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Table 3. Multivariate analysis of factors influencing OS in the subgroup of ATL patients receiving transplantation after MAC

Variable	No.	HR	95% CI	P
Age range at transplantation, y				
15-55	246	1.000		Reference
56-72	32	1.667	(1.051-2.643)	.030
Sex				
Female	120	1.000		Reference
Male	158	1.458	(1.053-2.019)	.023
Disease status at transplantation				
CR	95	1.000		Reference
Non-CR	159	2.071	(1.409-3.043)	< .001
Unknown	24	1.536	(0.822-2.870)	.178
PS				
0	102	1.000		Reference
1	120	1.322	(0.909-1.922)	.144
2-4	36	3.073	(1.920-4.919)	< .001
Unknown	20	1.109	(0.565-2.175)	.764
Relationship between recipient and donor				
HLA-matched related	96	1.000		Reference
HLA-mismatched related	21	1.165	(0.618-2.196)	.637
Unrelated	161	1.323	(0.920-1.902)	.131
Type of MAC				
TBI-based	208	1.000		Reference
BU-based	46	0.757	(0.475-1.206)	.242
Mel-based	21	1.388	(0.819-2.353)	.223
Others	3	0.666	(0.158-2.817)	.581

PS, relationship of the donor to the recipient, and preconditioning regimen. Among the 578 patients for whom multivariate analysis for OS was conducted (Table 2), when the HR for death of MAC recipients of a younger age (15-55 years) was determined as 1.000, the HRs of MAC recipients in the older age group (56-72 years) and RIC recipients in the younger and older age groups were 1.884, 1.239, and 1.443, respectively ($P_{interaction} = 0.072$; Figure 2A). When the HR for death of MAC recipients with CR at transplantation was determined as 1.000, HRs of MAC recipients with non-CR and RIC recipients with CR and non-CR were 2.117, 1.180, and 2.145, respectively ($P_{interaction} = 0.544$; Figure 2B). When the HR for death of MAC recipients with PS 0 at transplantation was determined as 1.000, HRs of MAC recipients with PS 1 and RIC recipients with PS 0 and 1 were 1.289, 0.839, and 1.429, respectively ($P_{interaction} = 0.268$), and HRs of MAC and RIC recipients with PS 2 to 4 were 3.166 and 4.351, respectively ($P_{interaction} = 0.115$; Figure 2C).

Multivariate analysis of factors influencing OS in the subgroup of ATL patients who had transplantation after MAC

Of the 280 ATL patients who received MAC, 1 patient was excluded because of missing data on the time from diagnosis to transplantation and one was excluded because of lack of data on HLA. Multivariate analysis was therefore conducted on 278 patients and included the variables of age, sex, disease status, PS, and relationship of the donor to recipient, which were found to have significantly affected OS in the entire subject population (Table 2). Also included was a sixth variable, the type of MAC (TBI, BU, Mel-based, or others). Of these 6 variables, 4 significantly affected OS, namely, older age (HR, 1.667; 95% CI, 1.051-2.643), male sex (HR, 1.458; 95% CI, 1.053-2.019), not in CR (HR, 2.071; 95% CI, 1.409-3.043), and worse PS (2-4 compared with 0; HR, 3.073; 95% CI, 1.920-4.919; Table 3).

Multivariate analysis of factors influencing OS in the subgroup of patients receiving transplantations after RIC

Of the 306 ATL patients receiving RIC, 3 were excluded because of lack of data on the time from diagnosis to transplantation, 2 were excluded because of receiving BMT and PBSCT together, and 1 was excluded because of lack of data on HLA. Thus, multivariate analysis on 300 ATL patients was performed using the following 6 variables: age, sex, disease status, PS, relationship of the donor to the recipient, and type of RIC (Flu + BU, Flu + Mel-based, or others). Of these, 4 significantly affected OS, namely, male sex (HR, 1.475; 95% CI, 1.100-1.978), not in CR (HR, 1.743; 95% CI, 1.249-2.432), worse PS (1 compared with 0; HR, 1.803; 95% CI, 1.293-2.516, 2-4 compared with 0; HR, 6.175; 95% CI, 3.908-9.756), and type of RIC (Flu + Mel compared with Flu + BU based; HR, 0.645; 95% CI, 0.453-0.918; Table 4).

Multivariate analysis of TRM and ATL-related mortality

Among the 586 ATL patients receiving allogeneic BMT or PBSCT, 14 could not be assigned to either the TRM or ATL-related mortality category because detailed information regarding cause of death was missing. The Fine and Gray proportional hazards model was applied to the remaining 572 patients to identify variables affecting TRM and ATL-related mortality, respectively. The variables included age, sex, disease status, PS, and relationship between recipient and donor, which was shown to significant affect OS in the entire patient population (Table 2), and the preconditioning regimen, namely, MAC or RIC. Among these variables, sex and PS were significantly associated with TRM. The HR for TRM of male patients was 1.383 (95% CI, 1.026-1.863). HRs for TRM of recipients with PS 1 and PS 2 to 4 compared with PS 0 were 1.509 (95% CI, 1.075-2.118) and 3.004 (95% CI, 1.915-4.714), respectively. Conversely, disease status, PS, and the preconditioning regimen were significantly associated with ATL-related mortality. HR for ATL-related mortality of recipients not in CR was

Table 4. Multivariate analysis of factors influencing OS in the subgroup of patients receiving transplantation after RIC

Variable	No.	HR	95% CI	P
Age range at transplantation, y				
15-55	122	1.000		Reference
56-72	178	1.127	(0.834-1.523)	.435
Sex				
Female	147	1.000		Reference
Male	153	1.475	(1.100-1.978)	.009
Disease status at transplantation				
CR	110	1.000		Reference
Non-CR	176	1.743	(1.249-2.432)	.001
Unknown	14	1.959	(0.998-3.843)	.051
PS				
0	117	1.000		Reference
1	140	1.803	(1.293-2.516)	< .001
2-4	38	6.175	(3.908-9.756)	< .001
Unknown	5	4.979	(1.849-13.409)	.001
Relationship between recipient and donor				
HLA-matched related	114	1.000		Reference
HLA-mismatched related	41	1.279	(0.836-1.959)	.257
Unrelated	145	1.237	(0.895-1.710)	.198
Type of RIC				
Flu + BU-based	165	1.000		Reference
Flu + Mel-based	86	0.645	(0.453-0.918)	.015
Others	49	0.854	(0.557-1.310)	.470

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Table 5. Multivariate analysis of TRM and ATL-related mortalities in patients receiving allogeneic HSCT

	TRM				ATL-related mortality			
Variable	No.	HR	95% CI	P	No.	HR	95% CI	P
Age range at transplantation, y								
15-55	116/362	1.000		Reference	93/362	1.000		Reference
56-72	79/210	1.403	(0.954-2.064)	.085	62/210	0.955	(0.658-1.385)	.810
Sex								
Female	75/262	1.000		Reference	66/262	1.000		Reference
Male	120/310	1.383	(1.026-1.863)	.033	89/310	1.226	(0.886-1.697)	.220
Disease status at transplantation								
CR	58/205	1.000		Reference	32/205	1.000		Reference
Non-CR	121/330	1.238	(0.906-1.691)	0.180	114/330	2.203	(1.469-3.302)	< .001
Unknown	16/37	1.507	(0.873-2.603)	0.140	9/37	1.511	(0.663-3.444)	.330
PS								
0	54/213	1.000		Reference	44/213	1.000		Reference
1	91/260	1.509	(1.075-2.118)	.017	74/260	1.272	(0.872-1.856)	.210
2-4	41/75	3.004	(1.915-4.714)	< .001	30/75	1.679	(1.035-2.723)	.036
Unknown	9/24	1.214	(0.614-2.403)	0.580	7/24	1.965	(0.802-4.818)	.140
Relationship between recipient and donor								
HLA-matched related	62/206	1.000		Reference	60/206	1.000		Reference
HLA-mismatched related	18/62	0.924	(0.532-1.606)	0.780	26/62	1.392	(0.873-2.220)	.160
Unrelated	115/304	1.429	(1.033-1.975)	.031	69/304	0.843	(0.589-1.209)	.350
Preconditioning regimen								
MAC	100/274	1.000		Reference	61/275	1.000		Reference
RIC	95/298	0.786	(0.538-1.148)	0.210	94/304	1.579	(1.080-2.308)	.019

2.203 (1.469-3.302). The HR for ATL-related mortality of recipients with PS 2 to 4 compared with PS 0 was 1.679 (95% CI, 1.035-2.723), and the HR of patients receiving RIC compared with MAC was 1.579 (95% CI, 1.080-2.308; Table 5).

Cumulative incidence of TRM and ATL-related mortality

Among the 572 ATL patients receiving allogeneic BMT or PBSCT, the cumulative incidence of TRM one year after transplantation was 32.7% (95% CI, 27.1-38.4) in MAC recipients and 29.2% (95% CI, 24.0-34.5) in RIC recipients. These figures at 3 years were 37.7% (95% CI, 31.8-43.6) and 33.3% (95% CI, 27.7-38.9), respectively (Figure 3). The cumulative incidence of ATL-related mortality 1 year after transplantation was 18.5% (95% CI, 14.1-23.4) for MAC and 25.0% (95% CI, 20.1-30.1) for RIC recipients and was 22.5% (95% CI, 17.5-27.9) and 33.2% (95% CI, 27.6-38.9), respectively, at 3 years (Figure 3).

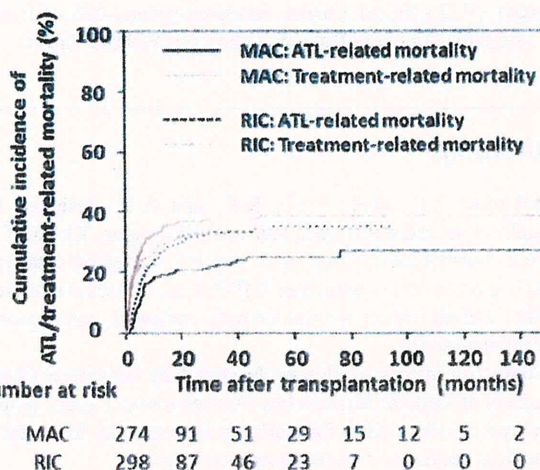

Figure 3. Cumulative incidence of ATL-related and TRMs in patients receiving BMT or PBSCT. Probabilities of ATL-related and TRMs in recipients of MAC or RIC were estimated using cumulative incidence curves to accommodate competing events.

Discussion

To the best of our knowledge, the present study is the largest retrospective study of ATL patients receiving allogeneic HSCT. Results showed that for allogeneic BMT or PBSCT for ATL, RIC was applied more frequently in older patients, as is reasonable and expected. RIC patients more often received PBSCT and had related donors. We surmise this was because RIC was initially proposed in the setting of PBSCT from HLA-matched sibling donors.[30]

The OS plot of ATL patients receiving allogeneic HSCT reached a plateau, leading to long-term survival of a subgroup of ATL patients. Recipients of CBT had a significantly worse prognosis than recipients of BMT or PBSCT, which was consistent with our previous report.[11] Direct comparison of transplantation outcomes between unrelated CBT and the other types of allogeneic HSCT was not possible because the selection of the graft source is an individual process strongly influenced by donor availability and the patient's ATL status. However, even considering such potential biases, the outcome of unrelated CBT seems clearly unsatisfactory. Thus, novel strategies to further improve the outcomes of unrelated CBT are warranted.

Among ATL patients receiving allogeneic BMT or PBSCT, multivariate analysis revealed 5 significant independent variables affecting OS, namely, age, sex, disease status, PS, and relationship between the recipient and donor. Of these factors, younger age, good ATL disease status, and PS at transplantation contributing to better OS were to be expected. The contribution to a better OS of HSCT from HLA-A, -B, and -DR–matched related donors also would be expected. The reason why the female sex was an independent favorable factor is not fully understood but is consistent with results of our previous study.[11] With respect to preconditioning, there was no significant difference in OS between MAC

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and RIC recipients. To further clarify the clinical significance of preconditioning in allogeneic BMT or PBSCT for ATL, we analyzed the interactions of preconditioning with age, disease status, and PS. There was a clear trend indicating that RIC contributed to better OS in older patients compared with MAC. In contrast, the associations between MAC and RIC to OS were almost similar even if ATL patients at transplantation were in CR or not. In general, when considering allogeneic HSCT for many other types of leukemia/lymphoma patients who are in non-CR, it seems more usual to apply MAC for those patients because MAC should have the more potent effect in eradicating residual leukemia/ lymphoma cells than RIC. However, the present study does not support this strategy at least in HSCT for ATL. The associations between MAC and RIC to OS were almost similar even when the PS at transplantation was 0, 1, or 2 to 4. In general, considering allogeneic HSCT for patients who have a worse PS, it seems to be more usual to apply RIC because RIC should be less toxic for recipients than MAC. However, the present study also does not support this strategy, at least in HSCT for ATL.

In the subgroup analyses stratified by MAC or RIC, older age was an independent unfavorable prognostic factor in MAC recipients, but not in RIC recipients. Female sex, good ATL disease status, and PS significantly contributed to better OS in both groups. Among MAC recipients, there was no significant difference in OS according to the type of MAC, but among RIC recipients, a Flu + Mel–based regimen contributed to better OS compared with a Flu + BU–based regimen. Although RIC regimens that contain alemutuzumab have been widely used in various parts of the world,[31] we had no data available as to whether any of the regimens used included alemtuzumab. Thus, we were not able to clarify the significance of the inclusion of alemtuzumab as a conditioning agent.

Multivariate analysis of variables contributing to mortality demonstrated that there was significantly more ATL-related mortality in RIC recipients. Although not statistically significant, a clear trend showed an association of increased TRM but not ATL-related mortality in older patients. Male sex was significantly associated with increased TRM, which might contribute to the better OS of female recipients. ATL patients not in CR had greater ATL-related mortality, but not TRM. A poor PS was significantly associated with both ATL-related mortality and TRM, but the association was closer with TRM. HSCT from unrelated donors was significantly associated with increased TRM but not with ATL-related mortality.

Cumulative incidence curves of TRM and ATL-related mortalities in MAC and RIC recipients showed characteristic features as illustrated in Figure 3. In comparison with the black lines indicating ATL-related mortality, the red lines showing TRM rise in the early phase after transplantation. Two solid lines for MAC had quite different trajectories, with TRM being greater than ATL-related mortality at any time after transplantation. In contrast, the 2 dotted lines for RIC nearly joined at 24 months after transplantation and were almost identical thereafter. Both lines for RIC were between those for MAC TRM and ATL-related mortality.

Currently, several promising new agents for ATL are being developed.[32-35] These novel treatments should increase the number of ATL patients with a sufficient disease control status and who have maintained a good PS who could become suitable candidates for transplantation. This would require further improvement in allogeneic HSCT for ATL as well as better rescue strategies for patients relapsing after HSCT. Although treatment by AZT/IFN-α[6] and/or alemutuzumab[34,36] are applied for ATL patients in many countries, none of these agents are currently approved in Japan for the treatment of ATL under the national health insurance. Therefore, there are currently no data on their clinical impact on outcome after allogeneic HSCT for ATL. We do expect, however, that the application of AZT/IFN and alemutuzumab would contribute to improved outcomes of HSCT for ATL.

Although this study reports significant novel findings for allogeneic HSCT for ATL patients, it also has inherent limitations common among observational retrospective studies. Eligibility for transplantation as well as choice of transplantation protocol, including the selection of MAC or RIC, was determined by the physicians at each institution. Regarding mortality analysis, it is not easy to determine whether death of an ATL patient after allogeneic HSCT is TRM or ATL-related mortality. This is partially because relapsed ATL patients sometimes achieve partial or complete remission on decreasing or discontinuing immunosuppressive agents, donor lymphocyte infusions, or chemotherapy, which can result in long-term remission and survival.[9,13,18]

In conclusion, allogeneic BMT or PBSCT not only with conventional MAC but also RIC is an effective treatment that results in long-term survival of selected patients with ATL. Posttransplantation outcomes are influenced by the recipient's age, sex, PS, disease status at transplantation, and the relationship between recipient and donor. Although no significant difference in OS between MAC and RIC recipients was observed, there was a clear trend that RIC contributed to better OS in older patients. Regarding results of analysis of mortality, RIC was more significantly associated with ATL-related mortality in comparison with MAC. More definitive conclusions on the role of allogeneic HSCT in the therapeutic algorithm for ATL will need to be drawn from well-designed prospective clinical trials.

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Authorship

Contribution: T.I., M.H., K.K., R.T., and A.U. designed the research, organized the project, and wrote the paper; T.I. and T.N. performed statistical analysis; H.S. and R.S. collected data from JSHCT; Y.M. collected data from JMDP; K.K. collected data from JCBBN; and all authors interpreted data, reviewed, and approved the final manuscript.

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Detection of Active Human Cytomegalovirus by the Promyelocytic Leukemia Body Assay in Cultures of PBMCs From Patients Undergoing Hematopoietic Stem Cell Transplantation

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A novel detection system was established previously for cells infected with the human cytomegalovirus (HCMV) in vitro that utilizes the unique IE1-dependent nuclear dispersion of promyelocytic leukemia (PML) bodies early in the HCMV replication cycle. This assay system, designated "the PML assay," makes use of the GFP-PML-expressing cell line SE/15, and allows real-time monitoring of infected cells by fluorescence microscopy without any staining procedures. A rapid and quantitative drug susceptibility testing was developed for low-titer clinical isolates propagated in fibroblasts in vitro. The present study sought to exploit the PML assay for evaluating in vivo status of HCMV without virus isolation. Progeny viruses were detected directly from peripheral blood mononuclear cells (PBMCs) infected in vivo obtained from hematopoietic stem cell transplantation recipients. The overall positivity of the PML assay tended to correlate with the levels of genomic DNA. Direct phenotypic susceptibility testing detected one ganciclovir (GCV)-resistant case among 19 samples, which was confirmed further by genomic and plaque reduction assays. However, in another patient with the sequence-proven mutant confirmed by sequencing, the progeny viruses exhibiting GCV-resistance were not detected. Studies on the isolated virus from the latter patient suggested the possibility that replication efficiency may differ between PBMCs and lesions infected in vivo, which may hamper the detection of GCV-resistant viruses by the PML assay, at least in this case. Taken together, the PML assay is sufficiently sensitive to monitor replication-competent HCMV directly from PBMCs infected in vivo, and provides a novel tool for comparing the characteristics of HCMV strains infected in vivo. ***J. Med. Virol. 84:479–486, 2012.***
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KEY WORDS: PML assay; GFP-PML; HCMV; clinical isolate; drug susceptibility testing; hematopoietic stem cell transplantation

INTRODUCTION

Despite recent advances in antiviral therapy, human cytomegalovirus (HCMV) remains a common viral pathogen that causes problematic infections, especially in patients undergoing hematopoietic stem cell transplantation and solid organ transplantation. The diagnosis and monitoring of HCMV in hematopoietic stem cell transplantation and solid organ transplantation recipients have advanced rapidly in the last two decades [Razonable et al., 2002; Baldanti et al., 2008], which has allowed the development of

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prophylactic or preemptive therapies for HCMV infection and disease [Griffiths and Boeckh, 2007]. The widely used HCMV diagnostic assays include the detection of pp65-positive leucocytes in antigenemia assays and quantitative PCR assays of viral DNA in blood [The et al., 1990; Grossi et al., 1995; van der Meer et al., 1996], both of which are based on the presence of virus or viral products in blood irrespective of the infectivity. Although pp65-guided preemptive therapy is highly effective in the management of HCMV infection and disease [Boeckh et al., 1996; Kanda et al., 2001; Asano-Mori et al., 2005], and is believed to reflect the activity of HCMV in vivo, insufficient sensitivity has been reported occasionally in patients with HCMV-associated gastrointestinal disease or HCMV retinitis [Boeckh and Ljungman, 2009]. On the other hand, genomic DNA evaluation by quantitative PCR is a highly sensitive method, although increased viral loads may include both replication-competent and -incompetent viruses and/or debris from infected cells [Razonable et al., 2002; Baldanti et al., 2008]. To improve patient management, rapid and accurate determination of drug susceptibility is important to distinguish apparently reduced sensitivity against antivirus drugs caused by severe immunosuppression. Some paradoxical phenomena, such as dissociation between antigenemia and the viral load, have been reported [Gerna et al., 2003]. Analysis of HCMV infectivity in vivo is important for understanding the molecular basis of these inconsistent phenomena, concomitant with currently available clinical testing. Although the shell vial assay is an alternative method for evaluating replication-competent viruses [Gleaves et al., 1984; Griffiths et al., 1984; Patel et al., 1995], it is labor-intensive for monitoring active HCMV in clinical specimens.

A novel detection system was established previously for cells infected with HCMV in vitro that makes use of SE/15 cells stably expressing GFP-PML [Ueno et al., 2006; Ueno and Ogawa-Goto, 2009]. In the nucleus of these cells, HCMV deposits its genome adjacent to promyelocytic leukemia (PML) bodies and starts IE transcription, which leads to disruption of the PML bodies mainly through de-SUMOylation of PML protein by the function of IE1 protein [Korioth et al., 1996; Ahn and Hayward, 1997; Muller et al., 1998]. Hence, visual assessment by fluorescence microscopy is sufficient for detection without the necessity for any further procedures, such as fixation and staining. The assay can detect progeny viruses of not only laboratory strains but also clinical isolates. These features permit continuous tracing over the time course of a single culture, and are particularly advantageous for clinical specimens, the amounts of which are often limited. Thus, it is expected that the use of GFP-PML-expressing cells will provide a quantitative method for monitoring replication-competent viruses as an alternative to the shell vial assay.

In the present study, a protocol for direct detection of HCMV progeny viruses is presented for evaluation of peripheral blood mononuclear cells (PBMCs) infected in vivo from patients undergoing hematopoietic stem cell transplantation, which enables susceptibility tests for anti-HCMV drugs without virus isolation procedures. In this article, the detection system using GFP-PML-expressing cells is designated the PML body assay ("the PML assay"), and positive cells exhibiting diffuse GFP-PML in their nucleus are designated "PML-positive" cells, which were triggered by infection of progeny viruses during in vitro cocultures.

MATERIALS AND METHODS

Patients and Clinical Specimens

The present prospective study was approved by the ethical committees at Saitama Medical Center of Jichi Medical University and Toranomon Hospital. After informed consent was obtained, 25 whole blood specimens from 20 individuals who underwent allogeneic hematopoietic stem cell transplantation from an HLA-matched or -mismatched donor at Saitama Medical Center of Jichi Medical University or Toranomon Hospital were evaluated. The patients were diagnosed as positive in HCMV antigenemia tests by the C7-HRP or C10/C11 method during routine surveillance. The patient characteristics are summarized in Table I.

Blood specimens (~15 ml) were aliquoted into three parts, comprising 200 μl for virus isolation in fibroblasts, 200 μl for DNA extraction and the remaining volume for PBMC preparation. PBMCs were prepared using Lymphoprep (Axis-Shield, Oslo, Norway) according to the manufacturer's instructions, and cultured in fibronectin-coated 24-well plates at a density of 0.6–2 × 10^6 cells/well. DNA was extracted from 200 μl of whole blood using QIAamp DNA blood mini kits (Qiagen, Hilden, Germany). Quantitative PCR of the genomic HCMV DNA was performed as described previously [Leruez-Ville et al., 2003].

TABLE I. Summary of Patients' Characteristics

Age, median (range)	50 (17–65)
Sex (male/female)	10/10
CMV serostatus before transplantation	
Recipient CMV-positive	19
Donor CMV-positive	10
Underlying disease	
AML	7
SAA	5
ATL	2
DLBCL	2
ALL	3
CML	1
Graft source	
Bone marrow	10
Peripheral blood stem cell	7
Cord blood	3
Donor type	
Matched related	3
Mismatched related	4
Unrelated	13

Cell Culture, Virus, and Reagents

The SE/15 cell line [Ueno et al., 2006] was used throughout this study. HCMV clinical isolates were propagated and titrated in human embryonic lung fibroblasts. Virus inoculums were prepared by ultrasonication of infected fibroblasts and stored in medium containing glycerol. Plaque reduction assays in fibroblasts or drug susceptibility tests estimated by SE/15 cells were carried out as described previously [Ueno et al., 2006]. Ganciclovir (GCV), foscarnet (FOS) (Sigma–Aldrich, St. Louis, MO) and cidofovir (a generous gift from Prof. E. De Clercq) were used as anti-HCMV drugs.

Protocol for In Vitro Infection of PBMCs by Clinical Isolates and Detection of Progeny Viruses by SE/15 Cells

PBMCs were prepared from EDTA-treated blood obtained from HCMV genome-negative volunteers and cultured in RPMI1641 medium containing 10% FBS. The protocol for the PML assay was determined using PBMCs infected in vitro as follows. PBMCs (4×10^5) were cultured in 24-well plates, and non-adherent cells were removed by washing with PBS. Subsequently, 200 µl of a clinical isolate inoculum was added and incubated for 2 hr. The cells were then extensively washed with PBS and cultured in RPMI1641 medium containing 10% FBS. At 3 days post-inoculation, SE/15 cells (5×10^4) were added after extensive washing with PBS, and maintained until 9 or 10 days post-inoculation. The numbers of PML-positive cells were counted daily by fluorescence microscopy. To characterize the cell types of the HCMV-infected cells, PBMCs infected in vitro were subjected to immunofluorescence analyses using antibodies against UL44 (Dako, Glostrup, Denmark) and CD14 (Novocastra Laboratories, Newcastle Upon Tyne, UK). UL44, an HCMV early gene antigen, was detected in the nucleus of CD14-positive cells (data not shown), confirming that monocytes/macrophages were the targets for the viruses. Among several reagents reported to be enhancers of HCMV replication in fibroblasts [West and Baker, 1990], dexamethasone (Dex) was found to be effective for progeny production in these cultures. Therefore, 5 µM Dex was used in the following experiments for cells infected in vivo.

Coculture of PBMCs Infected In Vivo With SE/15 Cells and Monitoring of Progeny Viruses

PBMCs ($0.6–2 \times 10^6$ cells/well) from allogeneic hematopoietic stem cell transplantation recipients were cultured in RPMI1641 medium containing 10% FBS. In Exp.0D, the coculture was started on the day of PBMC preparation and continued until day 6 or 7, while in Exp.3D, SE/15 cells were added on day 3 of PBMC culture and the culture was continued until day 9. During the cocultures, the numbers of PML-positive cells were monitored daily by fluorescence microscopy. In some experiments, cocultures were performed in the presence or absence of GCV or FOS.

Statistical Analysis

Fisher's exact test was used to compare the positive rates of the PML assay or virus isolation.

RESULTS

Detection of Progeny Viruses Produced From PBMCs Infected In Vivo

To test whether the PML assay detects progeny viruses produced by cells infected in vivo, PBMCs from patients with a variety of antigenemia levels after hematopoietic stem cell transplantation were used. Two series of experiments were carried out, designated Exp.0D and Exp.3D (Fig. 1A). In Exp.0D, the coculture started on the day of PBMC preparation, while in Exp.3D, SE/15 cells were added on day 3 of PBMC culture, and the numbers of PML-positive cells were monitored (Fig. 1A and B). In most cases, PML-positive cells began to appear from days 3 to 4 and peaked around day 5 in Exp.0D, while they peaked from days 7 to 9 in Exp.3D. Representative curves for Exp.0D and Exp.3D in the presence and absence of 5 µM Dex are shown in Figure 1C and D, respectively. More than 60% of the specimens tested responded to Dex treatment to various degrees.

The results of the PML assay together with the virus isolation are summarized in Table II. Overall, 20 of 25 (80%) samples were positive in either Exp.0D or Exp.3D. On the other hand, HCMV was successfully isolated in 11 of 25 (44%) samples using fibroblasts, and PML assays were positive in 10 of the 11 culture-proven HCMV-infected specimens. For comparison with the genomic DNA levels, the samples were categorized into two groups based on the copy numbers of the HCMV genome, namely low-grade ($<5 \times 10^3$ copies/ml) and high-grade ($\geq 5 \times 10^3$ copies/ml) groups. No viruses were isolated in the low-grade group, while viruses were successfully isolated from 64% of the high-grade group specimens.

In the high-grade group, 12 of 16 (75%) samples exhibited PML-positive cells in Exp.0D, which was significantly higher than the corresponding 2 of 8 (25%) samples for the low-grade group. Higher numbers of PML-positive cells were observed in the high-grade group in both Exp.0D and Exp.3D. However, a considerable number of high-grade group specimens exhibited undetectable or very low levels of PML-positive cells despite high levels of antigenemia and/or genome copy numbers. No significant difference was observed for the peak appearance day between the two groups.

Drug Susceptibility Test Using PBMCs Infected In Vivo

To establish a susceptibility test for anti-HCMV drugs in Exp.3D, PBMCs were cultured singly in the

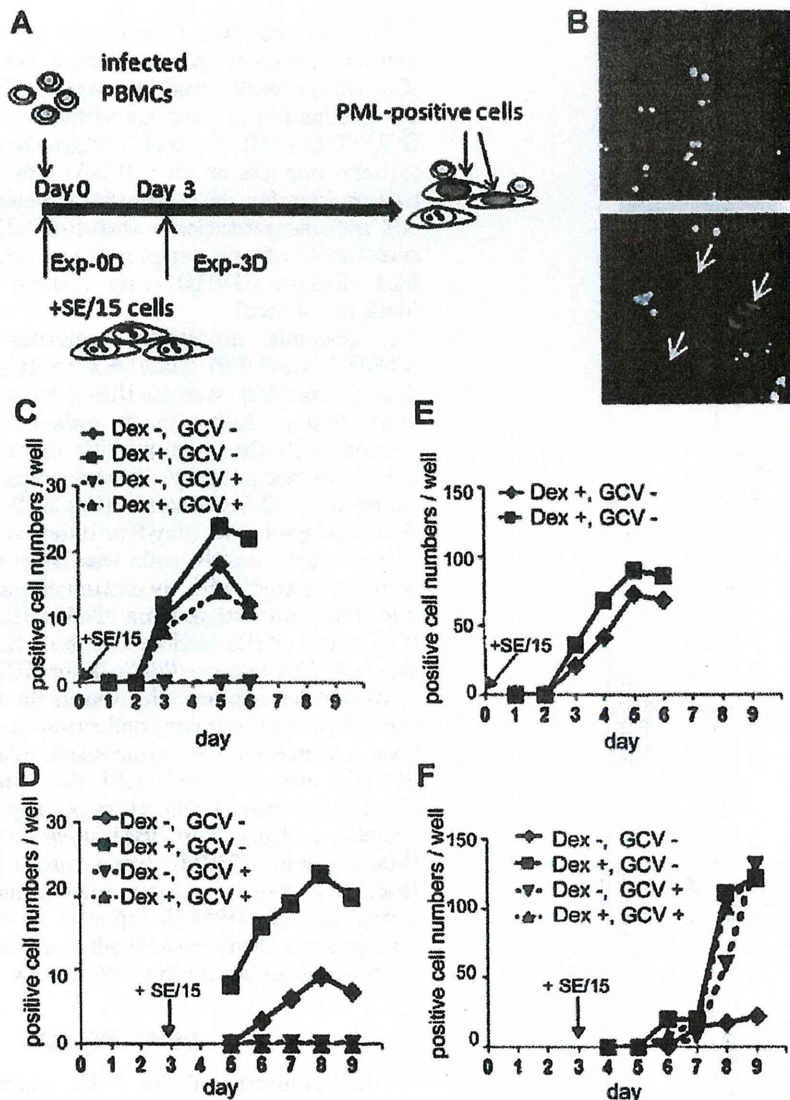

Fig. 1. Time-course profiles of progeny virus production from PBMCs obtained from hematopoietic stem cell transplantation recipients. A: Schematic representation of the PML assay using PBMCs infected in vivo obtained from patients after hematopoietic stem cell transplantation. B: Representative images of PML-positive cells (lower, shown by arrows) and PML-negative cells (upper) during the PML assay upon cocultures with the PBMCs infected in vivo. Images under fluorescence microscopy are shown. C–F: Exp.0D (C,E) and Exp.3D (D,F). In Exp.0D the coculture was started on the day of PBMC preparation. In panel C, the coculture was carried out in the presence or absence of GCV. In Exp.3D, SE/15 cells were added on day 3 of PBMC culture and the culture was continued until day 9 in the presence or absence of GCV. Representative curves for Exp.0D and Exp.3D in the presence and absence of 5 μM Dex are shown. C, D: #hsct-24; E, F: #hsct-14.

presence or absence of GCV for 3 days, followed by the start of coculture at day 3 (Fig. 1A). Addition of GCV inhibited the production of PBMC-derived progeny viruses in Exp.3D, but not in Exp.0D (Fig. 1C and D). The PML-positive cells in Exp.0D, even in the presence of GCV, were attributed to active viruses at blood sampling that had already passed through the phase of viral DNA replication, thereby escaping the inhibitory action of GCV that specifically targets viral DNA polymerase. Among the 19 samples tested for Exp.3D, six samples were determined to have apparent low sensitivity for GCV under the conditions used (Figs. 1F and 2A, Table III).

Genomic sequencing analyses revealed mutations conferring GCV-resistance in one patient (#hsct-17), who had been treated with GCV for more than 75 days. The mutations included C603W in the UL97 gene and T503I in the UL54 gene. Using an isolated virus strain from #hsct-17, an increased IC_{50} for GCV (21 μM) was confirmed, which was defined as a

TABLE II. Summary of the PML Assay With In Vivo Infected PBMCs and Virus Isolation

	The PML assay using in vivo infected PBMCs						Virus isolation in fibroblasts
	Exp.0D			Exp.3D			
	Positive % (positive/total)	Average peak appearance day	Median number of positive cells/ml blood (range)	Positive % (positive/total)	Average peak appearance day	Median number of positive cells/ml blood (range)	Positive % (positive/total)
Total	58 (14/24)	5.2	5.4 (0–68)	76 (19/25)	8.6	10.9 (0–141)	44 (11/25)
HCMV genome DNA							
Low grade ($5 \times 10^3 >$)[a]	25 (2/8)	5.5	0 (0–11)	63 (5/8)	8.3	3.0 (0–25)	0 (0/8)
High grade ($5 \times 10^3 \leq$)[a]	75** (12/16)	5.1	6.4 (0–68)	83 (14/17)	8.7	14.4 (0–141)	64*** (11/17)

[a]Copies/ml blood.
**$P < 0.05$ against low-grade group.
***$P < 0.005$ against low-grade group.

GCV-resistant level (Table III). On the other hand, neither apparent genomic mutations nor increased IC_{50} values were found in the other five cases, despite the production of progeny viruses in the presence of GCV (Table III). Several background factors reported to have impacts on the HCMV infection and disease had no bias for the apparent low susceptibility without genome mutations, including the donor HCMV serostatus before transplantation, acute graft-versus-host disease (GVHD) and relapse of antigenemia (data not shown).

A genomic mutation conferring GCV-resistance (L595S in the UL97 gene) was identified in another patient (#hasct-22), who had been treated with GCV for about 70 days. Although the antigenemia value jumped to over 7,000, the susceptibility test in Exp.3D was unable to detect progeny viruses produced from PBMCs exhibiting GCV-resistance (Fig. 2B). It was notable that a delayed peak (day 9 or later) with very low numbers of PML-positive cells was seen, which seemed to be uncorrelated with the extremely high levels of genomic DNA and antigenemia (Table III). In contrast, the IC_{50} value of the isolated virus in fibroblasts showed clear GCV-resistance (23.2 μM for GCV).

To further explore the reason for the discrepancy, the efficiencies of virus replication in vitro in different host cell species were addressed. When fibroblasts or PBMCs were infected with the same titers of the #hsct-22 isolate at the third passage, the replication speeds of #hsct-22 in PBMCs were apparently slower than those in a PBMC tropic control strain (isolate A) (Fig. 2C). Thus, an apparent inconsistency was observed for the PBMCs infected in vivo between the antigenemia levels/viral loads and progeny virus production estimated by the PML assay.

DISCUSSION

The hallmarks of the PML assay including high specificity, a simple procedure that enables sequential tracing, sensitive, and quantitative evaluation for low-titer clinical isolates have been verified by the previous study [Ueno et al., 2006]. The present study has demonstrated that the PML assay is sufficiently sensitive for direct monitoring of HCMV produced from PBMCs infected in vivo from hematopoietic stem cell transplantation recipients. In particular, the sequential tracing of the same culture has a clear advantage for analyzing the limited amounts of clinical specimens, where treatment by Dex enhanced viral replication in most of the PBMC specimens as in fibroblasts [West and Baker, 1990]. In addition, the diffuse nuclear pattern of GFP-PML has never been caused by infection with other herpes viruses including human HSV, VZV and HHV-6 (Ueno et al., unpublished observation), further confirming that the nuclear diffuse PML phenotype is HCMV IE-1 dependent [Ueno et al., 2006; Ueno and Ogawa-Goto, 2009].

In the present study, the overall positivity of the PML assay appeared to be correlated roughly with

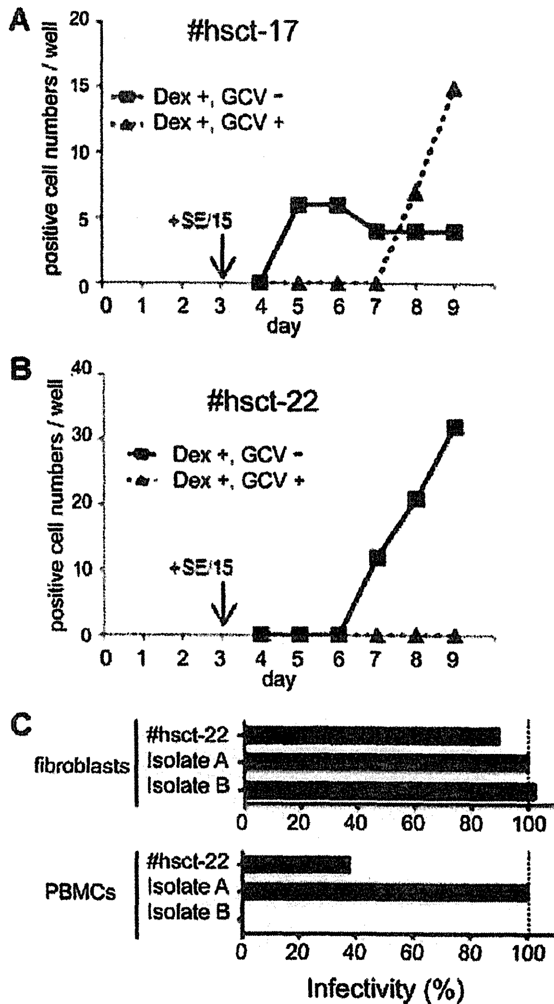

Fig. 2. Time-course profiles of progeny virus production and replication efficiency of sequence-proven GCV-resistant HCMV isolates. **A,B**: Progeny virus production was monitored in PBMCs from hematopoietic stem cell transplantation recipients who were diagnosed as being infected with GCV-resistant strains in a subsequent sequence analysis. SE/15 cells were added on day 3 of PBMC culture and the culture was continued until day 9 in the presence or absence of GCV. The data for Exp.3D are shown. A: #hsct-17; B: #hsct-22. **C**: The efficiencies of replication in fibroblasts and PBMCs were compared using the third passage isolate strain of #hsct-22, which had been propagated in fibroblasts. Isolate A: a positive control strain virus that can efficiently replicate in PBMCs; isolate B: a negative control strain virus that cannot replicate in PBMCs.

the levels of genomic DNA. Although the median numbers were increased modestly in the high-grade DNA group, the numbers of PML-positive cells were not well correlated with the DNA levels occasionally. Notably, there were several exceptional cases that showed apparent inconsistencies between the data from the PML assay and the viral loads. This tendency for inconsistency is elicited possibly by the different basis of the assays for evaluating the productive infectivity or the virus and/or viral products in blood [Razonable et al., 2002; Baldanti et al., 2008]. The levels of antigenemia or viral loads reflect the in vivo

TABLE III. Data of the PML Assay, Antigenemia, and Genomic DNA Levels, and Virus Characteristics Which Showed Low Susceptibility to GCV in the PML Assay

		HCMV Genome	The PML assay				Antigenemia		GCV treatment		Virus isolation	Genome analysis (blood)		IC50 (μM)		
			Exp.0D	Exp.3D	Susceptibility against											
Patient ID	Days post-transplantation at sampling	Copies/ml blood	Positive cells/ml blood	Positive cells/ml blood	GCV	FOS	Value[a] (day)	Assay method[b]	At sampling day	Duration (day)	In blood	UL54	UL97	GCV	FOS	CDV
#hsct-07	44	1.1×10^3	—	11.4	Low	S	261 (day 40)	A	+	16	–	—[c]	—[c]			
#hsct-09	63	5.8×10^3	—	17.0	Low		28 (day 61)	A	–	0	+	n.d.	n.d.	0.39	19	0.93
#hsct-12	163	1.7×10^4	33.3	10.9	Low	S	96 (day 159)	B	+	9	–	—	—			
#hsct-14	34	2.2×10^4	68.2	92.4	Low[#01]		79 (day 32)	A	+	14	+	n.d.	n.d.	0.33	64	1.3
#hsct-20	49	4.5×10^4	55.6	141.0	Low		14 (day 47)	B	+	14	+	n.d.	n.d.	0.46	178	1.8
#hsct-17	238	3.0×10^4	—	14.3	Low[#02]		61 (day 234)	A	+	77	+	T503I[d]	C603W[d]	21	81	4.4
#hsct-22	107	9.9×10^4	—	7.4	S[#03]		1,008 (day 107)	A	+	70	+	–	L595S[d]	23	92	2.8

S: sensitive; Low: low susceptibility assigned by the PML assay; n.d.: not determined.
#01, #02 and #03; time course profiles were shown in Figures 1F and 2A,B, respectively.
[a]Positive cell numbers/2 slides for data in C10/C11 assay or positive cell numbers/5×10^5 cells for data in C7-HRP assay were shown.
[b]A:C10/C11, B:C7-HRP.
[c]Sequence data showed no clear mutations conferring resistance, but partially contained unreadable ones probably due to heterogeneity of the template.
[d]Reported mutations conferring GCV resistance.