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Comparison of Heparin and EDTA Transport Tubes for Detection of Cytomegalovirus in Leukocytes by Shell Vial Assay, pp65 Antigenemia Assay, and PCR

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The anticoagulants heparin and EDTA were compared for inhibitory effects on the detection of cytomegalovirus from washed leukocytes in specimen transport tubes. Evaluation was made by the centrifugation/shell vial culture technique, the pp65 antigenemia assay, and PCR. For each assay, the results with heparin and EDTA were equivalent.

The centrifugation/shell vial culture technique (4), the pp65 antigenemia assay (10), and PCR (7, 8) are important methods for the rapid detection of cytomegalovirus (CMV) viremia. Typically, blood samples for these assays are collected and transported in tubes containing an anticoagulant. This study was performed to compare the effects of heparin and EDTA, both of which are widely used as anticoagulants for blood sample transport, on each of these assays.

Serial paired blood samples were collected from 10 patients who had undergone lung transplantation at Washington University Medical Center, St. Louis, Mo., during July 1992 to March 1993. Informed consent was obtained from each patient before samples were obtained. Blood obtained for CMV studies was drawn into non-silicone-coated blood collection tubes containing heparin or EDTA (Vacutainer; Becton Dickinson, Rutherford, N.J.). The heparin tubes were either those designed to accommodate 7 ml of blood (7-ml draw) containing 100 USP units of lithium heparin or 10-ml draw containing 143 USP units of lithium heparin. The EDTA tubes were 5-ml draw containing 0.05 ml of 15% K_3 EDTA. The interval from collection to processing in the laboratory was typically 3 to 6 h.

Upon arrival in the Virology Laboratory of the Washington University Department of Pediatrics, leukocyte suspensions were prepared from each tube by dextran sedimentation, lysis of erythrocytes, and washing of cells as previously described (3). Leukocyte suspensions were adjusted so that the concentration of cells was 2×10^6 to 8×10^6 per ml, and the concentrations of cells in suspensions prepared from the heparin and EDTA tubes of a single sample pair were adjusted to be identical. From each leukocyte suspension, aliquots of 0.25 ml were inoculated into two shell vial cultures containing MRC-5 human embryonic fibroblasts (ViroMed, Minneapolis, Minn.). The shell vial assay was performed by using the quantitative modification previously described (3), yielding results expressed as infectious centers per 10^5 leukocytes. Immunofluorescent-antibody staining of the shell vial cultures was performed at approximately 40 h after inoculation with two monoclonal antibodies specific for a CMV immediate-early antigen. The monoclonal antibody 9221 (DuPont Inc., Doraville, Ga.) was used on one coverslip, and the monoclonal antibody L14 (Ortho Diagnostic Systems, Raritan, N.J.) was used on the other. The pp65 antigenemia assay was performed with the CMV-vue kit (INCSTAR Corp., Stillwater, Minn.) as previously described (9), with the results expressed as positive cells per 10^5 leukocytes. PCR was performed on a subset of 19 samples from seven patients selected to include samples from periods when CMV viremia was either likely or unlikely on the basis of the time after transplantation. PCR was performed as previously described (9) on 10^5 cell equivalents of DNA from an alkaline lysate prepared from 5×10^5 to 1×10^6 leukocytes. All 19 samples were tested with primers that amplify the late gene encoding pp65 (8). All samples that were negative for late-gene DNA were also tested with primers that amplify the major immediate-early gene (7, 9). Finally, all samples that were negative for CMV DNA were tested for the presence of inhibitors of PCR by retesting them for late-gene DNA after the addition of 1 μl of a 10^{-2} dilution of a lysate of MRC-5 cells infected with the CMV Towne strain. PCRs were analyzed by electrophoresis on 3% NuSieve 3:1 agarose gels (FMC Bioproducts, Rockland, Maine) containing 0.5 μg of ethidium bromide per ml and were photographed with UV illumination.

The statistical significance of differences in the proportion of positive cultures or antigenemia assays according to anticoagulant were evaluated by McNemar's test. The 95% confidence interval of the difference in proportions was taken as the difference ± 1.96 times the standard error, when the standard error was calculated by using the following formula:

$$se(p_1 - p_2) = \frac{1}{n}\sqrt{b + c - \frac{(b - c)^2}{n}}$$

where *se* is the standard error; p_1 and p_2 are the proportions of samples transported in heparin and EDTA tubes, respectively, that were positive; n is the number of sample pairs (heparin and EDTA tubes); and b and c are the number of pairs in which only one of the two paired cultures was positive (1). The significance of differences between quantitative shell vial titers and antigenemia titers of samples transported in heparin and EDTA tubes was calculated by the paired t test. For all analyses, a P value of ≤ 0.05 was considered statistically significant.

A preliminary analysis of the results of shell vial culture assays revealed that the CMV monoclonal antibody used to

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TABLE 1. Effects of anticoagulant on the detection of CMV in leukocytes by shell vial culture, pp65 antigenemia assay, and PCR

Test	Sample pairs tested (*n*)	Heparin/EDTA results[a]			
		Pos/pos	Pos/neg	Neg/pos	Neg/neg
Shell vial culture	105	21	6	3	75
pp65 antigenemia assay	103	20	6	6	71
PCR	19	13	0	0	6

[a] Number of sample pairs showing the indicated pattern of PCR results after transport in heparin and EDTA tubes, respectively. Pos, positive; Neg, negative.

perform fluorescent-antibody staining had no effect on either the number of shell vials found to be positive or the titer of infectious centers (data not shown). Therefore, in subsequent analyses, the results of the shell vial cultures of each sample with the DuPont and Ortho monoclonal antibodies were combined and the shell vial culture for that sample was considered positive if any vial was positive.

The effects of anticoagulant on the detection of CMV by shell vial culture, pp65 antigenemia assay, and PCR are shown in Table 1. The small difference in the proportion of positive shell vial cultures from samples transported in heparin tubes compared with those in EDTA tubes (26 and 23%, respectively) was not statistically significant (95% confidence intervals of the difference, −3 to 9%; $P = 0.5$). For both the antigenemia assay and PCR, there was also no difference in the proportion of samples that were positive after transport in heparin or EDTA tubes (Table 1). The differences in shell vial titers (0.10 versus 0.07 infectious center per 10^5 leukocytes; $P = 0.23$) and in antigenemia titers (3.0 versus 2.6 positive cells per 10^5 leukocytes; $P = 0.7$) were also not statistically significant. For those samples that were positive by PCR, the intensity of ethidium bromide staining of the PCR product on an agarose gel was similar regardless of whether the samples had been transported in heparin or EDTA tubes (Fig. 1). All samples that were negative for late-gene DNA were also negative for immediate-early gene DNA. After the addition of infected cell lysate to each of the negative samples, late-gene DNA could be detected, suggesting the absence of gross PCR inhibitors in DNA prepared from leukocytes collected in either type of transport tube.

From these results, we conclude that commercial heparin and EDTA tubes are equivalent for use in shell vial cultures and pp65 antigenemia assays. We are confident that this study was capable of detecting an important difference, since both assays were performed in quantitative fashion, allowing comparisons of the levels of viremia and antigenemia rather than simply the proportions of samples that were positive. It should be noted that the manufacturer's instructions that accompany the CMV-vue kits used for the antigenemia assays in this study specify the use of EDTA tubes. This specification is the result of the use of EDTA tubes in the prelicensing studies of this kit that were submitted to the Food and Drug Administration (4a).

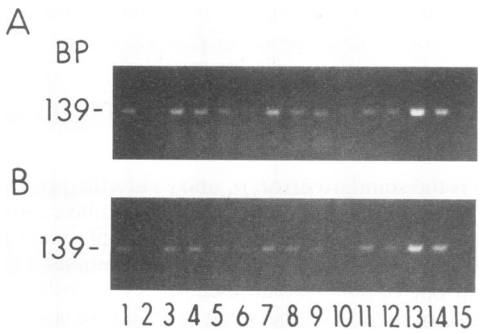

FIG. 1. Ethidium bromide staining of CMV late-gene PCR products amplified from washed leukocytes harvested from blood samples transported in collection tubes containing heparin (A) or EDTA (B). Positive and negative controls are in lanes 1 and 2 of each panel, respectively. Members of matched sample pairs are in the same lanes (lanes 3 to 15). BP, base pairs.

Our results also suggest that the use of heparin transport tubes does not interfere with the detection of CMV DNA in leukocytes by the PCR assay used in our laboratory. This result is consistent with that of Beutler et al., who found an inhibition of PCR performed with plasma but not with washed leukocytes (2). In contrast, Holodniy et al. (5) showed an inhibition of amplification of human immunodeficiency virus RNA and DNA from plasma and washed cells, suggesting that heparin might bind to cells and not be completely removed by washing. Since only Holodniy's study was quantitative, it is possible that the Beutler study and this one failed to detect a quantitative difference. Other studies showing an inhibition of PCR by heparin have involved the performance of PCR with plasma and/or the use of reverse transcription PCR to detect RNA (6, 11). Despite the lack of PCR inhibition detected in this study, the demonstrated capacity of heparin to inhibit *Taq* polymerase (2, 5) is of concern and suggests that heparin should be avoided as an anticoagulant in samples intended for quantitative PCR or PCR performed with plasma or serum.

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