

## **Level and Onset of CMV-pp65 Antigenemia as Determinants of Risk for CMV-Related Complications in Stem Cell Transplant Recipients**

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### **ABSTRACT**

This study was carried out to investigate if quantification of CMV antigen can be used to risk adapt the prophylaxis for CMV infection in stem cell transplant recipients in order to reduce the cost and the number of patients who are exposed to ganciclovir. Assay for CMV early antigen pp65 on circulating leukocytes was used to monitor early CMV infection in 54 consecutive patients undergoing peripheral blood stem cell transplantation (PBSCT). A total of 201 samples were examined for CMV antigen in peripheral blood leukocytes by the slide method and 40 samples were additionally investigated by flow cytometry. Of 54 patients examined, twenty five patients (46%) were positive for CMV pp65 antigen. Eleven of these patients developed CMV disease following PBSCT. Patients who suffered from CMV disease showed statistically significantly higher OT levels ( $p = 0.003$ ), higher frequency of complications ( $p = 0.008$ ) and unfavorable outcome ( $p = 0.002$ ). Patients who were positive for CMV antigen had a 4.5 times higher risk to develop GVHD. In multivariate analysis, we found that CMV pp65 antigen positivity was the only factor which was independently related to GVHD. On comparing the detection of antigenemia by the slide method to that by flow cytometry, the slide method was statistically significantly more sensitive in diagnosing patients who manifested with CMV-related disease ( $p = 0.01$ ). Thus, antigenemia can be used to assign PBSCT recipients at risk for CMV disease and risk adapted prophylaxis for CMV can be carried out according to the level of CMV positive leukocytes.

**Key Words:** PBSCT - CMV - GVHD - Pp65 antigenemia.

### **INTRODUCTION**

Primary HCMV infection in the normal population can cause a mononucleosis-like syndrome characterized by high fever, mild lym-

phocytosis and mild hepatitis [9]. This virus can cause severe disease in immunosuppressed patients and particularly so in seronegative transplant recipients. CMV pneumonitis in stem cell transplant (SCT) patients is often fatal [2]. Therefore, CMV-associated diseases in immunocompromised require immediate intervention.

Dramatic progress has been made in the past decade in the control of CMV infection with prophylactic preemptive ganciclovir therapy. Knowledge of the risk for CMV provides the rationale for providing antiviral protection during the early post BMT period. In the absence of antiviral prophylaxis, CMV reactivation and infection will occur in 80% of patients who were CMV seropositive prior to transplant and in 40% of seronegative patients, either because of the use of unscreened blood products [4] or seropositive stem cells [10]. Despite new therapeutic options, symptomatic CMV infection is a major cause of morbidity and mortality especially when interstitial pneumonia develops [13].

The strategy most widely used for CMV prophylaxis in the transplantation practice involves the administration of ganciclovir during the first 3 months posttransplant. This rationale for CMV prevention highly inflates the cost of stem cell transplantation affecting the patients' expenses. In addition, this protocol of antiviral

prophylaxis using ganciclovir for several months causes neutropenia that has a major impact on combat of infections in this population of patients [1]. Therefore, this study is carried out first to define patients at risk for CMV infection and then to assign the patients who will benefit most from CMV prophylaxis. In this study, we attempted to detect antigenemia by weekly monitoring SCT patients for CMV infection using both the slide-method and flow cytometry. We aimed at first to determine the best method for the clinical set-up to discriminate the group at high risk for whom prophylactic ganciclovir will be justified from the economical point of view.

### PATIENTS AND METHODS

*Patients' eligibility for SCT:* All patients were below 45 years of age, with a disease curable with PBSC transplantation. All patients received class I and II from a family HLA-identical stem cell donor.

*Patients and specimens:* Between May 1999 and October 2000, 54 consecutive patients undergoing allogeneic stem cell transplant at the National Cancer Institute, Cairo University and Nasser Institute were monitored weekly from week 1 to week 10 posttransplantation for CMV antigenemia. Inpatients were followed up weekly while outpatients were screened on every follow-up. Follow up was extended beyond 10 weeks for patients who were still at risk. The 54 PBSCT recipients were 18 children (below 18 years of age) and 36 adults with an age range from 2 to 45 years. They were 34 males and 20 females. SCT was undergone for a malignant disorder in 42 patients and for non-malignant disease in 12 cases. Chronic myeloid leukemia was the underlying disease in 23 of the SCT patients, followed by AML (n=10), MDS (n=5) and ALL (n=4). The non-malignant diseases were severe aplastic anemia in 8 patients, thalassemia in 2 and pure red cell aplasia in another 2 SCT recipients.

Patients undergoing allogeneic PBSCT were given 10 Gy total body irradiation and 120 mg/Kg cyclophosphamide when the original disease was CML and 16 mg/Kg busulphan and 120 mg/Kg cyclophosphamide for AML, ALL & MDS for conditioning. Antithymocyte globulin was added to the conditioning regimen for patients with severe aplastic anemia and thalassemia. Prophylaxis of GVHD consisted of cy-

closporine A 3 mg/Kg I.V. starting from day -1 and short course methotrexate on days 1, 3, 6 and 11. All patients received granulocyte colony stimulating factor post engraftment.

CMV infection was defined as detection of pp65 CMV antigen from peripheral blood leukocytes by the slide method. Serologic changes were not used to define CMV infection. We followed standard criteria to diagnose CMV disease [13]. No antiviral CMV prophylaxis was given routinely. CMV therapy was given only for patients who were diagnosed as CMV disease in the form of 5 mg of intravenous ganciclovir/Kg every 12 hours. Risk factors considered for CMV infection were age, sex, underlying disease, HLA-alleles, cyclosporine level, number of stem cells transplanted and pretransplant serological level of CMV IgG antibodies for donor and recipient.

Routine laboratory investigations carried out before transplant were complete blood picture, liver function tests (including ALT, AST, ALP, bilirubin and albumin), hepatitis B surface antigen (HbsAg), antibodies for hepatitis C virus (HCV) and CMV IgG antibodies by an ELISA technique. Patients who were reactive for HCV antibodies were further tested for the viral RNA by RT-PCR technique. Stem cells of the donor were evaluated for CD34+ cell count by flow cytometry. Follow-up samples for all patients were subjected to complete blood picture, liver function tests and cyclosporine level.

*CMV screening:* Beginning from week 1, CMV was routinely sought by screening for its matrix protein pp65 antigen by the slide method. Forty samples for 38 cases were examined by both the slide-method and flow cytometry. Detection of CMV antigenemia: leukocytes were isolated from heparinized blood. At least  $1 \times 10^6$  leukocytes were isolated, prepared onto poly-L-lysine coated slides and fixed using 3:1 methanol to acetone. Detection of CMV antigen by immunoperoxidase staining was performed according to Van der Bij et al. [19], utilizing the monoclonal antibody pp65 specific for CMV structural antigens (Biotest, Germany). An immunohistochemistry detection system utilizing amplified biotin-streptavidin reaction produced by Oncogene Science, Inc, (USA and Canada) was used. Human fetal fibroblasts (MRC-5 strain) infected with CMV strain AD169 were used as positive control for CMV infected cells. Infected cells presented with dark brown nucle-

ar staining. Results were reported as number of antigen positive cells per 200 cells. Results were reported as percentage.

*Detection of CMV antigen by flow cytometry (FC):* 1 ml of whole blood was hemolysed by FACS lysing solution (catalog number 349202) and then permeabilizing solution (catalog number 340457), from Becton and Dickinson (California, USA). Cells were then incubated with 5 µg of pp65 monoclonal antibody (Biotest, Germany) for 1 hour at 4°C. As a negative control, mouse IgG (Pharmigen, California, USA) was added to the cell suspension. Washing with phosphate buffered saline was followed by the addition of FITC-conjugated anti-mouse IgG. Cells were analyzed by an EPICS XL-MCL Coulter Flow Cytometer (Hialeah, Florida, USA) [8].

Comparison between means was done by the non-parametric *t*-test, namely the Mann-Whitney test. Comparison between positive and negative samples for the same patient was carried out by the Chi-square test or Fisher's exact test as the sample size was too small. Risk factors found to be statistically significant contributing to GVHD were entered in multivariate analysis. *p*-values were 2 sided and those less than 0.05 were considered statistically significant.

## RESULTS

A total number of 201 samples belonging to 54 PBSCT recipients were analyzed during the period of the study. In 9 of 54 patients enrolled in this study monitoring was stopped early because of death (n=8) or relapse (n=1).

*HBV and HCV:* HBsAg was positive in 11 patients (20.4%) and HCV viremia was detected by PCR in 7 patients (13%) prior to transplant.

*CMV serology:* Seropositivity for CMV IgG specific antibodies was detected pretransplant in 50 (92.6%) and 42 (77.7%) of SCT candidates and their donors, respectively. CMV infection was associated with higher levels of CMV antibodies in patients and/or their donors. Patients and their donors who had higher levels of CMV antibodies pretansplant showed a statistically significantly more positivity for CMV antigen posttransplant ( $p = 0.0094$  and  $0.0002$ , respectively). All patient and donor couples who were either seronegative or with low levels

of CMV antibodies (< 80 IU/ml) did not show antigenemia during the period of the study.

*CMV antigenemia:* Of 54 patients monitored for CMV antigenemia from week 1 to week 10, 25 (46.2%) were found to be positive. Eleven of these patients developed CMV disease. These patients manifested clinically by interstitial pneumonitis (IP) (n=7), CMV-enteritis (n=3) and failure of engraftment in one patient. The patients who suffered from CMV disease showed statistically significantly higher AST levels ( $p = 0.003$ ), higher frequency of complications ( $p = 0.008$ ) and unfavorable outcome ( $p = 0.002$ ).

According to time of detection of CMV antigen in their PMNLs, SCT patients were classified into 3 groups. The first group (n=6) was demonstrating high CMV antigenemia at a level of 15-20% at week one and showed further elevation at weeks 2 and 3, prior to any clinical manifestation. All manifested by early CMV disease and/or GVHD with rapid deterioration and death. The second group (n=8) had its CMV antigen positivity detectable also at week one but with levels of 5-6%. Clinical manifestations occurred between weeks 6 to 8 in these patients with coincident elevation in CMV antigenemia. The third group (n=11) was negative for CMV antigen at the beginning of the study, i.e. immediately posttransplant and the positivity was detectable at either week 3 or 4. Their clinical manifestations were evident beginning from week 8.

On the other hand, 29 SCT patients were negative for CMV antigenemia during the period of the study. Of these patients, 5 developed complications, being GVHD of the liver in 5 patients and bacterial pneumonia in one of them. Two of the patients who developed GVHD were HBsAg positive and one was HCV positive. Different characteristics of CMV positive patients versus negative are summarized in Table (1).

Twenty patients developed acute GVHD that was statistically related to male gender, high donor CMV antibodies, DR7 and CMV antigen positivity. To measure the independent effect of the significant factors on GVHD, these significant variables were entered into a stepwise logistic regression. CMV antigen positivity was the only factor which was independently related to GVHD. The risk of GVHD was 4.5

times more in patients with positive CMV than those who were negative during the study ( $p = 0.01$ ). Risk factors for GVHD are listed in Table (2).

On comparing the detection of CMV antigenemia by the slide method to flow cytometry, the 2 techniques agreed in 80% of the results. Discrepancy was observed in the samples of 3 patients. They were extremely leukopenic and suffered from CMV disease. The result of antigenemia in these patients was positive by the slide method (20-30%) and negative by flow cytometry (1%). On comparison of both techniques to the occurrence of CMV disease, it was found that the slide method was statistically significantly more going on with the clinical setting ( $p = 0.01$ ). Fig. (1) shows a CMV antigenemia positive case gated at neutrophils by the indirect FC technique.

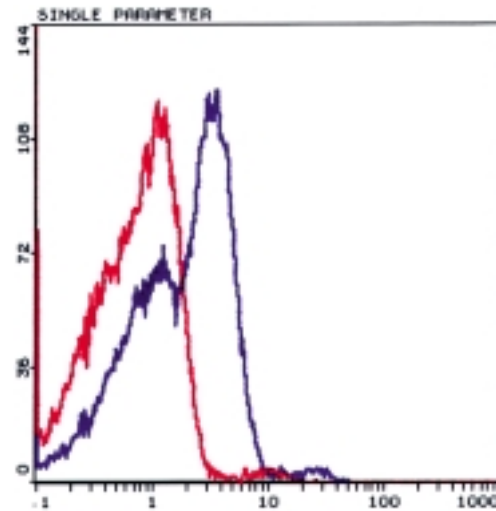


Fig. (1): Shows a positive case for CMV-pp65 antigen gated at leukocytes by the indirect flow cytometry assay.

Table (1): Characteristics of SCT recipients with and without CMV infection.

Characteristics	CMV negative patients n=29	CMV positive patients n=25	p-value
Age*, years	22.6±12.1	23.0±12.7	0.8
Sex:			0.02
Male	14 (48.2%)	19 (76.0%)	
Female	15 (51.8%)	6 (24.0%)	
Underlying disease, n (%):			0.40
CML	12 (41.4%)	11 (44%)	
AML, ALL, MDS	10 (34.5%)	9 (36%)	
Nonmalignant	7 (24.1%)	5 (20%)	
No. of stem cells transplanted*	7.5±3.6	6.4±3.2	0.1
CMV serology*:			
IU/ml			
Donor	60.3±57.7	135.2±69.6	< 0.01
Recipient	94.7±80.4	148.0±63.3	0.01
Cyclosporine*	253.5±205.0	193.5±96.3	0.80
OT*, IU/L	55.5±45.4	102.1±114.3	< 0.01
PT*, IU/L	38.9±25.5	66.0±78.9	0.03
ALP*, U/L	161.0±116.4	268.2±245.1	0.06
Bilirubin*, gm/L	1.9±3.9	2.2±2.5	0.08
Albumin*, gm/L	3.2±0.5	4.8±8.3	0.77
TLC*, x10	4.5±5.3	4.6±5.6	0.63
ANC*, x10	2.8±3.1	2.9±3.3	0.83
Hb*, gm/L	9.2±1.3	9.0±1.2	0.37
Platletes*, x10	80.1±77.8	69.6±62.6	0.69
Complications:			< 0.01
-	24 (82.8%)	5 (20.0%)	
+	5 (17.2%)	20 (80.0%)	
Outcome:			< 0.01
Alive	23 (79.3%)	7 (28.0%)	
Dead	6 (20.7%)	18 (72.0%)	

\* Values are expressed as mean ± standard deviation.  
p-values < 0.05 are considered significant.

Table (2): Comparison of different risk factors in relation to GVHD.

Parameters	GVHD-ve	GVHD+ve	p-value
Number	34	20	
Age*	20.5±11.8	27.3±11.8	0.063
Sex:			0.029
Male	17 (50%)	16 (80%)	
Female	17 (50%)	4 (20%)	
CMV-IgG*, IU/ml:			
Donor	81.9±77.7	113.3±62.8	0.043
Recipient	106.6±72.5	137.4±80.0	0.262
Number of PBSC*	7.7±5.6	7.1±3.7	0.967
Cyclosporine level*	212.3±157.2	242.7±171.2	0.515
Underlying disease:			0.339
CML	13 (38.2%)	10 (50.0%)	
ALL/AML/MDS	12 (35.3%)	8 (40%)	
Non-malignant	9 (26.5%)	2 (10%)	
DR7:			0.016
+	2 (5.9%)	6 (30.0%)	
-	32 (94.1%)	14 (70.0%)	
CMV-Ag:			0.007
+	11 (32.3%)	14 (70.0%)	
-	23 (67.7%)	6 (30.0%)	
HBsAg:			0.558
+	6 (17.6%)	5 (25%)	
-	28 (82.4%)	15 (75%)	
HCV:			0.666
+	5 (14.7%)	2 (10%)	
-	29 (85.3%)	18 (90%)	

\* Values are expressed as mean ± SD. p-values < 0.05 are considered significant.

## DISCUSSION

Cytomegalovirus-related disease is one of the most frequent infectious complications occurring after allogeneic PBSCT. Despite new therapeutic options, symptomatic CMV infection still has a high mortality rate, especially when interstitial pneumonitis develops [13]. In our study, we have found that CMV poses a great hazard for allogeneic SCT patients. We detected CMV antigenemia in 25 (46%) of 54 patients after allogeneic PBSCT. Eleven of these patients developed CMV disease. Complications were statistically significantly related to CMV infection, namely CMV-associated pneumonitis enteritis, or GVHD. On the other hand, only 5 patients of the 29 who were negative for CMV antigenemia during the monitoring period posttransplant developed GVHD. Furthermore, an unfavorable outcome following SCT was statistically significantly related to CMV infection, as 72% antigenemia positive patients died

due to these complications versus 20% of CMV negative cases ( $p = 0.001$ ). As regards level of detection of CMV antigen in leukocytes of PBSCT recipients, patients were found to either show high early positivity of 15-20% ( $n=6$ ), positivity within 5-6% ( $n=8$ ) or were negative ( $n=11$ ). It is noteworthy that the first group with high early level of CMV antigen manifested by early occurrence of CMV-related complications, rapid clinical deterioration and death.

Previously in the era of allogeneic BMT, CMV infection ranged from 45% to 80% in different studies [4,17]. With the use of allogeneic PB stem cells, it was expected that better immune reconstitution will lead to reduced CMV-related morbidity and mortality after allogeneic PBSCT [11]. Former studies reporting results on CMV reactivation and CMV disease in patients after allogeneic PBSCT generated conflicting results. On comparison of allogeneic BMT to PBSCT, it was found that patients receiving

PBSCT in allogeneic setting are at significantly higher risk of CMV-related complications (21% versus 62% in PBSCT recipients) [5]. Similarly, another study reported a higher risk of CMV infection in recipients of allogeneic PBSCT [11]. By contrast, other studies suggested lower risk for CMV disease after PBSCT [16]. The latter study did not state whether the patients underwent allogeneic or autologous PBSC transplants and did not take the risk factors for CMV infection into consideration. In a recent study comparing CMV antigenemia in BMT to PBSCT, CMV was positive in 56% of patients after BMT and 47% after PBSCT. In the latter study, 18 patients (11%) developed CMV disease, 14 of them were post-BMT and 4 were following PBSCT [18]. This, it is clear that the incidence of CMV infection and disease appears to vary considerably between centers.

In our study, risk factors for acquisition of CMV infection were male gender and higher CMV pretransplant CMV IgG antibodies of donors and recipients. The importance of seropositivity as a risk factor for acquisition of CMV infection is supported by the finding that CMV infection rates remained high in CMV seropositive recipients after the introduction of CMV negative blood support [4,17]. Similarly, Trenchel and his coworkers confirmed that seropositivity of the recipient prior to transplantation constitutes the strongest covariate in multivariate analysis for antigenemia, persisting antigenemia, CMV IgM seroconversion, CMV associated disease and CMV-IP [18]. In another study, the only risk factor for CMV antigenemia in BM recipients was seropositivity [14]. By contrast, it was reported that none of BMT recipients who were seronegative before transplantation and had a seronegative donor developed CMV pneumonia within the first year after transplantation [13]. Clearly, the problem of CMV infection currently exists only in recipients at risk for secondary or reactive CMV.

Acute GVHD continues to be the most important common complication associated with allogeneic transplants whether BM or SC grafts [14]. In our study, acute GVHD occurred in 20 patients (37%). Of these patients CMV infection was detected in 14. In the present study, GVHD was statistically significantly related to male gender, CMV seropositive SC donor, DR7 allele and positive CMV antigen. In multivariate

analysis, we found that CMV antigen positivity was the only factor independently related to GVHD. In agreement with our results, among 50 allogeneic BMT recipients 12 of the 23 patients with grade III-IV GVHD (52%) developed CMV antigenemia; whereas, none of 27 with or without grade Ia GVHD developed CMV antigenemia or CMV disease [12]. In addition, in a study on 118 BMT recipients, it was reported that patients with grade II-IV acute GVHD had 2.2 times the risk of antigenemia than those with no or only limited GVHD ( $p = 0.03$ ) [14].

Rapid, specific and sensitive methods are essential for early diagnosis of CMV infection in transplant patients as well as for monitoring antiviral therapy. One viral protein pp65 encoded by a gene which produces a virion structural protein accumulating during infection to be the most abundant CMV protein in productively infected cells. Detection of pp65 has served as a convenient marker for productive infection in seropositive transplant recipients because its abundance allows more sensitive detection than do assays for infectious CMV [1]. In our study, CMV antigenemia assay by the slide method was very reliable for the diagnosis of CMV infection and its positivity preceded the occurrence of CMV disease manifestations. In addition, level of positivity was a strong determinant of time of onset of CMV-related complications and more importantly, predicted severity of the disease. We found that in the group showing an early high level of CMV antigenemia, all patients developed early CMV-related complications and showed a high mortality rate.

In agreement with our findings, the assay for CMV early antigen pp65 on circulating leukocytes was reported to be sensitive and specific in detecting early CMV infection [14] and it gave highly sensitive results equivalent to PCR from whole blood and plasma PCR [7]. In the latter study, intensive surveillance continuing for > 6 months following transplantation was recommended as CMV disease was found to occur even in patients still receiving antiviral prophylaxis. In a recent study, it was concluded that antigenemia was the assay that better identified patients at risk of developing CMV disease in transplant patients when compared to quantitative PCR and a qualitative NASBA assay [6].

On comparing the immunoperoxidase staining for CMV antigen by the slide method (S-Ag) to flow cytometry antigenemia assay (FC-Ag), S-Ag showed higher sensitivity in detecting patients who developed CMV disease. This might be due to the fact that the 2 methods do not calculate the absolute number of PMNLs expressing antigen in the same way. Previous studies evaluating the use of flow cytometry to detect CMV infections in PBSCT recipients were either performed alone [8], or in comparison with culture technique [3,15] and analyzed few numbers of CMV positive patients [3,8]. These studies indicated that FC-Ag identification of CMV in PBSCT recipients correlated with isolation and had the advantage of rapid detection of CMV in blood samples [3,8,15].

One study compared shell vial cultures, quantitative PCR, S-Ag and FC-Ag in 25 SCT recipients and found FC-Ag results to correlate better with PCR and S-Ag than shell vial cultures. Discrepancies occurred between S-Ag and FC-Ag and were mainly observed for patients with active CMV infection during very early or late phases of the episode of infection [15]. Thus, detection of CMV antigen by flow cytometry gives rapid results, easier than the slide method and can be depended upon for the positive cases but negative cases need to be double checked by another method.

In view of our results, we conclude that CMV poses a high threat to the outcome of PBSCT recipients in our center and is strongly related to seropositivity of donor and recipient prior to transplant. In addition, risk adapted intervention can be tailored depending on the results of antigenemia.

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