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Use of a Modified Shell Vial Technique To Quantitate Cytomegalovirus Viremia in a Population of Solid-Organ Transplant Recipients

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A quantitative modification of the shell vial assay was used to investigate cytomegalovirus viremia in solid-organ transplant recipients. The level of viremia detected in 109 of 407 specimens ranged from 0.02 to 28 infectious foci per 100,000 leukocytes. By using a Poisson model, a technique was developed to determine 95% confidence limits for the measured levels of viremia. These confidence limits were used to determine the level of viremia that could be excluded by culturing a given number of cells. Longitudinal assessment of two transplant recipients revealed different patterns of viremia and demonstrated that significant disease sometimes occurred with low-level viremia. On the basis of the results of the studies, culture of at least 4 × 10^6 leukocytes is recommended for the sensitive detection of cytomegalovirus viremia.

Disease associated with human cytomegalovirus (CMV) infection is the most common infectious complication of immunosuppressed solid-organ transplant recipients (10). Although CMV can be isolated from a number of different sources, demonstration of viremia is generally considered to have the best correlation with clinically significant infection (1, 6–8). Because CMV in the blood is associated with leukocytes, the ability to culture CMV from the blood must depend on the number of leukocytes placed into culture and the proportion of leukocytes in the peripheral circulation that are productively infected with CMV. However, guidelines regarding the number of leukocytes required for optimal detection of CMV viremia by culture are lacking. To investigate these factors, we developed a quantitative modification of the shell vial culture (5) which we used to determine the frequency of leukocytes harboring infectious CMV in the peripheral blood of solid-organ transplant patients. The results defined the range of CMV viremia in this patient population and should be useful in maximizing the sensitivity of culture methods for detecting CMV viremia.

MATERIALS AND METHODS

Patients and leukocyte specimens. A total of 407 blood specimens were obtained from 48 transplant recipients. The organs transplanted were lung, 26 (54%); kidney, 17 (35%); liver, 4 (8%); and heart, 1 (2%). Blood leukocytes were isolated from heparinized whole blood by dextran sedimentation by a procedure modified from that of van der Bij et al. (13). Briefly, a maximum of 5 ml of blood was diluted to 10 ml with phosphate-buffered saline (PBS). Following the addition of 2 ml of 5% dextran (molecular mass, 250 kDa; Pharmacia; LKB Biotechnology, Piscataway, N.J.) in 0.9% NaCl, the suspension was mixed and incubated at 37°C for 10 to 20 min. The leukocyte-rich upper layer was removed, the cells were pelleted, and the remaining erythrocytes were lysed with a 0.8% ammonium chloride solution. The leukocytes were washed twice with Eagle's minimal essential medium supplemented with 10% fetal calf serum and counted electronically by using an S-Plus-Jr cell counter (Coulter Electronics, Hialeah, Fla.).

Isolation and quantitation of CMV from blood. For quantitation of CMV viremia, a modified centrifugation shell vial technique was used. Commercially prepared shell vials containing MRC-5 fibroblasts (Viro Med, Minnetonka, Minn.) were inoculated with 0.25 ml of leukocyte suspension adjusted with Eagle's minimal essential medium–10% fetal calf serum to obtain the appropriate number of cells. The number of leukocytes inoculated was dependent on the quantity recovered from the specimen. Using data from an initial pilot study (data not shown) in which a shell vial technique was used to quantitate CMV viremia, we determined that culturing of a total of 6 × 10^6 leukocytes would provide a high level of sensitivity. By dividing this total into four shell vials, including two each with 2 × 10^6 leukocytes and two each with 1 × 10^6 leukocytes, we avoided the toxicity to the cell monolayer sometimes seen when more than 2 × 10^6 leukocytes are inoculated into a single shell vial culture. Therefore, when the number of leukocytes was sufficient, four shell vials were inoculated with a total of 6 × 10^6 leukocytes. Fewer cells were inoculated when the number of leukocytes available was less than this total. The shell vials were centrifuged at 700 × *g* for 45 min, after which 2 ml of Eagle's minimal essential medium–10% fetal calf serum was added; this was followed by incubation for 40 h at 37°C. After fixation with acetone-methanol (1:1) for 10 min at −20°C, monolayers were stained in an indirect immunofluorescence assay to detect CMV infection of the monolayer. Murine monoclonal antibody 9221 (DuPont, Doraville, Ga.) (12), which is specific for the CMV major immediate-early antigen, was used as the primary antibody; this was followed by a fluorescein isothiocyanate-conjugated goat anti-mouse serum (Cappel, Organon Teknika, West Chester, Pa.). Stained coverslips were examined at ×200 magnification with a Leitz epifluorescence microscope, and the fluorescent foci were enumerated and expressed as the number of infectious centers (ICs) per 10^5 leukocytes. To construct approximate confidence intervals for the observed propor-

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TABLE 1. Level of viremia that can be excluded as a function of the number of leukocytes cultured

No. of leukocytes cultured	Level of viremia excluded by negative culture[a]
1×10^5	3.00
2×10^5	1.50
4×10^5	0.75
6×10^5	0.50
8×10^5	0.37
1×10^6	0.30
2×10^6	0.15
4×10^6	0.07
6×10^6	0.05
8×10^6	0.04

[a] Level of viremia expressed in ICs per 10^5 leukocytes that can be excluded with 95% confidence if the culture is negative for CMV (see Appendix).

tion of infected cells, the data were modeled as a Poisson process (9) (see also Appendix). An examination of the variance between replicates was compared with that predicted by a Poisson process, and an acceptable fit was observed. On the basis of the Poisson distribution, 95% confidence intervals were computed.

RESULTS

Of a total of 407 leukocyte specimens analyzed by the quantitative shell vial technique, 109 (27%) were positive for CMV. The level of viremia ranged from 0.02 to 28 ICs per 10^5 leukocytes, with a median value of 0.28 IC per 10^5 leukocytes. Notably, 69 (63%) of the positive specimens exhibited levels of <1.0 IC per 10^5 cells, including a group of 22 (20%) specimens that contained <0.1 IC per 10^5 cells.

It was possible to use the data from this study, along with calculated confidence limits, to predict the level of viremia that could be excluded with 95% confidence by culturing a given number of leukocytes. This is illustrated in Table 1, which shows the level of viremia excluded with 95% confidence by a negative culture as a function of the number of leukocytes inoculated into the culture. For example, a negative result from a culture containing 10^5 leukocytes would provide 95% confidence that CMV viremia at a level of 3 ICs per 10^5 cells or higher was not present. Such a culture would be of little value, since 85% of the positive cultures in this study were at levels lower than 3 ICs per 10^5 cells. In contrast, a negative result of a culture of 4×10^6 leukocytes would provide 95% confidence that CMV viremia at a level of 0.07 IC per 10^5 cells or greater was not present. Only 13% of the viremias detected in our study were beneath this level.

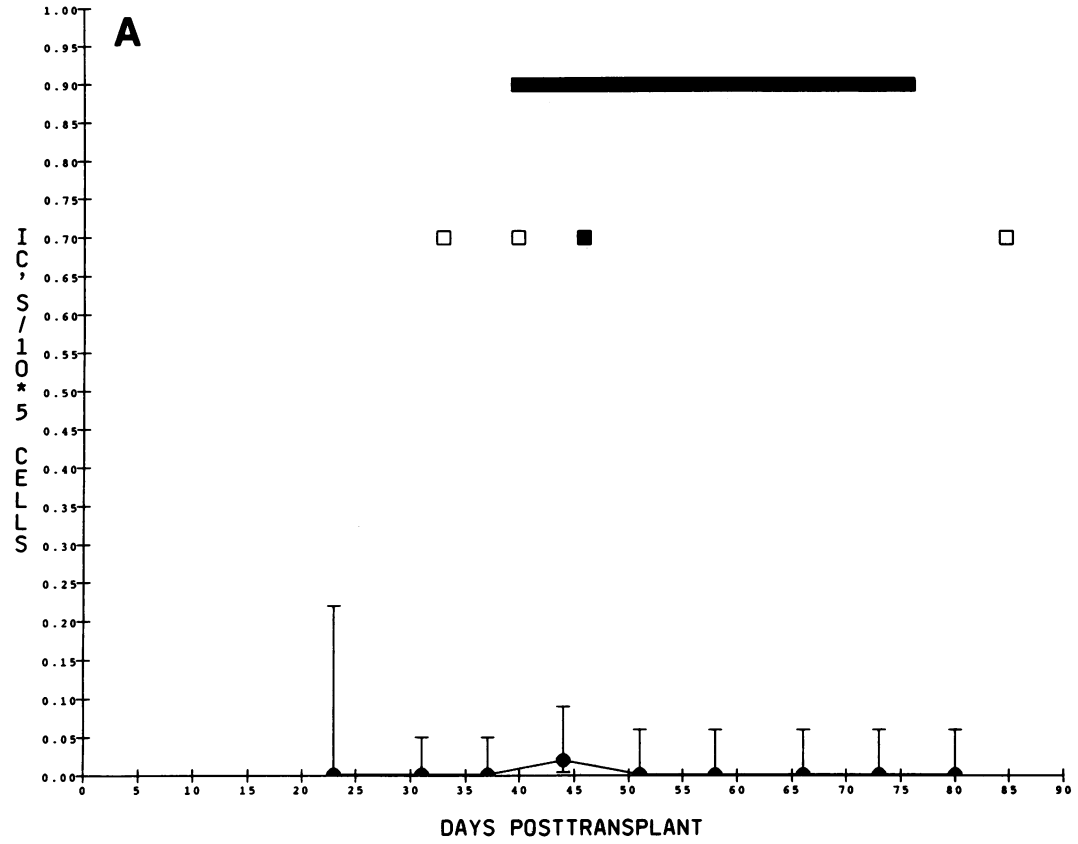
For illustrative purposes, the time course of CMV viremia in two patients during the early posttransplant period is shown in Fig. 1. Figure 1A shows data for a CMV-seronegative recipient of a lung from a CMV-seropositive donor. Analysis of serial blood specimens beginning 3 weeks after transplantation, including three specimens obtained before the initiation of ganciclovir therapy, yielded only a single positive culture, which had a very low frequency (0.02 IC per 10^5 leukocytes) of infected cells. The 95% confidence intervals of the negative samples suggest that the low level of viremia observed on one occasion (0.02 IC per 10^5 leukocytes) could not be excluded on those other occasions when no infectious centers were observed. Four days before the single positive culture, a clinical diagnosis of systemic CMV syndrome was made on the basis of the findings of fever and leukopenia. Therapy with ganciclovir was then initiated. Further evidence of clinically significant CMV infection was seen 4 days after the single positive blood culture, when CMV was isolated from a bronchoalveolar lavage specimen, and a transbronchial lung biopsy performed immediately after the bronchoalveolar lavage revealed evidence of CMV pneumonitis in the form of positive immunoperoxidase stains for CMV in the presence of an interstitial infiltrate consisting primarily of lymphocytes.

The patient whose data are presented in Fig. 1B was selected to illustrate a markedly different pattern of CMV viremia. Beginning 7 weeks after transplantation, this CMV-seropositive recipient of a lung from a CMV-seronegative donor had six of six positive blood cultures over a 1-month period. Quantitative culture demonstrated levels of viremia of up to 28 ICs per 10^5 leukocytes, a 1,000-fold-higher level of viremia than that seen in the previous patient. Despite the high level of viremia, this individual remained asymptomatic throughout the period without ganciclovir therapy. However, a lung biopsy specimen taken during the course of a routine bronchoscopy 11 weeks after transplantation revealed the presence of CMV pneumonitis, prompting the start of ganciclovir therapy.

DISCUSSION

We used a modified shell vial technique to quantitate the level of CMV viremia in blood specimens from a number of solid-organ transplant recipients. An important finding was that in such patients with posttransplant viremia, the frequency of circulating leukocytes detected by our assay as being productively infected with CMV was usually low. In these cases, failure to place an adequate number of leukocytes into culture would result in the failure to detect CMV viremia. Patients with low-level viremia included some with significant illness attributable to CMV.

Other investigators have also shown that CMV viremia is frequently of a low level. However, relatively few data exist concerning the relationship between the level of viremia and CMV disease. Using a plaque assay, Zaia et al. (14) found that the frequency of CMV-infected cells in polymorphonuclear leukocyte-enriched fractions from the blood of bone marrow transplant recipients is frequently less than 1.0 per 2×10^6 cells ($0.05/1 \times 10^5$ cells). The large number of cells required to detect many of these episodes of viremia (average of 20×10^6 cells) suggested that many of these viremias were of a low level. In that same study (14), two patients with the highest levels of viremia died from CMV disease, suggesting a link between higher levels of viremia and more severe disease. Using a standard tube culture method, Saltzman et al. (11) reported that the mean minimum number of cells required for detection of CMV viremia in a population of solid-organ and bone marrow transplant recipients is 1.6×10^5 for polymorphonuclear leukocytes and 2.3×10^5 for mononuclear cells. These numbers correspond to 0.62 and 0.43 infected cells per 10^5 cells, respectively. Gerna et al. (2–4), using a quantitative shell vial technique, reported frequencies of infectious centers in the blood of solid-organ transplant recipients and human immunodeficiency virus type 1-infected patients ranging from 1 to $300/2 \times 10^5$ cells (0.5 to $150/1 \times 10^5$ cells) in polymorphonuclear leukocyte-enriched fractions, with symptomatic CMV disease being seen only when viremia was >50 to 80 ICs per 2×10^5 cells. The levels of viremia detected in the present study, from 0.02 to 28 ICs per 10^5 leukocytes, were in general agreement


A
IC'S/10*5 CELLS
DAYS POSTTRANSPLANT

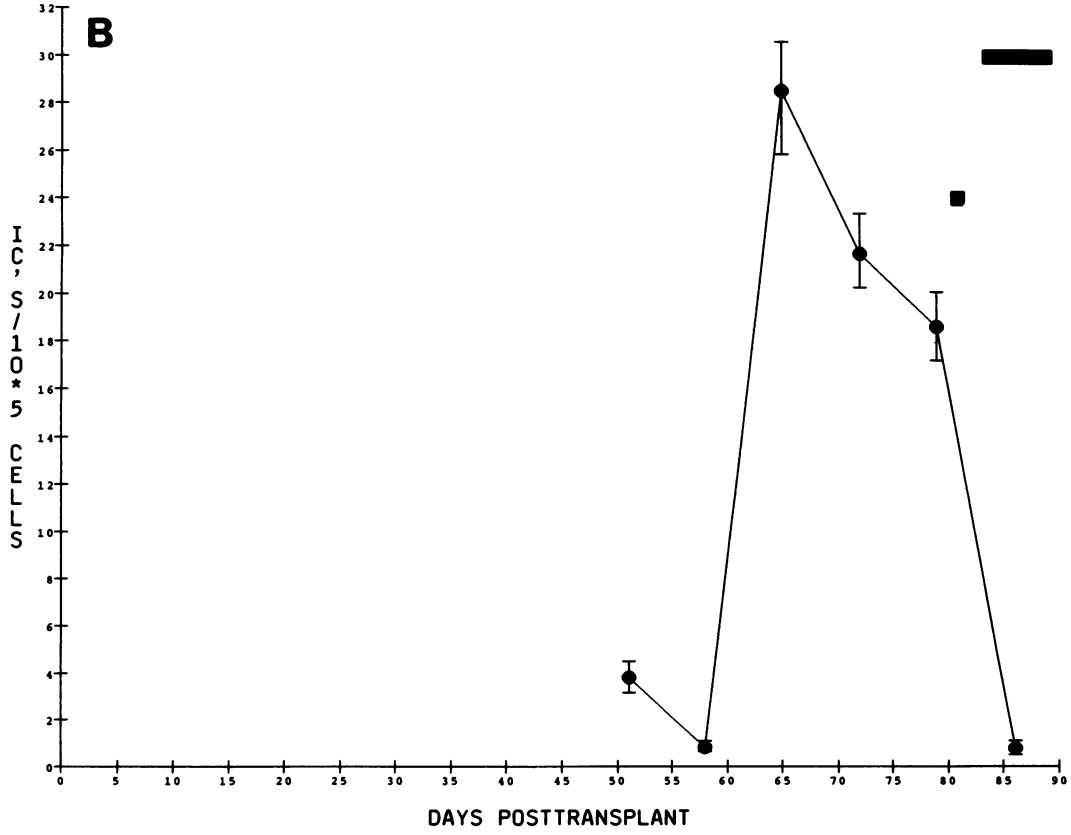

B
IC'S/10*5 CELLS
DAYS POSTTRANSPLANT

FIG. 1. Titers of CMV versus day posttransplant in serial blood specimens from two recipients of lung transplants. Solid circles represent levels of CMV (in ICs per 10^5 leukocytes) as determined by quantitative shell-vial culture. The vertical error bars indicate the 95% confidence intervals calculated on the basis of the Poisson distribution. Solid circles on the x axis without lower error bars indicate negative quantitative cultures. Open and filled squares represent the absence or presence, respectively, of CMV pneumonitis as demonstrated by histopathological examination of lung tissue obtained by transbronchial lung biopsy. The horizontal bars denote the periods of time during which the patient was treated with ganciclovir. (A) Results for a CMV-seronegative recipient of a lung from a CMV-seropositive donor. (B) Results for a CMV-seropositive recipient of a lung from a CMV-seronegative donor.

with those cited above. Our study did not address thoroughly the relationship between the level of viremia and clinically significant disease resulting from CMV infection. However, as illustrated in Fig. 1A, it is evident that significant disease can be associated with low levels of viremia. We are carrying out a longitudinal study of CMV infection after solid-organ transplantation. The study will address the relationship between the level of viremia and CMV disease and will consider factors such as the CMV serostatus of the donor and the recipient, the time period after transplantation when viremia is detected, immunosuppressive therapy, and the specific manifestations of CMV disease.

In comparing the levels of viremia in different samples, it is important to understand the precision of the level determined. We used the Poisson distribution to estimate for each culture the two-sided 95% confidence limits of the actual frequency of infected cells. It should be noted that the confidence limits derived are based only on the number of infectious centers detected and the numbers of leukocytes placed into culture. Thus, they provide statistical confidence limits that reflect the number of leukocytes cultured, but they do not take into account biological factors, such as conditions of specimen transport, that might introduce additional variability. The use of 95% confidence limits may be particularly valuable in interpreting negative cultures, since they provide a measure of the level of viremia that can be excluded in that culture with 95% confidence. Thus, if a culture was negative but only a low number of leukocytes had been cultured, the 95% confidence limit would show that the negative result did not exclude viremia at a frequency of infected cells seen in some patients with significant disease. The statistical analysis of our data shown in Table 1 allows us to make practical recommendations for how many leukocytes should be cultured in order to permit the sensitive detection of CMV viremia in solid-organ transplant recipients. The use of two shell vials, each with 2×10^6 leukocytes, would place an adequate number of leukocytes in culture and would also provide some safeguard against the loss of specimens because of cytotoxicity that, sometimes, for poorly understood reasons, may not affect all vials set up with the same specimen. The culture of more cells and/or the use of more shell vials might provide additional sensitivity and protection against loss owing to cytotoxicity, but it must be measured against the additional expense in materials and time. It may be particularly important to provide notification when the numbers of cells available for culture is inadequate to provide acceptable sensitivity.

APPENDIX

For the statistical characteristics of the number of foci observed, we assumed that they are distributed as a Poisson variable. Under such circumstances, the number of foci observed (k) follow the following probability distribution:

$$P(x = k) = \frac{e^{-\Theta}\Theta^k}{k!}$$

where Θ is the average number of foci per 10^6 cells. The obvious estimator for Θ is then $\hat{\Theta} = k/c$, where c is the number of cells (10^6). Using the relationships between the Poisson, χ^2, and the Γ distributions, the $1 - \alpha$ confidence interval can then be written as follows (5a):

$$\frac{\Gamma^{-1}(\alpha/2, k)}{c} < \hat{\Theta} < \frac{\Gamma^{-1}(1 - \alpha/2, k + 1)}{c}$$

where $k > 0$, and:

$$0 < \hat{\Theta} < \frac{-\ln(\alpha/2)}{c}$$

where $k = 0$.

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REFERENCES

1. **Cheeseman, S. H., R. H. Rubin, J. A. Stewart, N. E. Tolkoff-Rubin, A. B. Cosimi, K. Cantell, J. Gilbert, S. Winkle, J. T. Herrin, P. H. Black, P. S. Russell, and M. S. Hirsch.** 1979. Controlled clinical trial of prophylactic human leukocyte interferon in renal transplantation. Effect on cytomegalovirus and herpes simplex virus infection. N. Engl. J. Med. **300**:1345–1349.
2. **Gerna, G., M. Parea, E. Percivalle, D. Zipeto, E. Silini, G. Barbarini, and G. Milanesi.** 1990. Human cytomegalovirus viraemia in HIV-1-seropositive patients at various clinical stages of infection. AIDS **4**:1027–1031.
3. **Gerna, G., M. G. Revello, E. Percivalle, M. Zavattoni, M. Parea, and M. Battaglia.** 1990. Quantification of human cytomegalovirus viremia by using monoclonal antibodies to different viral proteins. J. Clin. Microbiol. **28**:2681–2688.
4. **Gerna, G., D. Zipeto, M. Parea, M. G. Revello, E. Silini, E. Percivalle, M. Zavattoni, P. Grossi, and G. Milanesi.** 1991. Monitoring of human cytomegalovirus infections and ganciclovir treatment in heart transplant recipients by determination of viremia, antigenemia, and DNAemia. J. Infect. Dis. **164**:488–498.
5. **Gleaves, C. A., T. F. Smith, E. A. Shuster, and G. R. Pearson.** 1984. Rapid detection of cytomegalovirus in MRC-5 cells inoculated with urine specimens by using low-speed centrifugation and monoclonal antibody to an early antigen. J. Clin. Microbiol. **19**:917–919.

5a. **Johnson, N. L., and S. Kotz.** 1969. Distributions in statistics: discrete distributions. Houghton Mifflin, New York.

6. **Marsano, L., R. P. Perrillo, M. W. Flye, D. W. Hanto, E. D. Spitzer, J. R. Thomas, P. R. Murray, D. W. Windus, E. M. Brunt, and G. A. Storch.** 1990. Comparison of culture and serology for the diagnosis of cytomegalovirus infection in kidney and liver transplant recipients. J. Infect. Dis. **161**:454–461.
7. **Meyers, J. D., P. Ljungman, and L. D. Fisher.** 1990. Cytomegalovirus excretion as a predictor of cytomegalovirus disease after marrow transplantation: importance of cytomegalovirus viremia. J. Infect. Dis. **162**:373–380.

8. **Richardson, W. P., R. B. Colvin, S. H. Cheeseman, N. E. Tolkoff-Rubin, J. T. Herrin, A. B. Cosimi, A. B. Collins, M. S. Hirsch, R. T. McCluskey, P. S. Russell, and R. H. Rubin.** 1981. Glomerulopathy associated with cytomegalovirus viremia in renal allografts. N. Engl. J. Med. **305**:57–63.
9. **Rosner, B.** 1986. Fundamentals of biostatistics, p. 88–89. *In* C. Lenter (ed.), Geigy scientific tables, 2nd ed. Duxbury Press, Boston.
10. **Rubin, R. H.** 1990. Impact of cytomegalovirus infection on organ transplant recipients. Rev. Infect. Dis. **12**:S754–S766.
11. **Saltzman, R. L., M. R. Quirk, and M. C. Jordan.** 1988. Disseminated cytomegalovirus infection. Molecular analysis of virus and leukocyte interactions in viremia. J. Clin. Invest. **81**:75–81.
12. **Shuster, E. A., J. S. Beneke, G. E. Tegtmeier, G. R. Pearson, C. A. Gleaves, A. D. Wold, and T. F. Smith.** 1985. Monoclonal antibody for rapid laboratory detection of cytomegalovirus infections: characterization and diagnostic application. Mayo Clin. Proc. **60**:577–585.
13. **van der Bij, W., J. Shirm, R. Torensma, W. J. van Son, A. M. Tegzess, and T. H. The.** 1988. Comparison between viremia and antigenemia for detection of cytomegalovirus in blood. J. Clin. Microbiol. **26**:2531–2535.
14. **Zaia, J. A., S. J. Forman, M. T. Gallagher, E. Vanderwal-Urbina, and K. G. Blume.** 1984. Prolonged human cytomegalovirus viremia following bone marrow transplantation. Transplantation **37**:315–317.