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Factors Influencing Detection of Quantitative Cytomegalovirus Antigenemia

MICHAEL BOECKH,* PATRICIA M. WOOGERD, TERRY STEVENS-AYERS, C. GEORGE RAY, AND RALEIGH A. BOWDEN

Program in Infectious Diseases, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104

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Of 20 blood specimens testing positive for cytomegalovirus antigen after immediate processing, 19 (95%) remained positive when kept at room temperature for 24 h before processing. Quantitative antigenemia decreased by an average of 44% after storage. Compared with acetone fixation, formaldehyde fixation showed improved readability, fewer artifacts, and a higher degree of sensitivity.

Several modifications of the cytomegalovirus (CMV) antigenemia assay have been proposed in recent publications, and some of these modifications are reported to increase the sensitivity of the assay, thereby potentially also altering predictive values and quantitative levels for the correlation with CMV disease (2, 3, 7). While the infectivities of blood specimens for culture inoculation appear to be preserved reasonably well for 48 h at 4°C (5), it is still controversial whether blood specimens can be stored for antigenemia testing (3, 8–10, 15). The purpose of the present study was to determine factors such as storage and the fixation method (i.e., acetone versus formaldehyde fixation) that may influence assay sensitivity.

Twenty milliliters of heparinized blood obtained from CMV-seropositive patients who underwent marrow transplantation was divided in two 10-ml tubes. The patients selected for these studies either were known to be antigenemia positive or were at high risk of being antigenemia positive. The contents of one tube were processed immediately (less than 2 h after the blood was drawn), while the contents of the other tube were divided into two portions, and 5 ml was held at room temperature (range, 20 to 22°C; all specimens) and 5 ml was placed on ice for 24 h (n = 11). In addition, in a subset of specimens, the leukocyte pellet was held in 0.2 ml of phosphate-buffered saline (PBS) at 4°C for 24 h. Processing of heparinized blood and preparation of cytospin slides were performed as described earlier (2, 14). Slides were fixed either in cold water-free acetone for 10 min (2) or by a formaldehyde-based staining method as described by Gerna et al. (3). Briefly, slides were placed in formaldehyde-sucrose solution (5% of F-1635 and 2% of S-9378 in PBS; both from Sigma Chemical Co., St. Louis, Mo.) for 10 min at room temperature, washed four times in PBS–1% fetal calf serum (FCS), and subsequently immersed in PBS–0.5% Nonidet P-40 (N-6507; Sigma)–10% sucrose–1% FCS for 5 min at room temperature for permeabilization. After washing the slides four times in PBS–1% FCS as described above and placing the slides in distilled water for 15 s, the slides were air dried and were either stained or stored at −20°C.

Immunofluorescence staining was performed as described earlier (2). We observed that rinsing with distilled water during the staining procedure may damage cell morphology. The reason for this phenomenon is unclear. The number of antigen-positive cells per slide was counted. The nonparametric sign test was used to detect differences between the methods.

Comparison of the results of formaldehyde fixation with those of acetone fixation is given in Table 1. The rate of artifacts by the two fixation methods was compared for 1,099 consecutive blood specimens. The artifact rate was higher with acetone fixation (30 of 642 specimens [4.7%]) than with formaldehyde fixation (6 of 457 specimens [1.3%]). Artifacts usually presented as nonspecific cytoplasmic staining. Because of the different staining pattern with formaldehyde fixation (i.e., bright nuclear staining), positive cells were easier to differentiate from artifacts by formaldehyde fixation than by acetone fixation.

The results of the storage experiments are given in Table 2 and detail only slides fixed with formaldehyde. Of the specimens for which additional slides were fixed with acetone, all remained positive, with a mean decrease of antigen-positive cells per slide of 42% (range, 0 to 75%) and 56% (range, 21 to 87%) when the slides were held at room temperature (n = 10) and on ice (n = 6), respectively. Three cell pellet specimens were stored at 4°C for 24 h. Although the specimens remained positive, the experiment was discontinued because of poor cell morphology.

A comparison of a 45-min incubation time at room temperature (2) versus incubation for 30 min at 37°C for both the monoclonal antibody and the fluorescein isothiocyanate conjugates (3) for 15 specimens from known antigenemia-positive patients showed that there was not a significant quantitative difference in quantitative antigenemia (P = 0.44).

Our results are in agreement with those of two studies comparing formaldehyde fixation, acetone-methanol fixation (3), and acetone fixation (7). We also found a bright nuclear staining pattern with formaldehyde fixation, whereas we found perinuclear staining when we used acetone fixation, and the number of fluorescing nuclei per positive specimen was slightly improved (Table 1). With formaldehyde fixation, even in the presence of nonspecific cytoplasmic staining, positive cells were easy to identify. Although formaldehyde fixation is somewhat more time-consuming (an additional 15 min), it offers the advantages of better readability and fewer artifacts. These factors are particularly important for routine use of a method in the clinical laboratory. We prefer immunofluorescence staining for routine use in the antigenemia assay. Immunoenzymatic techniques (immunoperoxidase, alkaline phosphatase, anti-alkaline phosphatase [1]) are also suitable for the detection of antigenemia and have the advantage of providing a

* Corresponding author. Mailing address: Program in Infectious Diseases, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104. Phone: (206) 667-4338. Fax: (206) 667-4411.

TABLE 1. Comparison of acetone and formaldehyde fixation methods

Antigenemia	Avg no. of positive cells/slide[a] (median [range])			No. of specimens[b]		
	Acetone	Formaldehyde	*P* value	A > F	A = F	F > A
≤5/slide[c] (*n* = 26)	2 (0–5)	3 (0–23)	0.05	4	8	14
6–10/slide[c] (*n* = 6)	7 (6–9)	8 (4–21)	ND[d]	2	2	2
>10/slide[c] (*n* = 12)	41 (15–123)	66 (9–213)	ND	3	0	9
All (*n* = 44)	5 (0–123)	6 (0–213)	0.02	9	10	25

[a] Cytospin slides were prepared in duplicate using 1.5×10^5 cells per slide.
[b] Number of specimens with a smaller, equal, and greater number of positive cells per slide with acetone (A) compared with formaldehyde (F) fixation.
[c] Categories were based on results with acetone fixation.
[d] ND, not determined because of low numbers.

permanent record; however, these techniques are more time-consuming (i.e., up to an additional 45 min) and no increased sensitivity has been observed (2, 3, 7).

Previous studies suggested that a delay of processing of blood specimens may result in a significant decrease in quantitative antigenemia (4) or may even result in false-negative results (8). In contrast, results of our study suggest that overnight storage only rarely results in the reaction of a positive specimen as negative, although the quantity of antigenemia may decrease by as much as 44% (Table 2). Nevertheless, every attempt should be undertaken to process the specimens without delay or at least to prepare the cytocentrifuge preparations on the same day. These slides can be stored at room temperature or at 4°C overnight, and staining can then be performed on the next day without a decrease in the level of quantitative antigenemia (data not shown). If overnight shipment cannot be avoided, this fact must be considered when interpreting the results. This may be particularly significant in low-positive specimens (i.e., fewer than five positive cells per slide), since it may result in false-negative results. Such a situation can occur early during CMV reactivation or during antiviral treatment. Specimens should be sent at room temperature for optimal preservation of the viral antigen (Table 2).

TABLE 2. Influence of storage of blood specimens on the antigenemia assay

Specimen	No. of positive cells/slide (% reduction on the basis of no storage) under the following storage conditions[a]:		
	No storage	24 h at room temp	24 h at 4°C
≤5 positive cells/slide (duplicate staining)[a]			
1	4/0[b]	1/1 (50)	ND[c] (ND)
2	2/1	2/1 (0)	ND (ND)
3	2/2	1/0 (75)	ND (ND)
4	2/0	0/0 (100)	1/0 (50)
5	2/0	1/0 (50)	0/0 (100)
>5 positive cells/slide (duplicate staining)[a]			
6	7/4	7/5 (0)	9/7 (0)
7	11/2	4/3 (46)	4/3 (46)
8	8/6	2/1 (79)	4/0 (71)
9	11/4	7/1 (47)	ND (ND)
10	18/7	4/3 (72)	5/1 (76)
11	18/11	10/3 (55)	3/ND (80)
12	19/12	6/7 (58)	8/5 (58)
13	26/8	13/8 (38)	12/1 (62)
14	25/17	22/18 (10)	ND (ND)
15	23/18	21/8 (29)	ND (ND)
16	30/15	17/0 (62)	ND (ND)
17	50/38	77/73 (0)	23/12 (60)
18	95/52	61/54 (29)	26/11 (75)
19	95/63	13/6 (88)	ND (ND)
20	129/126	142/110 (0)	ND (ND)
% Reduction			
Mean		44[d]	62[e]
Median		49	62
Range		0–100	0–100

[a] All slides were fixed with formaldehyde; acetone fixation did not change the results (data not shown).
[b] Results are for each slide (preparation of 1.5×10^5 cells per slide).
[c] ND, not determined, storage at 4°C was performed only for 11 specimens because of a limited amount of blood.
[d] $P = 0.0008$ for no storage versus storage at room temperature.
[e] $P = 0.007$ for no storage versus storage at 4°C; $P = 0.51$ for storage at room temperature versus storage at 4°C (not significant).

Numerous studies in transplant recipients and patients with AIDS have shown that the assay is a highly sensitive, specific, and predictive test for the rapid diagnosis of CMV in blood specimens (2, 4, 6, 7, 11–14). Additional advantages of the assay include the rapidity of processing and the lack of a requirement for cell culture, both of which result in lower costs and a low number of noninterpretable results. One of the most important features of the antigenemia assay is that it can be quantified and that high levels of antigenemia appear to correlate with CMV disease (2, 4, 6, 10–14). An application that is particularly promising is the use of quantitative antigenemia to predict CMV disease, thereby allowing the clinician to stratify the patient's risk for disease (2, 10, 12). Also, quantitative antigenemia may be useful for monitoring antiviral treatment and for predicting the recurrence of disease after an initial course of antiviral treatment (2, 3, 10, 13). However, breakpoints of antigenemia may be different between patient populations (2, 10, 12). For example, in renal transplant patients, an antigenemia level of more than 10 positive cells per slide is predictive of disease (10, 11), while in bone marrow transplant patients, levels of more than 1 positive cell per slide are highly predictive of subsequent disease (2). A concept that appears to be consistent in all patient populations is that rapidly rising levels of antigenemia are predictive of subsequent CMV disease (2, 4, 6, 10–13). Since quantitative antigenemia is increasingly used in the management of immunocompromised patients, standardization of the assay is needed. In this context, it appears important that future studies evaluating modifications of the assay (e.g., by using new monoclonal antibodies) should include a quantitative comparison with widely used antibodies and groups of patients with specific disease statuses in order to compare breakpoints and predictive values for symptomatic infection.

In conclusion, for the routine laboratory, our findings suggest that formaldehyde fixation with immunofluorescence staining is superior to acetone fixation because of the improved readability, fewer artifacts, and increased sensitivity obtained by formaldehyde fixation. Antigenemia remains detectable in 95% of specimens, including low-positive specimens, when processing is delayed for 24 h. However, immediate processing should be attempted for optimal results of quantitative antigenemia.

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