

## Effect of Delayed Specimen Processing on Cytomegalovirus Antigenemia Test Results

MARIE L. LANDRY,<sup>1,2\*</sup> DAVID FERGUSON,<sup>1</sup> SANDRA COHEN,<sup>1</sup> KARYN HUBER,<sup>1</sup>  
AND PATRICIA WETHERILL<sup>3</sup>

*Clinical Virology Laboratory, Yale New Haven Hospital,<sup>1</sup> and Departments of Laboratory Medicine<sup>2</sup> and Medicine,<sup>3</sup>  
Yale University School of Medicine, New Haven, Connecticut 06510*

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**Blood samples held at either 4°C or room temperature for 1 day had similar mean decreases in number of cytomegalovirus antigenemia-positive cells (52 to 55%) and similar false-negative test results (13 to 14%). After 2 days, samples held at 4°C showed no further decline, whereas samples held at room temperature had a mean 81% decrease in positive cells, a 32% false-negative rate, and a more marked deterioration in cell morphology.**

The development of the cytomegalovirus (CMV) antigenemia assay has been a significant advance in the diagnosis of CMV infection (3, 9, 10). The CMV antigenemia assay detects CMV pp65 antigens directly in peripheral blood leukocytes affixed to glass slides, by using either immunofluorescence or immunoperoxidase staining, with results available in 5 h. The antigenemia assay allows both rapid detection and quantitation of CMV in peripheral blood leukocytes and can be used to monitor the response to therapy.

However, the test has a limitation: it is recommended that samples be processed within 6 h of collection (3). Delays in processing can reduce the number of positive cells detected and can lead to false-negative results (3, 7, 11). This is a particular problem for laboratories that receive samples transported over a distance, such as regional reference laboratories and many commercial laboratories, because samples are commonly received 1 to 2 days after collection. The goal of this study was to better define the impact of holding temperature, holding medium, antiviral therapy, and protease inhibitors on quantitative and qualitative CMV antigenemia test results when processing is delayed for 1 to 2 days.

A total of 43 blood samples from 32 human immunodeficiency virus-infected patients and one bone marrow transplant recipient were examined in this study. Blood samples in EDTA-treated tubes were submitted for CMV antigenemia assay to the Clinical Virology Laboratory at Yale-New Haven Hospital. Upon receipt, specimens were separated into aliquots. One aliquot was processed within 6 h of collection (day 0) to determine the baseline CMV antigenemia value. Only samples positive at baseline were further evaluated. The remaining aliquots were held under different storage conditions and processed on days 1 and 2 after receipt. The CMV antigenemia assay was performed as previously described (5). Leukocytes obtained by dextran sedimentation were applied to slides (100,000 cells per slide) by cytocentrifugation (Shandon, Inc., Pittsburgh, Pa.). Cells were fixed with 5% paraformaldehyde and stained with Clonab CMV monoclonal antibodies (Biotest Diagnostics, Denville, N.J.) followed by affinity-purified fluorescein isothiocyanate-labeled sheep antimouse immunoglobulin G (Fab) (Organon Teknika-Cappel, Malvern, Pa.). The results were expressed as the sum of the numbers of CMV

antigen-positive cells detected on two slides. One slide from each time point and storage condition was also stained with Wright-Giemsa stain and examined by an experienced hematology technologist without knowledge of the storage conditions. Protease inhibitors were obtained from Boehringer Mannheim Corporation (Indianapolis, Ind.). The inhibitors utilized were those suggested by the company for use with animal tissues and were added to whole blood to give the recommended final concentrations as follows: Pefabloc SC, 1 mg/ml; EDTA, 1 mmol/liter; leupeptin, 10 µg/ml; pepstatin, 10 µg/ml; and aprotinin, 1 µg/ml.

To evaluate the effect of storage temperature, 24 aliquots of whole blood from 19 patients were stored at room temperature (RT) and 4°C and processed for detection of CMV antigenemia on days 1 and 2 after receipt (Table 1). One day after collection, 81 and 83% of the samples held at 4°C and RT, respectively, showed a decrease in the number of CMV antigen-positive cells. By day 2, 82% of the samples at 4°C and 95% of those at RT showed decreases in CMV antigenemia. The overall mean reduction in the number of positive cells was similar for samples held at RT or 4°C at day 1, but it was greater for blood samples held at RT at day 2 ( $P < 0.01$  [ $t$  test]). Likewise, the number of false-negative results was similar for RT and 4°C at day 1 but greater for RT at day 2 (Table 2); however, this difference was not statistically significant ( $P < 0.25$  [Fisher's exact test]). Examination of Wright-Giemsa-stained cells held at either temperature showed progressive deterioration with longer storage. However, the morphology of polymorphonuclear leukocytes held at 4°C was better preserved and facilitated the detection of CMV antigen-positive cells, especially at day 2 (data not shown). Thus, for the remainder of the study, all samples were held at 4°C.

Seven samples collected from patients on ganciclovir and foscarnet were identified (Table 1). Taken in total at both time points and temperatures, the percentage of reduction in antigenemia for samples obtained from patients on therapy was greater than that for those not on therapy ( $P < 0.001$  [ $t$  test]). The number of positive cells at baseline for samples obtained from patients on antiviral therapy (mean, 336) was significantly greater than that for samples from patients not on therapy (mean, 179) ( $P < 0.05$ , Wilcoxon's nonparametric test). Thus, as shown in Table 2, samples obtained from patients on antiviral therapy remained positive despite a greater decline in positive cells. Of note, nuclear staining for pp65 antigen was fainter in the presence of anti-CMV therapy.

To evaluate the effect of holding medium, leukocytes from

\* Corresponding author. Mailing address: Department of Laboratory Medicine, Yale University School of Medicine, 333 Cedar St. P.O. Box 3333, New Haven, CT 06510. Phone: (203) 785-3475. Fax: (203) 785-7340.

TABLE 1. Effect of delayed processing and storage temperature on CMV antigenemia test results

Patient	No. of positive cells detected (% reduction from baseline) at:				
	Baseline (day 0 [RT])	Day 1		Day 2	
		RT	4°C	RT	4°C
1	3	0 (100)	0 (100)	ND <sup>a</sup>	ND
2	2	1 (50)	0 (100)	0 (100)	1 (50)
3	6	1 (83)	2 (67)	ND	ND
4	6	6 (0)	5 (17)	1 (83)	3 (50)
5	7	0 (100)	4 (43)	0 (100)	0 (100)
6	10	10 (0)	26 (0)	29 (0)	27 (0)
7	11 <sup>b</sup>	5 (55)	4 (64)	3 (73)	6 (45)
8	15	7 (53)	6 (60)	1 (93)	0 (100)
9	15	6 (60)	18 (0)	0 (100)	11 (27)
10	21	5 (76)	20 (5)	0 (100)	15 (29)
11	42	26 (38)	65 (0)	0 (100)	20 (52)
12	48 <sup>b</sup>	14 (71)	14 (71)	9 (81)	22 (54)
13	59	24 (59)	ND	ND	ND
	34 <sup>b</sup>	17 (50)	ND	8 (76)	ND
14	32	15 (53)	ND	0 (100)	ND
	77	64 (17)	57 (26)	32 (58)	138 (0)
	227	247 (0)	331 (0)	35 (85)	69 (70)
15	167	56 (66)	67 (60)	ND	ND
	133 <sup>b</sup>	53 (60)	15 (89)	12 (91)	58 (56)
16	1,140 <sup>c</sup>	2 (100)	26 (98)	ND	ND
	253 <sup>d</sup>	0 (100)	8 (97)	8 (97)	48 (81)
17	733 <sup>b</sup>	227 (69)	260 (65)	54 (93)	385 (47)
18	847	935 (0)	0 (100)	673 (21)	509 (40)
19	1,500	501 (67)	940 (37)	191 (87)	1,518 (0)
Mean % reduction		55 <sup>e</sup>	52	81 <sup>e,f</sup>	47 <sup>f</sup>

<sup>a</sup> ND, not determined.<sup>b</sup> Ganciclovir treatment.<sup>c</sup> Foscarnet treatment.<sup>d</sup> Treatment with both ganciclovir and foscarnet. Note that samples obtained from patients on these drugs showed greater antigenemia reduction with storage ( $P < 0.001$  [ $t$  test]).<sup>e</sup> The difference between day 1 and day 2 was significant for samples held at RT ( $P < 0.01$  [ $t$  test]).<sup>f</sup> At day 2, the difference between RT and 4°C was significant ( $P < 0.01$  [ $t$  test]).

14 samples from 12 patients were separated by dextran sedimentation on the day received, added to 2.0 ml of viral transport medium or Hanks balanced salt solution, stored at 4°C, and then processed on days 1 and 2 for detection of CMV

TABLE 2. Effect of storage temperature and antiviral therapy on false-negative CMV antigenemia test results

Day after collection	Anti-CMV treatment <sup>a</sup>	No. of false-negative results/no. tested (%) at <sup>b</sup> :	
		RT	4°C
1	No	2/17 (12)	3/15 (20)
	Yes	1/7 (14)	0/6 (0)
Total		3/24 (13) <sup>c</sup>	3/21 (14) <sup>c</sup>
2	No	6/13 (46)	2/12 (17)
	Yes	0/6 (0)	0/5 (0)
Total		6/19 (32) <sup>d</sup>	2/17 (12) <sup>d</sup>

<sup>a</sup> Ganciclovir or foscarnet.<sup>b</sup> Whole blood stored at RT or 4°C.<sup>c</sup> Difference between RT and 4°C not significant ( $P < 1.00$  [Fisher's exact test]).<sup>d</sup> Difference between RT and 4°C not significant ( $P < 0.25$  [Fisher's exact test]), and difference between day 1 and day 2 RT not significant ( $P < 0.20$  [Fisher's exact test]).

antigenemia. Leukocytes stored in Hanks balanced salt solution showed poor cell morphology and greater CMV antigen degradation; thus, use of Hanks balanced salt solution was promptly discontinued. Cells separated and held in viral transport medium showed slightly greater reduction in the number of antigen-positive cells than cells in unseparated whole blood (47 versus 39% reduction on day 1, and 52 versus 37% reduction on day 2), but the differences were not significant ( $P < 0.60$  and  $P < 0.50$ , respectively [ $t$  test]). Prompt cell separation and storage in viral transport medium also did not improve the false-negative rate (data not shown).

Lastly, a selection of protease inhibitors was added on day 0 to a total of five whole-blood samples from five patients. At both days 1 and 2, all samples containing protease inhibitors had antigenemia reductions of 99 to 100%, whereas whole-blood samples without inhibitors had mean reductions of 27% at day 1 and 53% at day 2. Examination by Wright-Giemsa staining revealed more disrupted cells and extracellular material in protease-treated cells.

The CMV antigenemia assay is a rapid and sensitive test that uses technology readily within the capability of a clinical laboratory. Results can be reported either qualitatively or quantitatively, with quantitation providing additional information to help clinical interpretation and monitoring of treatment. The suitability of the CMV antigenemia test for regional reference laboratories given the need for processing within 6 h of collection remains an unanswered question.

Two recent papers have addressed the effect of delayed processing on CMV antigenemia results. In one paper (1), the effect of holding heparinized blood samples from bone marrow transplant recipients at RT versus 4°C for 24 h was examined. As in our study, only samples positive at baseline were further evaluated. The authors examined 20 samples and found RT to be superior to 4°C at 24 h, although the differences were not significant. Most samples remained positive at 24 h, and the mean reductions in antigen-positive cells were 44% at RT and 62% at 4°C compared with baseline values. As in our study, some samples showed increases in antigen-positive cells. None of these samples was obtained from patients on antiviral therapy. Boeckh et al. (1) also reported cell deterioration when cell pellets were stored in phosphate-buffered saline, similar to our experience with Hanks solution.

In a subsequent paper (8), aliquots from all samples submitted during the test period were retested after being held at 4°C for 1 day. RT storage was not evaluated. Interestingly, 8 of 40 samples (20%) positive at baseline were negative on retesting the next day, whereas 9 previously negative samples were positive after 1 day of storage. These samples were reported to have low numbers of antigen-positive cells. Calculations of the mean number of positive cells after 1 day of storage compared with baseline showed only a 13% reduction, but this average included samples positive only after storage. Some samples were obtained during antiviral therapy, but the effect of treatment on the results was not mentioned.

In the present study, information on holding blood samples for up to 2 days was obtained because samples frequently arrive at reference or commercial laboratories 2 days after collection. The percentage of reduction in antigen-positive cells after 1 day of storage in our study was similar to that found by Boeckh et al. (1), averaging about 50%. Also in agreement with Boeckh et al. (1), significant differences were not identified between RT and 4°C for either reduction in CMV antigen-positive leukocytes or the number of false-negative specimens at day 1. However, by day 2 after collection, blood samples held at RT showed a significantly greater decline in CMV antigen-positive cells, poor cell morphology, and more false-negative results. Importantly, samples held at 4°C showed no significant change between day 1 and day 2.

The present study is the first to address the effect of antiviral treatment on CMV antigenemia with stored samples, and although the false-negative rate was not increased, a significantly greater decline in the number of positive leukocytes in the presence of ganciclovir or foscarnet was noted. However, further study is needed, because the number of samples studied was small and significant variation was observed.

Unfortunately, attempts in our laboratory to better preserve CMV antigenemia levels, by either early cell separation and holding in viral transport medium or addition of protease inhibitors to whole blood, were not successful. Neutrophils, where most of the pp65 CMV antigen is expressed (3, 9, 10), are considered fragile cells that are easily damaged by improper handling and prone to rapid degeneration on storage (4). The effect of temperature, anticoagulants, various cell separation procedures, and length of storage on neutrophil

function in vitro have been well studied (2, 4, 6). Because storage of neutrophils at 0 to 4°C leads to impaired locomotion and chemotaxis in vitro (4, 6), holding of neutrophils at RT is standard in blood banks and transfusion on the day of collection is recommended. For CMV antigenemia, preservation of morphology, number, and integrity of cells may be more important, and these may be better maintained at 4°C than at RT (2).

In summary, despite significant decreases in CMV antigen-positive cells, the majority of whole-blood samples remained positive after 2 days. Storage of whole-blood samples in EDTA for 1 day at either 4°C or RT yielded essentially equivalent results. After a 2-day delay, storage at 4°C was superior; cell morphology was better preserved and antigenemia results were comparable to those obtained after 1 day of storage. Because samples for viral cultures are routinely shipped at 4°C and CMV isolation is commonly performed, as well as the antigenemia assay, holding and shipping all viral samples at 4°C would simplify specimen handling. To avoid misinterpretation of decreasing antigenemia levels, quantitative results are reported in our laboratory only for samples processed within 6 h of collection.

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