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# An Increase in the Number of Polymorphonuclear Leukocytes Inoculated on Shell-Vial Culture Increases the Sensitivity of This Assay in the Detection of Cytomegalovirus in the Blood of Immunocompromised Patients

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*A prospective study was conducted comparing the sensitivity of the pp65 antigenemia assay (AGA) to that of the shell-vial culture (SVC) inoculated with increasing quantities of polymorphonuclear leukocytes (PMNLs) in the detection of cytomegalovirus (CMV) in peripheral blood. From the cellular suspension, three SVCs were inoculated with 200,000, 400,000, and 800,000 PMNLs, respectively. Of the 201 patients studied, 67 (31.9%) had positive results in one of the two analytic tests (AGA or SVC). In this group, 13 (19.4%) presented a negative AGA assay; 13 (19.4%) an AGA of 1; 13 (19.4%) an AGA of between 2 and 5; and 28 (41.8%) an AGA with a value >6 PMNL-positive × 100,000 PMNLs. The SVC inoculated with 200,000 PMNLs detected the presence of CMV in 42 cases (62.6%); 55 (82%) with 400,000; and 64 (95.5%) with 800,000. Statistically significant differences were observed between the isolation capacities of the SVC inoculated with 200,000 and 400,000, and the SVC inoculated with 800,000 PMNLs (p = 0.0001). In the comparison of the overall sensitivity of the AGA with that of the SVC with 200,000, the AGA was found to be significantly more sensitive (p = 0.0052). When comparing with the SVC with 400,000 PMNLs, the two techniques were found to be equally sensitive; and in the comparison with the SVC with 800,000, the culture displayed a greater detection sensitivity (p = 0.0023). According to these results, it seems evident that the increase in the absolute number of PMNLs inoculated in the SVC leads to a significant increase in the sensitivity of the SVC in the detection of low-level viremia by CMV.* © 1998 Elsevier Science Inc.

## INTRODUCTION

Infection by cytomegalovirus (CMV) is one of the main causes of morbidity and mortality in inmunocompromised patients, particularly in solid-organ transplant recipients and AIDS patients (Francisci et al. 1995; Rubin 1990). Although, in these patients, CMV may be isolated from various clinical samples, the confirmation of its presence in peripheral blood (viremia) is generally considered one of the most reliable markers of clinical significance (Meyers et al. 1990; Pillay et al. 1993).

The use of the shell-vial culture assay (SVC), with results available in 18 to 24 h, has encouraged the use of cultures of peripheral blood in the diagnosis of viremia by CMV (Gleaves et al. 1985; Patel et al. 1995). A new diagnostic technique recently has been

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introduced, the antigenemia assay (AGA), which quantifies the viral load of CMV by means of the detection of the pp65 antigen in the polymorphonuclear leukocytes (PMNLs) of peripheral blood (Landry and Ferguson 1993; Van der Bij et al. 1988).

In the majority of studies comparing the sensitivity of the AGA with that of the SVC in the detection of CMV in peripheral blood, fixed volumes (0.2 to 0.3 mL) or set quantities (200,000 PMNLs) of the cell suspension are inoculated into the SVC. As a result, in general, the AGA seems to have a greater sensitivity in the detection of CMV (Erice et al. 1992; Landry and Ferguson 1993). Nevertheless, it is logical to suppose that the addition of greater quantities of PMNLs to the SVC, exceeding the number of cells tested in the AGA (200,000 PMNLs), would increase the capacity to isolate CMV in those patients with a low level of viremia (Buller et al. 1992; Storch et al. 1994).

The aim of this study was to compare the sensitivity of the conventional AGA with that of the SVC inoculated with increased quantities of PMNLs.

## MATERIALS AND METHODS

Over a period of 1 year, we studied patients with clinically suspected CMV infection or disease (fever, leukopenia, hepatitis, retinitis, local infections).

We used the method of sedimentation in a dextran solution for leukocyte extraction, following the recommendations of Gerna et al. (1992), with minor modifications (Reina et al. 1996). Briefly, 3 mL of heparinized whole blood was mixed with 1 mL of 6% dextran solution in saline. Following incubation at 36°C for about 30 to 45 min, 1 mL of supernatant rich in PMNLs were collected and mixed with 3 mL of phosphate-buffered saline (PBS) (pH 7.2) and centrifuged at 200 × *g* for 10 min at room temperature. The supernatant was discarded, and the pellet was resuspended in 4 ml of PBS. The sample was divided into two parts of 2 mL each. The part intended for culture was mixed with 2 mL of PBS and centrifuged. The supernatant was discarded, and the pellet was resuspended in 1 mL of PBS and the cells were counted.

For the quantitative study, we used only those patients who, after the processing of the blood, had a count >1,500,000 PMNLs/mL. The PMNLs were inoculated into three MRC-5 shell-vials (Vircell, Granada, Spain); one with 200,000 PMNLs, one with 400,000 PMNLs, and one with 800,000 PMNLs. The MRC-5 shell-vials were incubated at 36°C for 18 to 24 h, and then stained by an indirect immunofluorescence assay method with a monoclonal antibody against the HCMV p72 antigen (Argene Biosoft, France).

The sample intended for quantitative antigenemia was mixed with 1 mL of erythrocyte lysis reagent ($0.8\%\ NH_4Cl$) that was allowed to act for 2 to 3 min at room temperature, after which 1 mL of PBS was added, and the sample was centrifuged for 10 min at 200 × *g*. After centrifugation, the pellet was washed again with PBS and centrifuged. The final pellet was resuspended in 1 mL of PBS, and the cells were counted. Cytospins of PMNLs preparation were obtained by centrifugation of $2 \times 10^5$ cells onto glass slides at 700 rpm for 7 min. The slides were fixed with Formalin for 10 min, washed in PBS, and allowed to dry. After fixation, slides were stained by an indirect immunofluorescence assay with a murine monoclonal antibody directed against HCMV pp65 (Monofluokit CMV; Diagnostics Pasteur, France). Slides were read and fluorescent cells counted under fluorescence microscopy at ×40. The quantitative antigenemia results were expressed as the number of pp65-positive PMNLs/200,000 total leukocytes.

Statistical analysis was carried out on results of different comparison by performing the Student *t* test on paired data. All probability values are two-tailed and considered significant if less than 0.05.

## RESULTS

Over the study period, 201 patients were analyzed. Of the 70 renal transplant recipients, 33 (47.1%) had positive results in one of the two tests (AGA or SVC); 31 in the SVC (93.9%); and 26 in the AGA (78.7%). Thirty-four of the 131 AIDS patients gave positive results: 33 (97%) were SVC positive and 27 (79.4%) were AGA positive. A total of 67 (31.9%) patients presented with positive results in one of the two assays.

The overall values detected in the AGA and the results of the SVC obtained according to the quantities of PMNLs inoculated are shown in Table 1. Of the 67 positive patients, 13 (19.4%) had a negative AGA (<1 PMNL positive/200,000 PMNLs), 13

**TABLE 1** Overall Results Obtained in the Comparison of the AGA with the SVC Inoculated with Increasing Quantities of PMNLs

| AGA Value[a] | No. | No. of PMNLs Inoculated in the SVC | | |
|---|---|---|---|---|
| | | 200,000 | 400,000 | 800,000 |
| 0 | 13 | 3 (23)[b] | 6 (46.1) | 13 (100) |
| 1 | 13 | 6 (46.1) | 10 (76.9) | 11 (84.6) |
| 2–5 | 13 | 9 (23) | 13 (100) | 13 (100) |
| >6 | 28 | 24 (85.7) | 26 (92.8) | 27 (96.4) |
| Total | 67 | 42 (62.6) | 55 (82) | 64 (95.5) |

[a] No. of PMNL positive/200,000 total leukocytes.
[b] No. of SVC positive (%).

(19.4%) an AGA value of 1; 13 (19.4%) an AGA value between 2 to 5; and 28 (41.8%) an AGA value of >6.

The SVC inoculated with 200,000 PMNLs detected the presence of CMV in 42 cases (62.6%), with 400,000 PMNLs in 55 (82%), and with 800,000 PMNLs in 64 (95.5%) cases. Statistically significant differences were observed between the detection capacity of the SVC inoculated with 200,000 PMNLs and that with 400,000 ($p = 0.0036$); between that with 200,000 and that with 800,000 ($p = 0.0001$); and between that with 400,000 and 800,000 ($p = 0.0052$).

When inoculating the SVC with the same quantity of PMNLs as used for the AGA (200,000), a higher statistically significant sensitivity in the detection of CMV in peripheral blood was obtained with the AGA ($p = 0.0052$). Doubling the quantity of PMNLs inoculated on the SVC provided an equal sensitivity in the two tests. The inoculation of 800,000 PMNLs on the SVC resulted in a significant sensitivity increase in of the SVC compared to the AGA ($p = 0.0023$).

Of the 13 cases with negative AGA, 3 (23%) were detected in the SVC with 200,000 PMNLs, 6 (46.1%) in the vial with 400,000 and 13 (100%) in the vial with the maximum number of PMNLs. The three cases with positive AGA and negative SVC corresponded with AGA values of 1, 1, and 16 PMNL-positive × 200,000 total PMNLs, respectively.

## DISCUSSION

In the majority of studies comparing the sensitivity of the AGA in the detection of CMV in peripheral blood to that of the SVC, the AGA appears to be more sensitive (Buller et al. 1992; Erice et al. 1992). There are two possible reasons for these results. First, many positive AGA with negative cultures are from patients receiving antiviral treatment, which produces negative results in the culture far more rapidly than in the AGA test (Gerna et al. 1991). Second, as has been mentioned, the inoculation of set volumes or quantities of the PMNL suspension on the SVC limits the real sensitivity of this method (Erice et al. 1992; Landry and Ferguson 1993).

As previously reported by Buller et al. (1992) the inoculation of large quantities of PMNLs permits reaching the maximum isolation capacity of the SVC without detecting notable toxic effects on the monolayers, if this quantity is divided between four different vials. However, at least in our experience, it is very difficult to obtain such high numbers of PMNLs in immunocompromised patients, such as those infected with HIV.

To include a higher number of patients in the study, we limited the number of PMNLs inoculated to a maximum of 800,000/vial. In the group of patients with negative AGA (<1 PMNL positive × 200,000 leukocytes), the inoculation of the same quantity of PMNLs on the SVC led to the detection of three patients (23%) with viremia. In these cases, it may be that the low viral load does not permit an homogenous distribution of the PMNLs infected with CMV among those used for the AGA and the SVC. However, on adding greater quantities of PMNLs to the SVC, we observed a significant increase in the detection capacity (sensitivity). The 13 patients with negative AGA and positive SVC, with the inoculation of 800,000 PMNLs/vial, represent 9.7% of all patients with negative antigenemia (13/134), corresponding to patients with CMV viremia that would not have been detected with the AGA test.

A comparison of the overall sensitivity of the AGA and the SVC in the detection of CMV with the same quantities of PMNLs studied (200,000) shows the AGA to have been significantly more sensitive than the SVC. These results may be due to the inclusion of patients on suppressive-specific antiviral treatment, which does not permit the growth of CMV in cell culture.

In those patients with positive AGA with low values (between 1 and 5), we found that the addition of larger quantities of PMNLs to the SVC lead to a significant increase in the CMV isolation. However, the overall comparison of the results obtained in the SVC and AGA tests with 400,000 PMNLs showed that the two techniques had the same sensitivity in the detection of CMV. When comparing the sensitivity of the AGA with that of the SVC inoculated with the maximal number of PMNLs (800,000), we observed that the initial results were inverted. In this case, the SVC was found to be significantly more sensitive than the AGA in the detection of CMV in patients with either positive or negative AGA.

It seems logical that the addition to the SVC of a quantity of PMNLs greater than that of the AGA will lead to an increase in its capacity to detect the presence of low level CMV viremia (Buller et al. 1992; Storch et al. 1994). Our results confirm this hypothesis and, in concept, are in agreement with the studies performed by Buller et al. (1992), even though we used lesser quantities of PMNLs, thus enabling us to study a larger patient population. In our study, only eight patients (3.9%) were detected with a quantity equal to or greater than that recommended by this author. Our results appear to contradict the results previously reported by Storch et al. (1994), where only 4% of the blood samples analyzed presented quantities less than $6 \times 10^6$ PMNLs/mL.

Our results suggest that to obtain the maximum diagnostic yield from the SVC in the detection of CMV viremia, inoculation of the greatest possible quantity of PMNLs is necessary. In our study, after

the inoculation of 800,000 PMNLs, we detected 100% of the viremic samples with negative antigenemia and 95.5% of all of the positive samples. With these quantities of PMNLs, hardly any toxic effects on the cellular monolayers were found. Using this procedure, we have ascertained that the SVC may be as, or more, sensitive than the AGA in the detection of CMV in blood. Moreover, the SVC is an easier, faster, and more available method in most of the laboratories as compared to AGA. The quantitation of the number of PMNLs inoculated in the SVC permits quantification of the viral load present in the blood through the number of infectious foci obtained in the SVC (Gerna et al. 1992), which is the basis of the quantitative shell-vial culture.

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