient had mild disease that was documented by one shell vial culture of a biopsy specimen while both conventional tube cultures and histology remained negative. One additional patient with GI disease with preceding excretion from urine for 15 days

![Fig 1. Kaplan-Meier product limit estimates of the probability of developing CMV antigenemia (---) versus a positive shell vial centrifugation culture (-----) in patients not receiving prophylactic ganciclovir after engraftment \((N = 30)\) \((P = .0006)\).](image-url)

Table 2. Sensitivity and Specificity of the Antigenemia Assay Versus Viremia in Blood Specimens

<table>
<thead>
<tr>
<th>Specimens in Patients</th>
<th>Positive by Antigenemia</th>
<th>Positive by Conventional Cultures</th>
<th>Viremia*</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Specimens ((N = 570))</td>
<td>Positive Negative</td>
<td>Positive Negative</td>
<td>Positive Negative</td>
</tr>
<tr>
<td>Negative</td>
<td>14†</td>
<td>1‡</td>
<td>11</td>
</tr>
<tr>
<td>Positive</td>
<td>69</td>
<td>486</td>
<td>62</td>
</tr>
</tbody>
</table>

*Viremia detected by conventional cultures or shell vial and conventional cultures (see text).
†CytoSpin preparation of separated blood was made after 24 hours, which may have influenced the result.
‡Centrifugation cultures the time of onset indicates when cultures where taken, not when result was available (processing time not considered).

**Fig 1. Kaplan-Meier product limit estimates of the probability of developing CMV antigenemia (---) versus a positive shell vial centrifugation culture (-----) in patients not receiving prophylactic ganciclovir after engraftment \((N = 30)\) \((P = .0006)\).**

![Graph](image-url)
had antigenemia detected 30 days before the onset of disease.

Viremia was detected in 10 patients, with seven patients having viremia as first evidence of infection, two having viremia coincident with disease, and one after previous excretion from throat. In five of the seven patients with viremia as first evidence of infection, viremia was detected by both shell vial centrifugation cultures and conventional cultures, and antigenemia preceded viremia by a median of 8 days (range 6 to 14 days) (Table 3). Of the two patients who had viremia detected by conventional cultures with negative or toxic shell vial cultures, one had antigenemia coincident with viremia and the other had antigenemia 7 days after viremia. Both patients subsequently developed pneumonia, but antigenemia was the first evidence of infection because of the long processing time of conventional cultures.

Eighteen of 22 patients with CMV infection documented by shell vial centrifugation cultures had positive conventional cultures. Four patients with positive shell vial cultures but negative conventional cultures had excretion from urine (two patients), excretion in throat (one patient), and a gastric biopsy (one patient).

**Influence of ganciclovir.** Of four patients treated for viremia, one had no further cultures while the other patients had persistent cultures for 1, 2, and 3 weeks, respectively, after start of ganciclovir. Antigenemia persisted for 7, 12, 14, and 25 days, respectively. Four of six patients treated for excretion from urine or throat had no further positive cultures while antigenemia persisted for 0, 7, 13, and 22 days, respectively. One patient treated with ganciclovir for excretion in throat had persistent cultures for 40 days and persistent antigenemia for 69 days while on ganciclovir and subsequently developed both CMV pneumonia and GI disease after day 100 posttransplant and died. Another patient treated for excretion in urine developed gastrointestinal disease 15 days after initiation of ganciclovir with persistent antigenemia for 18 days.

Of 11 patients treated for disease, one (9%) cleared antigenemia by day 7, and three patients (27%) cleared antigenemia by day 14 after initiation of therapy. Three additional patients cleared antigenemia by day 21 (two died, one survived), and two patients had persistent antigenemia for more than 21 days (32 and 37 days). Three patients died of CMV disease between day 14 and 21 after diagnosis without cessation of antigenemia.

In contrast to the pattern of antigenemia in association with ganciclovir treatment, a rapid cessation of virus excretion was observed in patients with disease regardless of the clinical outcome (60% at day 7, 70% at day 14, and 100% at day 21 after starting treatment).

**Correlation of antigenemia with activity of CMV infection.** The highest levels of antigenemia were detected in patients with pneumonia, viremia, or GI disease (Fig 2). There was no statistically significant difference between these groups. Patients with excretion from urine or throat had significantly lower levels of antigenemia than patients with pneumonia or viremia ($P < .05$). Patients with initial levels of antigenemia of more than 1 positive cell per 50,000 cells were more likely to develop disease or viremia (positive predictive value 75%). However, four patients with low initial antigenemia (1 or less positive cell per 50,000 cells, i.e., 2 or less per duplicate testing) also subsequently developed disease. Two of these patients developed GI disease, one pneumonia, and one both GI disease and pneumonia with maximum levels of 6, 9, 46, and 50 antigen-positive cells per 50,000 leukocytes, respectively.

**DISCUSSION**

In this study we have shown that detection of CMV antigenemia is a sensitive, specific, and rapid test for early diagnosis of CMV infection in patients undergoing allogeneic marrow transplantation. The median time of onset of antigenemia and shell vial cultures was day 47 and 55 after transplant, respectively ($P = .0006$). All six patients who developed CMV pneumonia without preceding excretion and two of three patients who developed GI disease without preceding excretion had antigenemia detected before rapid cultures by a median of 10 and 7 days, respectively ($P = .0002$). Considering the time required for processing of shell vial cultures (up to 2 days), the result of antigenemia was available substantially earlier.

This study confirms and extends previously published data on the antigenemia assay in other transplant settings.6-11 It has been reported in organ transplant and AIDS patients that the antigenemia assay is more sensitive than rapid immunofluorescence culture methods. In these studies, heart, liver, and kidney transplant recipients were monitored after transplant with antigenemia detected 2, 6, and 25 days, respectively, before rapid cultures.5-11 However, there are also reports describing the antigenemia assay of being of limited value.20-22 The lack of detection of antigenemia in the study by Miller et al may have been related in part to technical problems because more than half of the specimens were processed only the day after
collection. Because more information is available on the specificity of the MoAbs used in this assay, it appears to be essential to use certain antibodies specific to CMV lower matrix protein pp65 (C 10 and C 11) and to consider certain technical aspects of the assay to avoid pitfalls.

In this study, we compared immunoperoxidase and immunofluorescence staining techniques. Because of less artifacts (ie, endogenous peroxidase background) and the shorter processing time, the immunofluorescence technique appeared preferable. In addition, the sensitivity of the assay using immunofluorescence may be higher in low-positive specimens. This observation is based only on a small number of specimens but is in agreement with a recently published study by Gerna et al. One limiting factor of the antigenemia assay in marrow transplant patients may be the availability of sufficient numbers of cells in patients with late engraftment or graft failure. In this study, even with low counts in most of the cases, at least one slide could be stained.

Three of 50 patients had a positive antigenemia test without positive cultures. In two of these patients the dissociation between antigenemia and cultures may have been caused by early institution of ganciclovir. A dissociation between antigenemia and cultures was also reported in kidney, liver, and heart transplant recipients. In these studies, dissociation between antigenemia and cultures occurred either in patients with ganciclovir treatment or with low numbers of antigenemia. An important question is whether a positive antigenemia test without positive cultures represents a false-positive result or reflects a higher sensitivity of the assay. Comparative studies using both the polymerase chain reaction (PCR) and the antigenemia assay suggest that antigenemia is more sensitive. Gerna et al, who monitored heart transplant recipients by using both antigenemia assay and PCR, reported that all positive antigenemia results could be confirmed by PCR.

The observation that antigenemia is more sensitive than rapid cultures may be important for prevention strategies in marrow transplant patients. Major progress has been made in prevention of CMV-related death by initiation of antiviral treatment based on excretion or a positive BAL. In a double-blind placebo-controlled study at our institution we were able to show that institution of ganciclovir after excretion from urine, throat, or blood led to a significant reduction of CMV disease. Schmidt et al, who started ganciclovir based on detection of CMV in BAL at day 35 after transplant, also reported a reduction of CMV disease by using this strategy. Although treatment of patients with excretion or with a positive BAL was highly effective in prevention of disease among these patients, a substantial number of patients in these studies developed CMV disease without preceding excretion or BAL positivity (12% to 13% of all patients). One strategy would be to administer ganciclovir to all CMV seropositive patients undergoing allogeneic transplantation. This strategy exposes a substantial number of patients to a drug that is potentially marrow toxic (30% to 41% of patients). Another possible approach would be to use antigenemia as the indicator to start antiviral treatment. Our results suggest that the use of antigenemia as a test for starting antiviral therapy would have detected the 12% of patients who developed disease coincident with or before excretion. This strategy may also delay the initiation of ganciclovir prophylaxis and could eliminate its use from patients who do not reactivate CMV, and would thereby limit it to patients at highest risk for disease. In our most recent study (data not shown), engraftment occurred at a median of day 24 while first antigenemia was detected at a median of 47 days. Therefore, the average delay of start of ganciclovir based on this strategy would be at about 2 to 3 weeks. Several studies have shown that viremia is the most significant risk factor for CMV disease while excretion from urine and throat is not predictive for subsequent disease. Antigenemia alone or in combination with viremia may prove to be sufficient to identify patients at risk for disease, thereby eliminating the need to screen patients for excretion from urine and throat. We are currently studying this approach in a controlled prospective study.

A significantly higher number of antigen-positive cells was found in patients with CMV pneumonia, GI disease, and viremia than in patients with excretion from urine or throat or with antigenemia only ($P < .05$) (Fig 2), suggesting that antigenemia is a marker for the activity of CMV infection. It has been shown in organ transplant and AIDS patients that there is a correlation between activity of CMV infection and the maximum number of antigen-positive cells. A comparison of our study with this data is difficult because differences in definitions of disease, infection, patient population, and technical variations (ie, number of cells per slide). Nevertheless, our study is in agreement with the association of clinically severe disease with high levels of antigenemia. Another interesting question is the significance of low positive test result, ie, one single positive cell per slide. In this study, the positive predictive value of an initial test of more than 1 positive cell per 50,000 cells was higher when compared with all positive results, regardless of the number of positive cells (75% vs 68.4%). However, four patients with low initial antigenemia subsequently developed disease, suggesting that even low levels of antigenemia may be significant in patients after allogeneic marrow transplantation. In this situation, one strategy to identify patients at risk for disease may be to repeat the test after a few days because patients with disease appear to have rapidly increasing antigenemia.

In patients treated with ganciclovir for either excretion or disease, rapid and complete cessation of virus excretion was seen after 3 weeks and thereafter regardless of the clinical outcome. This observation is in good agreement with published reports in marrow transplant patients. In contrast to the rapid effect on virus excretion, cessation of antigenemia appeared related to both disease activity and outcome. Among excretors one patient had prolonged antigenemia and subsequently developed both pneumonia and GI disease after day 100 posttransplant and finally died of CMV disease. In addition, three of five patients who died of CMV disease did not clear antigenemia before death while all cultures were negative in these patients. This might indicate that prolonged antigenemia is associated
with a poor prognosis. Because negative cultures are not necessarily associated with effective antiviral therapy, antigenemia might be a better marker for monitoring antiviral treatment and for identifying patients who need prolonged treatment. Recent reports suggest that PCR may also be suitable for monitoring antiviral treatment, although it may increase the number of patients treated earlier because of its sensitivity.

In conclusion, we have shown that detection of CMV antigenemia is a sensitive, specific, and rapid method for early detection of CMV infection in patients undergoing allogeneic marrow transplantation. Our results suggest that it may be a more sensitive diagnostic test for monitoring patients after marrow transplantation than rapid immunofluorescence culture methods. Whether the antigenemia test is useful in prophylaxis strategies as an indicator for early institution of antiviral therapy for prevention of CMV disease must be determined in controlled prospective studies.

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Cytomegalovirus antigen detection in peripheral blood leukocytes after allogeneic marrow transplantation

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