Heightened Peripheral Blood Lymphocyte CD69 Expression is Neither Sensitive nor Specific as a Noninvasive Diagnostic Test for Renal Allograft Rejection

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Abstract. It has been reported that acute allograft rejection is associated with heightened expression of the peripheral blood lymphocyte (PBL) early activation marker CD69 and that this may serve as a potential biomarker of rejection. This study sought to determine whether PBL CD69 expression correlates with both acute clinical and subclinical renal allograft rejection as well as clinically apparent cytomegalovirus (CMV) infection. Flow cytometric determination of PBL CD69 expression was performed at the time of clinical and protocol biopsies in 45 renal transplant recipients. Nineteen patients also underwent weekly monitoring of PBL CD69 expression for the initial 15 wk after transplantation. Simultaneous screening for CMV viremia was performed with a semiquantitative PCR assay. No differences were seen in either CD4+ or CD8+ lymphocyte CD69 expression between the biopsy diagnoses. CMV viremia however, independent of rejection, was associated with greater CD69 expression on CD8+ lymphocytes (17.8 ± 10.4% versus 9.6 ± 4.8%; P < 0.0001) but not CD4+ lymphocytes. No individuals experienced clinical CMV disease. Weekly monitoring of PBL CD69 expression did not change coincident with the diagnosis of rejection; however, CMV viremia coincided with a substantial rise in the proportion of CD8+69+ lymphocytes in a number of individuals. Thus, PBL CD69 expression is neither sensitive nor specific for the noninvasive diagnosis of renal allograft rejection. Furthermore, clinically apparent CMV viremia is associated with heightened expression of this activation marker on CD8+ lymphocytes. This latter finding suggests that clinically apparent CMV viremia may be a potential confounder for biomarkers of rejection that examine peripheral blood lymphocytes. mkarpinski@hsc.mb.ca

The development of sensitive and specific noninvasive biomarkers of rejection would benefit kidney transplant recipients considerably. At present, the diagnosis of rejection can only be made by allograft biopsies, which are costly, inconvenient, and carry a small risk of complications (1–3). Biopsies furthermore, do not allow for frequent monitoring of allograft function, which may be advantageous, as rejection can develop in allografts before overt evidence of graft dysfunction (i.e., subclinical rejection) (4–7). Unfortunately, to date, studies evaluating biomarkers have only been performed in the setting of clinically apparent acute rejection. The question remains whether candidate biomarkers are sufficiently sensitive to detect subclinical rejection.

Another concern pertaining to biomarkers is that of specificity. Confounding factors, such as infection, may represent significant obstacles for biomarker development. Immunocompromised transplant recipients are at increased risk of infection, which may be either predominantly isolated to the graft (e.g., BK viral nephropathy) or systemic. Cytomegalovirus (CMV) infection, when monitored by sensitive techniques, is highly prevalent posttransplant and may act as a significant confounder for peripheral blood biomarkers of rejection. Prospective studies demonstrate that >60% of kidney transplant recipients demonstrate CMV viremia posttransplant (8,9).

A number of noninvasive techniques for diagnosing rejection have been investigated in recent years, although none have yet reached clinical application. Recently, two groups have reported that heightened peripheral blood lymphocyte (PBL) expression of the cell surface molecule CD69 correlates with the diagnosis of acute renal and cardiac allograft rejection (10,11). CD69 is a C-type lectin expressed on T lymphocytes and natural killer cells and is one of the earliest surface molecules expressed upon T cell activation (12). Moreover, infiltrating CD8+69+ lymphocytes have been shown to play a role in renal and cardiac allograft rejection, and our own biopsy studies have demonstrated abundant CD69+ lymphocytes in allograft infiltrates in both subclinical and clinical acute rejection (13,14).

The objectives of the current study were twofold: to determine whether flow cytometric determination of PBL CD69...
expression would reliably detect both clinical and subclinical renal allograft rejection, and to determine whether clinically inapparent CMV infection may confound this potential peripheral blood biomarker of rejection.

Materials and Methods
This study protocol was approved by the University of Manitoba Faculty of Medicine Research Ethics Board. The patient population consisted of 45 HLA nonidentical adult renal transplant recipients who underwent transplantation in the Manitoba Transplant Program between 1999 and 2001. All individuals were treated with the same immunosuppressive protocol consisting of basiliximab, cyclosporine microemulsion, mycophenolate mofetil, and prednisone. Basiliximab was administered at a dose of 20 mg on both day 0 and day 4 posttransplant. Cyclosporine was dosed to achieve trough levels of 400 ng/ml in the first month and subsequently adjusted to lower the trough level by 25 ng/ml per month until a trough of 150 to 200 ng/ml was achieved and maintained.

Allograft Biopsies and Peripheral Blood Collection for Determination of Peripheral Blood Lymphocyte CD69 Expression
Since 1990, almost all kidney transplant recipients in the Manitoba Transplant Program have undergone protocol biopsies at 1, 2, 3, and 6 mo posttransplant in addition to clinically indicated biopsies. With informed consent, 3 to 5 ml of peripheral blood was obtained from study participants on the day before or on the day of an allograft biopsy. A total of 131 allograft biopsies with concurrent blood collections were available for analysis in the 45 study participants. One hundred seventeen biopsies were protocol biopsies, and 14 were clinically indicated. Biopsies were scored according to the Banff 97 criteria (15). Subclinical rejection was diagnosed when the allograft biopsy demonstrated rejection of at least Banff type la severity (acute score $\geq 12$) and the serum creatinine was $\leq 110\%$ of baseline values. All acute and subclinical rejections were treated with a tapering course of high-dose prednisone. No steroid resistant rejections were observed.

In addition to blood collections at the time of biopsy, weekly blood collections for prospective monitoring of PBL CD69 expression were gathered in a subset of 19 study participants over the initial 12 to 15 wk posttransplant.

Immunostaining of Peripheral Blood Lymphocytes for the Determination of CD69 Expression
All determinations of PBL CD69 expression were performed on the day of blood collection. For the assay, 100 $\mu$l of whole blood was extracted from an EDTA tube and incubated with fluorescein monochlonal antibodies specific for human CD4 (CD4 IgG1 FITC), CD8 (CD8 IgG1 perCP), and CD69 (CD69 IgG1 PE), as well as the appropriate isotype controls (all antibodies and isotype controls were obtained from Becton Dickinson Immunocytometry Systems, San Jose, CA). After a 15-min incubation at room temperature with the fluorescent antibodies, 2 ml of FACS Lysis Solution (Becton Dickinson, San Jose, CA) was added to lyse red blood cells, and the mixture was then incubated in the dark for another 10 min. Samples were then subsequently centrifuged at 1500 rpm for 12 min, the supernatant was discarded, and the remaining cell pellet was resuspended in 2 ml of phosphate-buffered saline (PBS) with 0.1% sodium azide. After two further wash steps, the final cell pellet was resuspended in 500 $\mu$l of PBS with azide and then temporarily refrigerated at 4°C until flow cytometric data acquisition was performed.

Flow Cytometric Determination of Peripheral Blood Lymphocyte CD69 Expression
All flow cytometric data acquisition was performed with a FACScalibur instrument and analyzed using the accompanying CellQuest software system (Becton Dickinson, San Jose, CA). Lymphocytes were gated on the basis of light scatter characteristics, and at least 10,000 events through the scatter gate were obtained for each patient sample. CD4$^+$ and CD8$^+$ lymphocytes were identified by gating on CD4 FITC and CD8 perCP bright populations. In a concurrent assay, a CD3$^+$ lymphocyte purity of $\geq 98\%$ of these gated populations was confirmed with co-staining of a CD3 PE IgG1 antibody (Becton Dickinson). The percentage of CD4$^+$ and CD8$^+$ lymphocytes expressing CD69 (i.e., CD4$^+$ 69$^+$ and CD8$^+$ 69$^+$) were determined as described previously (10). That is, the fluorescence staining intensity of the IgG1 PE isotype control for CD69 was first determined for CD4$^+$ and CD8$^+$ lymphocytes for each patient sample, immediately after which the percentage of CD4$^+$ and CD8$^+$ lymphocytes displaying CD69 PE IgG1 fluorescence greater than the cutoff intensity determined by the isotype control was considered to be the %CD4$^+$ 69$^+$ and %CD8$^+$ 69$^+$. In addition, to examine for differences in the degree of CD69 expression upon CD4$^+$ 69$^+$ and CD8$^+$ 69$^+$ lymphocytes displaying CD69 PE IgG1 fluorescence intensity of CD4$^+$ 69$^+$ and CD8$^+$ 69$^+$ lymphocytes was obtained from the flow cytometric data. The mean of logarithmically acquired data was expressed as a linear value (i.e., 1 to 10$^4$) as opposed to a channel value (i.e., 1 to 1024).

Determination of CMV Viremia
As per the clinical practice in our program, study participants underwent screening for CMV infection with a CMV PCR assay. An attempt was made to obtain weekly serum for CMV screening for the initial 15 wk after transplantation and at the time of any allograft biopsy. The PCR assay used was a semiquantitative assay developed at the Manitoba Cadham Provincial Laboratory and accredited by the College of American Pathologists. The assay is performed onuffy coat specimens, incorporates custom-made primers, and uses standard amplification and hybridization techniques (see Appendix for detailed methods). A simultaneous internal control (human beta-globin) is incorporated in a multiplex PCR fashion. Positive results are reported as either “low level CMV detected” or “CMV detected.” The threshold for a low level result has deliberately been set high to avoid detection of extremely low levels of CMV DNA, and both results have been shown to demonstrate exceptionally high sensitivity and specificity for CMV infection.

Study participants were considered CMV DNA–positive if the PCR was either “low level CMV detected” or “CMV detected.” No individuals demonstrating CMV DNA positivity during the course of the study were only transiently positive (i.e., $<2$ wk). PCR screening for CMV DNA was not performed for seronegative recipients who had received a kidney from a seronegative donor (i.e., D−/R−, n = 11 patients). It has been shown that such individuals demonstrate an extremely low prevalence of CMV infection posttransplant (<5%) (8,9,16). D−/R− study participants were thus assumed to be CMV PCR–negative.

Recipients becoming CMV PCR–positive posttransplant were given oral ganciclovir 1 g thrice daily for 3 mo as preemptive CMV prophylaxis therapy. No episodes of clinical CMV disease were observed during the course of the study. Specifically, no individuals
developed invasive CMV disease (e.g., hepatitis, gastroenteritis, pneumonia), and none developed a coincident clinical CMV viral syndrome (i.e., fever, leukopenia, thrombocytopenia).

Statistical Analyses

Statistical analyses were performed using Statview 5.0 software (SAS Institute Inc., Cary, NC). Data are presented as mean ± SD. The t tests, ANOVA analysis, and tests of least significant difference (e.g., Fisher’s PLSD) were applied to comparisons of continuous variables, and tests of association (e.g., χ2 test and Fisher’s exact test) were applied to comparisons of categorical variables.

In patients undergoing weekly monitoring of PBL CD69 expression, evidence of contemporaneous changes in CD69 expression upon CD4⁺ or CD8⁺ lymphocytes and the development of rejection were analyzed using multiple tests of association incorporating several categorical criteria, including increases of %CD69⁺ of ≥5%, 10%, or 15% above baseline values, as well as increases of %CD69⁺ expression of ≥1 or 2 SD above baseline values.

Results

Peripheral Blood Lymphocyte CD69 Expression and Histologic Diagnoses

The demographic profile of the study population is presented in Table 1. A total of 131 biopsies and concurrent determinations of PBL CD69 expression were performed in the 45 study patients (Table 2). Of the 14 clinical biopsies, one biopsy revealed cyclosporine toxicity and 13 biopsies were performed in cases of acute rejection. Eleven of these exhibited Banff type I histology (Ia, n = 4; Ib, n = 7), and two cases exhibited Banff type IIa histology. All 15 biopsies revealing subclinical rejection demonstrated Banff type I histology (Ia, n = 8; Ib, n = 7).

There were no significant differences in CD69 expression in either CD4⁺ or CD8⁺ lymphocyte populations between the different biopsy diagnoses (Table 3). Furthermore, when rejections were stratified according to histologic severity, no significant differences were observed among normal biopsies and Banff type Ia, Ib, or IIa rejections (% CD4⁺/CD69⁺: 5.5 ± 3.4, 4.8 ± 3.2, 4.1 ± 3.2, and 8.8 ± 6.8, respectively [P = NS]; %CD8⁺/CD69⁺: 13.2 ± 8.8, 9.7 ± 4.4, 11.2 ± 6.2, and 18.3 ± 15.2, respectively [P = NS]). Likewise, no significant differences were detected in the mean fluorescence intensity of CD69 expression upon either CD4⁺/CD8⁺ or CD8⁺/CD69⁺ lymphocytes between the different biopsy diagnoses (data not shown).

Peripheral Blood Lymphocyte CD69 Expression and CMV Viremia

PCR results for CMV DNA were available for 77 biopsies and were assumed to be negative in a further 31 biopsies performed in the 11 CMV D−/R− recipients (total CMV PCR results with concurrent biopsies; n = 108). Thirty-three biopsies were performed in individuals testing positive for CMV viremia at the time of the biopsy (n = 15 patients), and 75 biopsies were performed in individuals where the CMV PCR was negative or assumed to be negative (i.e., D−/R− recipients).

There was no significant difference in CD69 expression on CD4⁺ lymphocytes between CMV PCR-positive and PCR-negative patient samples (Table 4). In contrast, CMV viremia was associated with a significant increase in CD69 expression on CD8⁺ lymphocytes. This was irrespective of the relative quantity of CMV DNA detected in the semiquantitative PCR assay (“low level CMV detected” %CD8⁺/CD69⁺ = 16.5 ± 11.3 versus “CMV detected” %CD8⁺/CD69⁺ = 18.5 ± 10.1; P = NS). To exclude the possibility that the patients testing CMV PCR-positive at the time of a biopsy were a select group with heightened CD8⁺/CD69⁺ expression before the development of CMV viremia, their CD8⁺/CD69⁺ expression both before transplantation and <1 wk posttransplant were compared with that obtained at the time of viremia. These individuals showed similar degrees of CD8⁺/CD69⁺ expression before and early after transplantation to individuals without CMV viremia at the time of biopsy (10.0 ± 4.9 and 11.4 ± 5.0 versus 9.6 ± 4.8; P = NS) and significantly lower expression than they demonstrated at the time of CMV viremia (P < 0.001).

Next, the CMV PCR status and the presence or absence of rejection were considered together. CMV PCR results were available in 23 of 28 biopsies exhibiting rejection and 85 of 103 biopsies without rejection (Table 2). Patients without CMV viremia but who were experiencing rejection (n = 14) did not exhibit heightened CD8⁺/CD69⁺ expression compared with individuals without CMV viremia who had normal biopsies (Table 5). In contrast, patients without rejection who were CMV PCR-positive exhibited heightened CD8⁺/CD69⁺ expression over that of all other groups (P < 0.03).

Identical findings of heightened CD8⁺/CD69⁺ expression with CMV viremia but not rejection were observed when D−/R− individuals were excluded from all analyses (data not shown).

Weekly Monitoring of PBL CD69 Expression

Nineteen of the 45 study participants underwent weekly flow cytometric monitoring to determine whether a rise in PBL CD69 expression would precede or coincide with the onset of rejection. An attempt was made to perform regular CMV PCR screening in these individuals; however, due to a lack of adequate CMV screening in two patients, results were only interpretable in 17 cases. There was no evidence of a rise in either %CD4⁺/CD69⁺ or %CD8⁺/CD69⁺ lymphocytes immediately before or at the time of a rejection episode in patients who

Table 1. Patient demographics

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<th>Study Population (n = 45)</th>
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<tr>
<td>Donor (Cad/LD)</td>
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<tr>
<td>Recipient Age</td>
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<tr>
<td>Peak AHG-PRA &gt;10%</td>
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<td>CMV D−/R−</td>
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* Cad, cadaver; LD, living donor.
remained CMV PCR-negative posttransplant. However, in a number of individuals, the development of CMV viremia appeared to be paralleled by a considerable rise in %CD8+69+ expression, independent of the presence or absence of rejection. Three illustrative cases of weekly PBL CD69 monitoring are presented in Figure 1.

Discussion

The only method of reliably diagnosing renal allograft rejection at present is by needle biopsy; although biopsies provide information crucial for patient management, they remain somewhat inconvenient, costly, and invasive (1–3). The development of noninvasive biomarkers of renal allograft rejection would benefit both patients and clinicians.

The ideal biomarker of rejection would be sensitive enough to detect subclinical and mild forms of clinical rejection and specific enough to avoid confounding by other conditions arising in transplant recipients (e.g., infection). In this study, we have demonstrated that a candidate biomarker, the expression of the peripheral blood lymphocyte early activation marker CD69, failed to meet both these criteria, as it did not correlate with the diagnosis of either clinical or subclinical allograft rejection; furthermore, it was increased during asymptomatic CMV infection.

The lack of correlation between CD69 expression and rejection observed in our study contrasts with a recent report in heart allograft recipients in which heightened expression of CD69 in peripheral blood lymphocytes was observed in patients with more severe types of rejection detected in surveillance cardiac biopsies (10). A similar correlation was recently reported in abstract form in renal transplant patients with clinical rejection episodes (11). It is possible that in our study the lack of correlation between CD69 expression and rejection is due to the relatively mild nature of rejections in the study cohort and that more severe rejections, such as those observed in the aforementioned reports, may be more consistently associated with heightened PBL CD69 expression. The majority of clinical rejections observed in our patients were of Banff type I severity, and only 2 of 13 clinical rejections demonstrated Banff type II histology. It bears noting however that this cohort of patients is likely representative of other renal transplant populations wherein a large proportion of acute rejections are of a similar degree of severity. The 1995 Efficacy Endpoints Conference indicated that >40% of biopsy proven acute rejections were of type I severity with some centers reporting a substantially greater proportion (17).

Moreover, a biomarker of rejection must ideally be capable of detecting very early rejection to permit timely intervention aimed at minimizing allograft injury and fibrosis. Biomarkers that only become positive with clinically severe rejections may allow irreversible allograft injury to occur before treatment can be initiated. Protocol biopsy studies indicate that a substantial proportion of transplant recipients with stable graft function have histologic criteria for rejection (i.e., subclinical rejection) (4–7). Molecular analysis of these biopsies reveals inflammatory programs similar to clinical acute rejections; furthermore, follow-up studies indicate that these inflammatory infiltrates are associated with subsequent allograft scarring and impaired function (5,6,14,18–20). There have been no studies to date...
Figure 1. Weekly monitoring of PBL CD69 expression.
that have evaluated biomarkers for the detection of subclinical rejection. The current study indicates that periodic monitoring of peripheral blood lymphocyte CD69 expression will not meet this important objective, despite the fact that considerable intragraft infiltration with CD69+ lymphocytes occurs in both clinical and subclinical rejection (13,14). A plausible explanation for the absence of heightened peripheral blood lymphocyte CD69 expression in either subclinical or clinical acute rejection may be that a large proportion of alloreactive CD69+ lymphocytes are in fact sequestered within renal allografts.

The finding that clinically apparent CMV infection is associated with an increased proportion of activated CD8+ peripheral blood lymphocytes merits further discussion. This finding has been previously noted (21,22). Recently, Engstrand et al. (23), by staining CD8+ lymphocytes with an HLA class I tetramer and immunodominant CMV peptide, observed that a large proportion of circulating CD8+ lymphocytes (up to 15%) may demonstrate specificity to even a single CMV peptide. Such observations must be considered when evaluating potential biomarkers of rejection, particularly when one considers the high prevalence of CMV viremia posttransplant (8,9). Recent biomarker studies for rejection have focused on evidence of CD8+ lymphocyte activation through the detection of cytotoxic gene transcripts in the peripheral blood (e.g., perforin, granzyme B, and FasLigand) (24–26). The finding that CMV viremia is associated with a greater proportion of circulating activated CD8+ lymphocytes suggests that other markers of lymphocyte activation, including cytotoxic gene transcripts, may also be influenced by clinically apparent CMV infection. Indeed, circulating CMV specific CD8+ lymphocytes are known to exhibit an effector phenotype, producing granzyme B and perforin (27,28). Confounding by asymptomatic CMV viremia has not been excluded by many studies evaluating peripheral blood biomarkers of rejection. Studies to date have either considered only clinically apparent infection or deliberately excluded patients with CMV infection (24–26).

As in this study, noninvasive tests of rejection have largely focused on the detection of lymphocyte activation, which may of course also occur as a result of non-allogeneic factors (e.g., CMV viremia). Rejection however is driven by HLA disparities, and biomarkers specific for alloimmune responses may avoid such errors. The ELISPOT technique, for example, quantifies cytokine production from primed alloreactive lymphocytes activated through either the direct or indirect pathways of allorecognition, correlating cytokine production with rejection (29,30). The possibility that such an allospecific biomarker may not be confounded by infection is intriguing. Alternatively, the specificity of a biomarker may potentially be enhanced by identifying a panel of candidate molecules for rejection through bioinformatic approaches integrating genomic (i.e., chip arrays) or proteomic analyses (31–33). Irrespective of what technique is applied, future studies should be designed to identify rejection at its earliest stage, ideally incorporating protocol biopsies as a gold standard (i.e., to identify subclinical rejection).

Finally, it is possible that urinary biomarkers of rejection, such as urinary flow cytometry, spectrographic analysis, and molecular analysis of cytotoxic gene transcripts, are not confounded by common infections such as CMV viremia and that these may be more specific than techniques that examine the peripheral blood (34–37). It bears noting however that allograft infections such as pyelonephritis are not uncommon posttransplant, and recent studies on the natural history of BK viral nephropathy indicate that 18 to 26% of individuals demonstrate BK viruria in the initial months posttransplant (38–40). The development of urinary biomarkers of rejection must also take into account these potential confounders.

In conclusion, we have demonstrated that monitoring of PBL CD69 expression does not reliably identify the presence of either clinical or subclinical renal allograft rejection, despite the abundant presence of CD69+ lymphocytes in rejecting allograft infiltrates. Furthermore, clinically inapparent CMV viremia is associated with an increased proportion of circulating CD8+ lymphocytes expressing this activation marker. The latter finding raises concern regarding the specificity of other potential biomarkers of rejection that examine peripheral blood lymphocytes. Until noninvasive techniques for diagnosing rejection are proven to be sensitive, reliable, and convenient, clinicians will have to continue to rely on allograft biopsies to diagnose clinical acute rejection and survey the graft for the presence of subclinical rejection.

Acknowledgments

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Appendix: CMV PCR Methodology

Specimens

Leukocytes were separated from 5 to 10 ml of heparinized blood within 4 h of being drawn. Briefly, the whole blood was mixed with a volume of 6% dextran equivalent to 20% of the specimen volume. After standing for 20 min at 35°C, the plasma fraction containing the leukocytes was removed and washed. Leukocytes were counted, adjusted to between 10^6 and 6 x 10^6/ml and resuspended in lysis buffer (50 mM KCL, 10 mM Tris-HCL [pH 8.3], 2.5 mM MgCl2, 0.45% NP40, and 0.45% Tween 20) containing 100 μg/ml proteinase K. This mixture was incubated at 56°C for 1 h. After incubation, the proteinase K was inactivated by heating to 95°C for 10 min.

PCR

Primers were synthesized from published sequences (41,42). The CMV primers targeted a 152-bp sequence located in the EcoRI fragment D region of the genome (41). A second set of primers, multiplexed as an internal control, amplified a 268-bp fragment of the single-copy human β-globin gene (42). This was used to demonstrate sample sufficiency and lack of inhibition. DNA was amplified from 5 μl of the processed leukocyte suspension using 2 U of Taq DNA polymerase (Invitrogen) previously neutralized by an equal volume of TaqStart antibody (Clontech), 150 nM of each CMV primer, 16.5 nM of each β-globin primer, 200 μM of each deoxynucleoside.
triphosphate, 2% formamide and 1.5 mM MgCl₂ in a total reaction volume of 100 μl. Amplification proceeded in a Perkin Elmer TC-1 thermal cycler (version 2.1) for 45 cycles, beginning with a 2-min pre-PCR incubation at 95°C. The following 20 cycles consisted of 94°C for 1 min and 60°C for 2 min. The remaining 25 cycles consisted of 90°C for 1 min, 58°C for 1 min, and 72°C for 30 s. Final extension occurred for 7 min at 72°C followed by refrigeration at 4°C. PCR products were separated and visualized on a 2% agarose gel containing 200 ng of ethidium bromide per ml and documented using a digital Kodak EDAS 290 system.

Interpretation
Co-amplification of the 268-bp β-globin sequence was required for reporting of any CMV result. A band at 152 bp denoted the presence of CMV DNA, which was routinely detectable at five copies per PCR reaction. The result was expressed semiquantitatively by incorporating a standard, which indicated the approximate sensitivity of conventional culture and shell vial analyses. Band intensities below this threshold were reported as “low level CMV detected.”

References


