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Comparison of Two Leukocyte Extraction Methods for Cytomegalovirus Antigenemia Assay

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We carried out a prospective, parallel, and blind study on 113 blood samples from immunocompromised patients in order to compare two leukocyte extraction methods (6% dextran sedimentation and Polymorphprep separation) for cytomegalovirus (CMV) antigenemia assay. CMV was detected in 38 samples by antigenemia assay (34 by dextran sedimentation and 35 by Polymorphprep separation). No differences either in the number of positive specimens ($P = 1$) or in the mean CMV-positive cell counts ($P = 0.41$) were observed between the two leukocyte extraction methods. In conclusion, the two methods performed equally well for this assay.

The quantitative antigenemia (AGM) assay for cytomegalovirus (CMV) has emerged in recent years as a valuable tool for the diagnosis and prognosis of CMV disease and for monitoring patients at risk of CMV disease (12, 16). Although the test is rapid, sensitive, and easy to perform, it is not sufficiently standardized and technical factors may affect its performance (16). One such factor is the leukocyte extraction method.

The standard AGM assay is based on dextran leukocyte extraction, but other extraction methods are available (2, 7), such as the one involving Polymorphprep solution. Better culture results have been reported by the Polymorphprep method than by methods that use inoculated blood cell suspensions extracted by dextran sedimentation (2). In addition, the AGM assay performed on a mixed leukocyte blood fraction obtained by the Polymorphprep method allows for the detection of higher numbers of positive samples than is possible by isolation in culture (3). However, no comparative studies of the various leukocyte extraction methods for the AGM assay have been carried out. We carried out a prospective, parallel, and blind study to compare, both qualitatively and quantitatively, the efficiency of the method with Polymorphprep extracting solution against the generally used method with dextran extraction for the AGM assay under the routine conditions of a diagnostic laboratory.

Specimens. We studied 113 consecutive heparinized blood samples (10 ml) from 72 patients at risk of CMV infection (25 human immunodeficiency virus-infected patients, 44 transplant recipients [12 heart, 16 liver, 8 kidney, and 8 bone marrow recipients], and 3 miscellaneous immunocompromised patients). All of the specimens were processed within 4 h after the blood samples were obtained.

Methods. For the dextran extraction method, a 5-ml aliquot of whole blood was mixed with 1.5 ml of 6% dextran solution in saline (Macrodex; Pharmacia, Uppsala, Sweden). After sedimentation for 30 min at 37°C, the polymorphonuclear leukocyte (PMNL)-enriched supernatant was collected, washed, and centrifuged ($500 \times g$, 10 min). Contaminating erythrocytes in the sediment were lysed with 1 ml of a chilled 0.8% NH_4Cl solution. After another washing and centrifugation step, the cells were resuspended in 1 ml of phosphate-buffered saline (PBS).

For the Polymorphprep (Nycomed Pharma AS, Majorstua, Norway) extraction method, we followed the manufacturer's instructions. The remaining 5-ml aliquot of the heparinized blood sample was carefully layered over 3.5 ml of this solution (sodium metrizoate, 13.8% [wt/vol]; dextran 500, 8.0% [wt/vol]), and the mixture was centrifuged at $475 \times g$ for 30 min at 20°C. After that, we obtained two leukocyte bands (mononuclear cells in the top band and PMNLs in the lower one) which were harvested, mixed, washed, and centrifuged ($500 \times g$, 10 min). The cells were finally resuspended in 1 ml of PBS. A lysis step with the NH_4Cl solution was not done with the Polymorphprep method.

Both (dextran and Polymorphprep) leukocyte extracts were counted in an automatic hematological counter, and the cell concentration was adjusted to 10^6 cells per ml. Then, 200 μl (2×10^5 cells) of each adjusted suspension was spotted onto a slide by means of a cytocentrifuge (Cytospin 3; Shandom Scientific, Runcorn, England) at 700 rpm for 7 min, air dried, and fixed in a solution containing 5% formaldehyde and 2% sucrose in PBS. The slides were stained by an indirect immunofluorescence assay with a commercially available monoclonal antibody directed against the CMV pp65 antigen (Monofluokit CMV; Pasteur Diagnostics, Marnes-la-Coquette, France). The slides were screened at a magnification of ×160 and were confirmed at a magnification of ×400. Quantitative blind readings were always performed immediately by an experienced observer. Only cells showing well-defined nuclear fluorescence were recorded, and the results were expressed as the number of CMV-positive cells per 10^5 total leukocytes.

Following the methods currently used in our laboratory, dextran leukocyte extracts (not treated to lyse the erythrocytes) were also used for conventional and shell vial cultures. They were processed in MRC-5 fibroblasts in accordance with standard procedures (5, 14). Briefly, the vials were inoculated with approximately 4×10^5 leukocytes, centrifuged at $700 \times g$, incubated at 37°C overnight, and then stained with a monoclonal antibody directed against the 72-kDa immediate-early CMV antigen (E13; Biosoft, Varilhes, France). The same inocula were used for tube cultures, which were incubated at 37°C for 15 days (6), and were checked 4 days per week for the typical cytopathic effect.

Statistical analysis. The McNemar test was used to compare the differences in positive specimens. To compare the mean

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TABLE 1. Comparison of two different leukocyte extraction methods for the AGM assay

Extraction method	AGM assay-positive samples (n = 38)		No. of CMV-positive cells/10^5 leukocytes	
	No.	% of total	Mean	Range
Dextran	34	89.5	9.00	1–>250
Polymorphprep	35	92.1	8.46	1–>250

positive cell counts, the two-tailed Student t test for paired samples was used after carrying out analysis of variance. A P value of <0.05 was considered to be statistically significant.

Table 1 provides the results of the study. Overall, CMV was detected in 41 samples; this was confirmed in 31 samples by culture techniques and 38 samples by the AGM assay (at least by one of the extraction methods). Consequently, the results for 10 AGM assay-positive specimens were not confirmed by any of the culture techniques. This occurred either in samples with low antigenemia counts (8 of 10 samples showing less than 10 CMV-positive cells per 10^5 leukocytes) and in specimens from treated patients (8 of 10 specimens, including the only 2 samples with antigenemia counts higher than 10 antigen-positive cells per 10^5 leukocytes). On the other hand, in three blood samples CMV was detected only by conventional culture or the shell vial assay.

Among the samples with AGM assay-positive results, four samples were positive only when the Polymorphprep extraction procedure was used and three were positive only when the dextran procedure was used. All of these seven discordant samples showed very low CMV-positive cell counts (1 or 2 cells per 10^5 leukocytes). Six of the seven samples were confirmed to be positive by culture methods. The remaining sample was from a known viremic bone marrow transplant patient and was obtained during ganciclovir therapy. No statistical differences were observed between the numbers of specimens found to be positive when the two leukocyte extraction methods were used. (P = 1; McNemar test). Nor were differences in the mean number of CMV-positive cell counts observed when the two extraction methods were used (P = 0.41; two-tailed Student's t test). Twelve samples showed higher positive cell counts by the dextran extraction method; in 16 samples, higher counts were observed with Polymorphprep extracts; in the remaining 10 samples the same counts were found by the use of both methods.

Detection of CMV in blood leukocytes (CMV viremia) is considered a reliable marker of disseminated CMV infection and may predict invasive CMV disease (9, 10, 13). Therefore, rapid and sensitive methods for the detection of CMV viremia are essential for identifying patients at risk of developing invasive CMV disease who may benefit from early antiviral therapy. Despite technical improvements such as the shell vial technique, there is no completely satisfactory culture method for the detection of CMV in blood samples (8). On the other hand, both sensitivity and rapidity can be achieved by the AGM assay, as shown in the literature (2, 11, 12, 16).

The standard AGM assay is based on dextran leukocyte extraction (16). It mainly obtains the PMNL population, while Polymorphprep extracts a mixed leukocyte fraction consisting of PMNLs and mononuclear cells. PMNLs are the most common site of active CMV genome expression and replication during viremia and, probably, the most important cells for the dissemination of CMV in immunocompromised patients (1, 4). Mononuclear cells (mainly monocytes) are also commonly infected and play an important role in CMV persistence and latency (1, 15). Consequently, and bearing in mind the data reported in comparative studies of the efficiency of culture results with different leukocyte suspensions (2, 7), better results could be expected for the AGM assay when the Polymorphprep solution was used for leukocyte extraction. However, we did not observe statistical differences either in the number of CMV-positive samples detected or in the mean CMV-positive cell counts by the two leukocyte extraction methods used in the present study. Although we found no overall differences between the two extraction methods, we observed discrepancies in CMV-positive cell counts in some particular specimens. We hypothesize that these discrepancies might be due to the intrinsic variability of the AGM assay. Of course, performing tests with duplicate or multiple slides could reduce discrepancies, but this practice should be analyzed from the point of view of cost-effectiveness.

Although we did not try specifically to address the performance of the AGM assay in comparison with those of culture techniques, our study demonstrates again that the former is more sensitive, regardless of the leukocyte extraction method. This higher degree of sensitivity of the AGM assay is in accordance with data from the literature (2, 11, 12, 16). In our study, culture techniques (shell vial and conventional culture) detected CMV in 75% of all positive samples, whereas the AGM assay detected CMV in 93% of them. If we consider culture techniques to be the standard, the sensitivity of the AGM assay is 110% by the dextran extraction procedure and 113% by the procedure with Polymorphprep extracts. In our opinion, this high degree of sensitivity cannot be attributed to the lower degree of specificity of the assay, because the 10 samples positive only by the AGM assay were from patients with demonstrated viremia and were obtained during antiviral therapy for CMV disease.

In conclusion, the two extraction methods perform equally well for the AGM assay both in terms of detecting positive specimens and in terms of quantitative results. Polymorphprep extraction is more expensive and, perhaps it is more cumbersome to carry out than the dextran extraction method (the dextran method is the procedure that we use). On the other hand, extracts obtained by the former method could be used to inoculate cultures with better results, as reported in the literature (2, 7). This possibility is being evaluated in our laboratory. Each laboratory should choose the leukocyte extraction method that best suits its particular work routines.

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