JOURNAL OF CLINICAL MICROBIOLOGY, May 1992, p. 1232-1237 Vol. 30, No. 5
0095-1137/92/051232-06$02.00/0
Copyright © 1992, American Society for Microbiology

Comparison of Different Immunostaining Techniques and Monoclonal Antibodies to the Lower Matrix Phosphoprotein (pp65) for Optimal Quantitation of Human Cytomegalovirus Antigenemia

GIUSEPPE GERNA,* M. GRAZIA REVELLO, ELENA PERCIVALLE, AND FULVIA MORINI

Virus Laboratory, Institute of Infectious Diseases, Istituto di Ricovero e Cura a Carattere Scientifico Policlinico S. Matteo, University of Pavia, 27100 Pavia, Italy

Received 9 October 1991/Accepted 14 February 1992

The main parameters of immunostaining techniques, i.e., the type of fixative, immunocytochemical reaction, and quality of monoclonal antibodies (MAbs), for quantitation of human cytomegalovirus (HCMV) antigenemia in peripheral blood polymorphonuclear leukocytes (currently performed by the indirect immunofluorescence or immunoperoxidase reaction by using MAbs to HCMV pp65) were investigated in order to optimize procedural steps and reagents. Significantly better results (in terms of the number of positive cells) were obtained on multiple cytospin preparations from heart transplant recipients with HCMV viremia when we used (i) formalin instead of methanol-acetone fixation and (ii) the indirect immunofluorescence reaction instead of the immunoperoxidase reaction, the avidin-biotin complex method, or the alkaline phosphatase antialkaline phosphatase procedure. In addition, comparison of the staining capabilities of three MAbs to pp65, which were developed in the laboratory and which were reactive to different epitopes of the protein, with a commercially available MAb (Clonab CMV) for determination of HCMV antigenemia showed that, while individual MAbs did not provide better results, the pool of MAbs detected a significantly higher number of positive peripheral blood polymorphonuclear leukocytes than Clonab CMV did. In addition, the sensitivity of the pool in detecting patients with low levels of viremia ($<5/2 \times 10^5$ cells inoculated) as antigenemia positive was 100%, whereas the sensitivity of Clonab CMV was 47%. No differences in the specificities between the two MAb preparations were observed.

In the last few years, a major breakthrough in the rapid diagnosis of systemic human cytomegalovirus (HCMV) infections in immunocompromised patients has been achieved by the development of a diagnostic assay that quantitates HCMV antigenemia; i.e., it allows the direct determination and quantitation of peripheral blood leukocytes and, namely, polymorphonuclear leukocytes (PMNLs) that express nuclear viral proteins (12, 15, 16). This assay was based on the use of a pool of two monoclonal antibodies (MAbs), which was made commercially available through Biotest (Dreieich, Germany) as Clonab CMV. The viral protein recognized by Clonab CMV and predominantly expressed in PMNLs was initially referred to as an immediate-early (IE) protein (16), but it was subsequently identified in our laboratory as the lower matrix phosphoprotein pp65 (10). The invaluable usefulness of determining HCMV antigenemia in immunocompromised patients (those who have received organ transplants and those with AIDS) has repeatedly been demonstrated, in particular, with respect to monitoring HCMV infections and antiviral treatment (2, 6).

In the present study, we investigated the multiple parameters involved in the assay for determination of HCMV antigenemia, i.e., the type of fixation (methanol-acetone versus formalin), the type of immunocytochemical procedure (indirect immunofluorescence assay [IFA] versus immunoenzymatic techniques), and in particular, the type of pp65 MAbs (use of individual MAbs directed to different epitopes of pp65 versus a pool of MAbs). Results indicate that significant improvements in the assay were achieved by using (i) formalin instead of methanol-acetone fixation; (ii) IFA instead of immunoperoxidase (IPA) or other immunoenzymatic techniques; and (iii) a pool of pp65 MAbs that were reactive to different epitopes instead of individual MAbs.

MATERIALS AND METHODS

Preparation of PMNL cytospins. One milliliter of 6% dextran solution (molecular weight, 70,000) in saline was added to 5.0 ml of freshly collected heparinized blood. Following incubation at 37°C for about 30 min, the supernatant rich in PMNLs was collected and centrifuged at 200 × *g* for 10 min at room temperature. The supernatant was discarded and the pellet was resuspended in phosphate-buffered saline (PBS; pH 7.2). Following centrifugation, if the pellet was heavily contaminated with erythrocytes, the cells were lysed with 0.8% NH_4Cl (1.0 ml for 2 min). After centrifugation, the pellet was washed again with PBS and centrifuged. The final pellet was resuspended in 1.0 ml of PBS and the cells were counted. The cell concentration was adjusted to 2×10^6/ml. Cytospins of dextran-enriched PMNL preparations were obtained by centrifugation of 2×10^5 cells onto glass slides (100 μl per slide) at 90 × *g* for 3 min by using a cytocentrifuge (Cytospin 2; Shandon Southern Products Ltd., Runcorn, United Kingdom). Slides were air dried for at least 30 min.

Fixation procedures. Two methods of fixation were then used during the optimization process of the assay. Initially, cytospin preparations were fixed with a cold mixture (−20°C) of methanol-acetone (1:2) for 5 min (12). Slides were then air dried and were stained immediately or were stored at −80°C. Subsequently, the fixation procedure was changed by using formalin as a fixative and 0.5% Nonidet P-40 for permeabilization of fixed cells (9). In detail, slides were fixed

* Corresponding author.

with 5% paraformaldehyde–2% sucrose in PBS for 10 min at room temperature. Following three washings with PBS–1% fetal calf serum, the cells were permeabilized with 0.5% Nonidet P-40–10% sucrose–1% fetal calf serum in PBS for 5 min at room temperature and were then washed three times with PBS–1% fetal calf serum. Following air drying for at least 20 min, slides were ready for staining or storage at −80°C.

IFA method. For the IFA method, cytospin preparations were first reacted for 30 min at 37°C with optimal dilutions of the different MAbs and then, following the washings, with a fluorescein-conjugated $F(ab')_2$ goat anti-mouse immunoglobulin G (IgG) fragment (Fc fragment, gamma chain specific; Organon Teknika, Cappel Laboratories, West Chester, Pa.) optimally diluted in PBS–0.0005% Evans blue. Counterstaining was essential for removing nonspecific background staining. Cells were never left dry after the washings. Cells were finally mounted onto slides, and positive cells were counted under a UV microscope by using a ×40 oil immersion objective.

IPA method. For the IPA method, slides were incubated with MAbs for 30 min at 37°C in the first step of the reaction. Then, following the washing step, endogenous peroxidase activity was removed by incubating cytospin preparations with methanol-H_2O_2 (0.3%) for 25 min at 37°C. Finally, slides were incubated with a peroxidase-conjugated $F(ab')_2$ goat anti-mouse IgG fragment (Fc fragment, gamma chain specific; Cappel Laboratories) for 30 min at 37°C. Chromogen substrate solution (diaminobenzidine-H_2O_2 in 0.05 M Tris [pH 7.6]) was then added, and the solution was incubated for 5 min at room temperature. Cells were then mounted onto slides, the slides were read under a light microscope, and the number of positive cells (those that showed brown-stained nuclei) were counted.

ABC method. In some experiments, during the second step of the indirect immunoenzymatic reaction, slides were incubated for 30 min at 37°C with a biotinylated antibody to mouse IgG, which was then detected by the avidin-biotin peroxidase complex (ABC) procedure (Vectastain ABC kit; Vector Laboratories, Burlingame, Calif.). The chromogenic substrate was the same as that used in the IPA procedure; and the staining patterns of positive cells were similar, but the staining intensities were greater by the ABC method.

APAAP method. For the alkaline phosphatase antialkaline phosphatase (APAAP) method, fixed slides were first reacted with MAbs for 30 min at room temperature. Following washing with Tris-buffered saline (0.05 M; pH 7.6), slides were incubated with rabbit anti-mouse immunoglobulin as a link antibody (Dakopatts a/s, Glostrup, Denmark) for 30 min at room temperature, washed an additional three times with Tris-buffered saline, and then incubated again with the APAAP immune complex (Dako APAAP kit; Dakopatts) for 30 min at room temperature. The enzyme label was developed according to the instructions of the manufacturer. Cells were then mounted onto the slides and scored. A bright red color was observed in the nuclei of positive PMNLs.

MAbs. MAbs to HCMV pp65 were developed in the laboratory and were characterized as reported previously (4, 5, 10). Clonab CMV (Biotest) was used for comparison during development of the assay. In addition, MAbs that were reactive with the major IE1 protein p72 (MAb 5D2) and a late protein (MAb 5A11) were developed in the laboratory (4), as were MAbs to the major DNA-binding protein p52 (11).

Statistical analysis. Statistical analysis was carried out on results of different comparisons by performing the Student t test on paired data. In Table 1, ranges rather than standard deviations were reported, given the great dispersion of numerical data.

RESULTS

Fixation of cytospin preparations. When we used Clonab CMV and IFA, comparison between methanol-acetone and formalin fixation showed that the latter procedure gave significantly better results in terms of the number of positive cells, and it greatly improved the intensity of staining and the patterns of nuclear fluorescence (Table 1). Parallel examination of six positive samples fixed by either procedure showed that the mean number of positive cells was significantly greater with formalin fixation than with methanol-acetone fixation ($P < 0.01$). In addition, with formalin fixation the degree of nuclear staining was mostly 4+ fluorescence and the pattern was evenly bright, whereas with methanol-acetone fixation the fluorescence intensity ranged from 1+ to 4+ and the staining pattern was uneven and, often, only perinuclear.

Comparison of IFA and IPA methods using Clonab CMV. The IPA technique, which was initially proposed as the test of choice for the determination of HCMV antigenemia (15, 16), was replaced by the IFA test in our laboratory (13), since the latter neither raised the problems of nonspecific staining nor required treatment for removal of endogenous peroxidase activity. However, to our knowledge a comparative study between the IFA and IPA reactions for quantitation of HCMV antigenemia has not yet been performed. Thus, in this study we compared IFA (Fig. 1A) and IPA (Fig. 1B) reactions on a group of 137 blood samples collected from 55 immunocompromised patients. The study was carried out by using Clonab CMV. Of the 137 samples examined, 61 gave concordant positive and 65 gave concordant negative results, while 11 gave discordant results. However, all of the 11 samples that gave discordant results (6 positive by IFA only and 5 positive by IPA only) contained <5 infected cells of the 2×10^5 cells examined. All these samples were from previously or subsequently symptomatic patients with positive viremia in blood samples that were taken a few days before or after.

Comparison of the 61 concordant positive samples showed a mean number of positive PMNLs significantly greater by the IFA reaction than by the IPA reaction ($P < 0.01$) (Table 1). Since treatment for removal of endogenous peroxidase activity could be a reason for such a difference, 10 pp65-positive blood samples were examined by IFA in parallel prior to and following treatment with methanol-H_2O_2. Results confirmed that such a treatment was responsible for decreasing the number of cells positive by the IFA method to the levels achieved by the IPA method. In addition, treatment decreased the intensity of nuclear staining by 1+ to 2+ fluorescence. When the ABC method (Fig. 1C) was used on 12 blood samples, the number of positive cells again was significantly lower than that given by the IFA method ($P < 0.05$) (Table 1).

Comparison of pp65 MAbs versus Clonab CMV by IFA. On the basis of the results reported above, IFA was selected as the method of choice for direct detection of pp65 in peripheral blood PMNLs. In the meantime, a panel of four MAbs to pp65 (MAbs 4C1, 4D5, 2A6, and 1C3; all IgG1) was developed in the laboratory, and competitive enzyme-linked immunosorbent binding assays showed that while two of them (MAbs 4C1 and 4D5) reacted with the same epitope, the remaining two (MAbs 1C3 and 2A6) recognized two

TABLE 1. Selection of optimal parameters for determination of HCMV antigenemia[a]

Parameter	Fixative	Method	MAb	No. of pp65-positive samples examined	No. of pp65-positive cells detected (range)	Statistical analysis		Staining degree
						t	*P*	
Type of fixative	Formalin	IFA	Clonab CMV	6	273 (207–268)	+9.35	<0.01	4+
	Methanol-acetone				154 (98–216)			1–4+
Immunocytochemical method	Formalin	IFA	Clonab CMV	61	110 (1–800)	+3.63	<0.01	4+
		IPA			57 (1–360)			3+
		IFA	Clonab CMV	12	322 (53–1,000)	+2.78	<0.05	4+
		ABC			159 (22–500)			5+
		IFA	Pool[b]	12	199 (12–600)	+2.22	<0.05	5+
		APAAP			142 (5–400)			5+
MAb	Formalin	IFA	Clonab CMV[c]	5[d]	135 (19–268)	NA[e]	NA	4+
			2A6		156 (21–280)	−1.31	>0.05	4+
			1C3		161 (22–288)	−1.44	>0.05	4+
			4C1		40 (5–73)	+2.71	<0.05	2–3+
			Pool		212 (35–371)	−3.43	<0.05	5+

[a] Vertical lines indicate the type of comparison.
[b] The pool was a mixture of three MAbs (2A6, 1C3, and 4C1) that were reactive with different epitopes of pp65.
[c] The Clonab CMV was compared with each of the three MAbs and the pool of the three MAbs.
[d] The same five samples were examined in duplicate with each MAb.
[e] NA, not applicable.

additional epitopes (Fig. 2). Since only the Clonab CMV was used in previous tests, the staining capabilities of the MAbs developed in house versus that of the Clonab CMV were investigated by the IFA method. MAbs were used at the optimal immunoglobulin concentrations (30 to 80 μg/ml) determined on a series of HCMV-positive cytospin preparations, either individually or as a pool (Table 1). Comparison was performed on a group of five blood samples from heart transplant recipients with various levels of HCMV antigenemia. Statistical analysis of comparisons between Clonab CMV and each of the three in-house-developed MAbs as well as the pool of MAbs showed that while MAbs 2A6 and 1C3 gave comparable results and MAb 4C1 yielded a lower number of positive cells, the pool of the three MAbs gave significantly higher positive cell counts with respect to those obtained with the Clonab CMV ($P < 0.05$) (Table 1). However, 65 of 110 heart transplant recipients with levels of viremia of >10 infected cells were consistently found to be positive for antigenemia by using both the pool of MAbs and the Clonab CMV. On the other hand, in 15 patients with low levels of viremia (<5 infected cells), the pool showed a greater sensitivity in detecting positive patients (15 of 15; 100%) than the Clonab CMV did (7 of 15; 47%). On the basis of these results, use of Clonab CMV was discontinued and the pool of MAbs was confirmed to provide better results than those provided by individual MAbs on additional samples (Table 2).

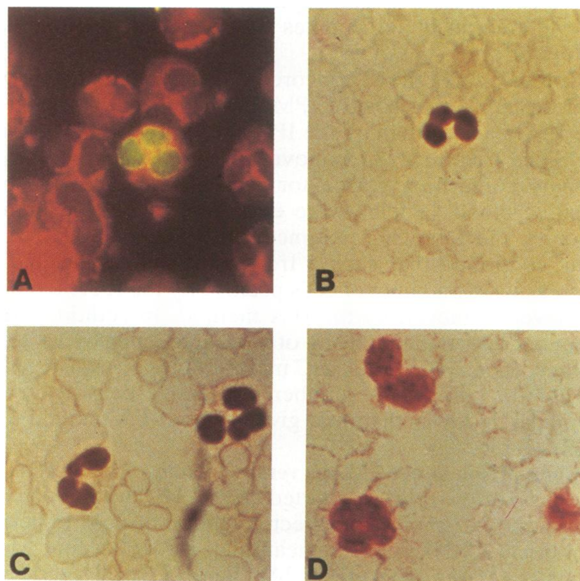

FIG. 1. Immunostaining of PMNL cytospin preparations by using pp65 MAbs and the IFA method (A), the IPA method (B), the ABC method (C), and the APAAP method (D). Magnification, ×1,000.

Comparison of the APAAP and IFA methods. Following selection of the IFA method and the pool of pp65 MAbs as the most sensitive test system for the detection of HCMV-positive PMNLs, an APAAP procedure was tested (Fig. 1D) against the IFA method to determine whether the sensitivity of the assay could be improved further. Twelve blood samples were run in parallel (Table 1). Again, the mean cell count obtained by the IFA method was greater than that obtained by the APAAP method, and the difference was statistically significant ($P < 0.05$). On the basis of these results, the IFA method remained the test of choice for routine testing of HCMV antigenemia.

Specificity controls. The major specificity control was represented by consistent negative results for antigenemia in >1,000 viremia-negative blood samples taken from both immunocompetent and immunocompromised subjects by using either the MAb pool or the Clonab CMV. The only

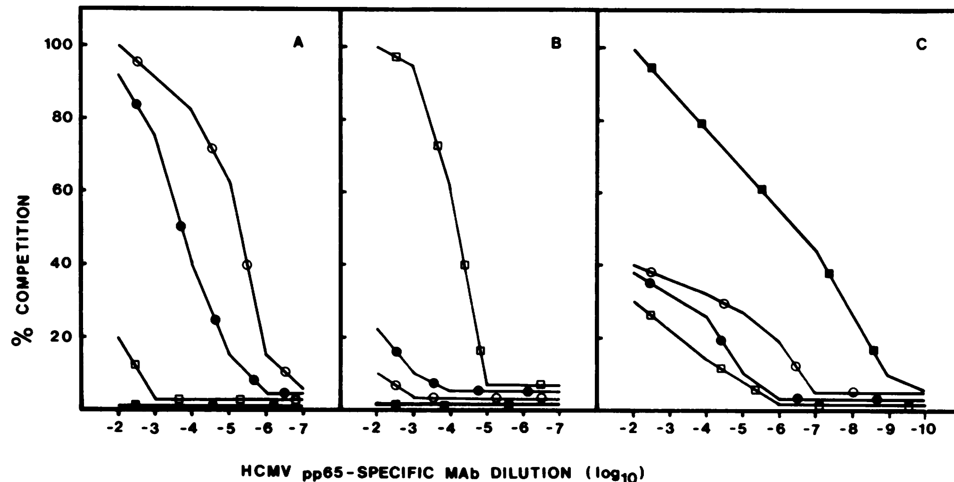

FIG. 2. Enzyme-linked immunosorbent competitive binding assay with peroxidase-labeled MAbs 4C1 (A), 2A6 (B), and 1C3 (C). The competing antibodies were 4C1 (○), 4D5 (●), 2A6 (□), and 1C3 (■).

conditions under which viremia-negative samples could be considered antigenemia positive were represented by (i) first days of antiviral treatment and (ii) low levels of antigenemia (<5 HCMV-positive PMNLs per 2×10^5 cells examined). However, in both cases, results had to be considered specific since they were obtained during the course of an active HCMV infection in blood.

Search for IE, early, and late antigens in PMNLs. We recently presented data showing the presence of IE1 transcripts in PMNLs (18). In this study, by examining seven blood samples from as many viremic heart transplant recipients, we found that six samples with a mean number of pp65-positive PMNLs of 265.2 (range, 60 to 426) were also positive for p72 (Fig. 3A), with a mean number of positive cells of 26.3 (range, 7 to 51) (Table 2). Thus, 9.9% of pp65-positive PMNLs were p72 positive. However, evaluation of the degree of staining showed that, while pp65-positive PMNLs stained 4+, p72-positive PMNLs faintly stained only from ± to 1+. The pattern of p72 staining consisted of very fine granules that were evenly distributed inside the entire area of the nucleus. In addition, three of six pp65- and p72-positive samples also reacted with a MAb directed to a HCMV late capsid protein (Fig. 3B), with the number of positive PMNLs being comparable to that of p72-positive cells in two of the three samples (Table 2). Although the staining pattern was very weak, it consisted of a few large dots of fluorescence scattered throughout the nucleus. No reactivity was found in four samples tested with four individual p52 MAbs and a pool of the same MAbs (Table 2).

DISCUSSION

In the present study, we attempted to optimize the test procedure for antigenemia by examining the following parameters: (i) type of fixative, (ii) type of immunocytochemical staining procedure, and (iii) type of MAbs. First, replacement of methanol-acetone fixation with 5% formalin followed by permeabilization with 0.5% Nonidet P-40 led to two major improvements: more intense and even staining of pp65-positive cells and a significantly greater number of positive cells. Second, comparison of the IFA method versus immunoenzymatic techniques (IPA, ABC, and APAAP methods) showed that significantly better results were obtained by the IFA method. It seems likely that this result may be attributed to treatment for removal of endogenous

TABLE 2. Quantitative determination of HCMV antigenemia by the IFA method by using different MAbs

Blood sample no.	No. of HCMV-positive cells by using MAbs to[a]:							
	pp65					IEA (MAb 5D2)	EA (MAb 4B3)	LA (MAb 5A11)
	2A6	1C3	4C1	Pool[b]	Clonab CMV			
24094	21	22	5	35	19	ND[c]	ND	ND
24288	239	238	83	268	ND	ND	ND	ND
24310	148	153	40	263	163	14	0	0
24346	87	122	50	141	ND	7	ND	0
24373	41	45	31	60	ND	21	0	0
24396	309	329	75	426	ND	51	ND	2
24439	59	57	15	65	32	0	ND	ND
24443	275	288	70	330	197	20	0	45
24474	280	287	73	371	268	45	0	56

[a] IEA, immediate-early HCMV antigen; EA, early HCMV antigen; LA, late HCMV antigen. MAb 5D2 is an MAb to the IE HCMV antigen p72 (4). MAb 4B3 to the EA HCMV antigen p52 as well as a pool of four different p52 MAbs (11) gave same negative results on the same samples tested. MAb 5A11 is an MAb to a late HCMV antigen (4).
[b] Pool of MAbs (2A6, 1C3, and 4C1).
[c] ND, not determined.

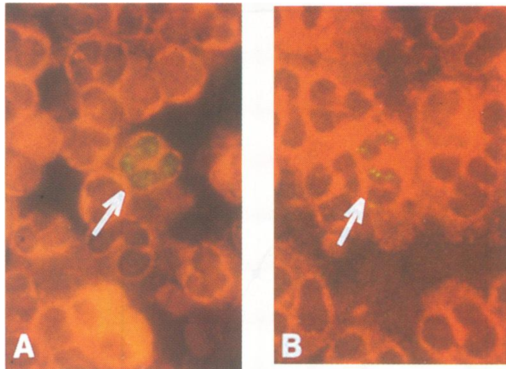

FIG. 3. IFA staining of PMNL cytospin preparations by using MAbs to the IE protein p72 (A) and a late protein (B). The nuclei of positive cells are indicated by arrows. Magnification, ×1,000.

enzymatic activities in PMNLs when immunoenzymatic procedures were used. In addition, it must be stressed that neither the APAAP nor the ABC method displayed any greater sensitivity in comparison with that of the IPA method. Recently reported data (1) obtained by the APAAP method were confirmed in this study. However, it appears that there are no sufficient reasons for proposing APAAP as the method of choice for determination of HCMV antigenemia (1). Finally, it was found that whichever technique was used, a delay of 6 h in the transport of blood samples to the laboratory decreased the viral antigen detection rate by at least 80% (3), thus explaining the reported negative results in heart transplant recipients (8).

A major requirement in the optimization of the assay for HCMV antigenemia was the selection of suitable MAbs and, in particular, a pool of MAbs that recognized different epitopes of the HCMV lower matrix phosphoprotein. From a panel of MAbs developed in our laboratory, we selected three MAbs that recognize different epitopes. The three selected MAbs could not be tested against the commercially available Clonab CMV, since the latter was available only as a pool of cell culture supernatants. Of the three MAbs, two gave a comparable number of positive PMNLs when the MAbs were tested individually, whereas one MAb stained a significantly lower number of positive cells. However, when they were used as a pool, the three MAbs showed a synergistic effect, giving the best results in terms of the number of positive PMNLs detected. In addition, the pool gave a significantly higher number of positive cells in comparison with that obtained from the Clonab CMV. However, this difference in positive cell count did not result in an overall increased sensitivity of the pool in detecting patients positive for antigenemia with levels of viremia of >10 infected cells per 2×10^5 PMNLs inoculated. We previously reported that there is a significant correlation between levels of antigenemia and viremia (4). It was also shown that a dissociation between a positive antigenemia and a negative viremia occurs as a rule in the first days of antiviral treatment and sporadically when levels of viremia are <5 infected cells per 2×10^5 cells inoculated. The latter finding was attributed to sampling variability (4). However, it is noteworthy that, in this study of 15 samples that were positive for viremia at a low level, all 15 (100%) were found to be positive by using the MAb pool, whereas only 7 of 15 (47%) were positive by the commercially available Clonab CMV. Since low levels of viremia and antigenemia are not associated with clinical symptoms, as reported previously (4, 6), the slightly lower sensitivity of the Clonab CMV does not appear to be of major clinical importance.

When MAbs to the IE and late antigens were tested on highly positive cytospin preparations, only a few (≤10%) PMNLs were found to be positive for the relevant HCMV antigens. In addition, the degree of staining was very faint, and a very careful and time-consuming microscopic observation was required to detect positive cells. Thus, use of MAbs directed to HCMV proteins other than pp65 was not useful for the routine determination and quantitation of HCMV antigenemia, as has also been confirmed by other investigators (8, 14). It has been shown that complete and infectious virus may be detected in PMNLs (7, 17). However, it remains to be determined whether the infectious virus present in PMNLs is derived from the uptake of infected cell debris by phagocytosis or represents new viral progeny produced in the cells.

Results of this study led to the following conclusions. (i) Optimal fixation of cytospin preparations of PMNLs is obtained by using formalin and then permeabilization with a nonionic detergent, (ii) the IFA method appears to be superior to immunoenzymatic techniques because of the lack of treatment for removal of endogenous enzymatic activities, and (iii) a pool of MAbs reactive to different epitopes of HCMV pp65 represents a better probe than individual MAbs for the detection and quantitation of HCMV antigenemia both in terms of the number of positive cells and the number of positive patients with low levels of viremia.

ACKNOWLEDGMENTS

This work was partially supported by contract 7205-10 from the Ministero della Sanità, Istituto Superiore di Sanità, Progetto Nazionale AIDS 1992, and by contract 91.01178.PF70 from the Consiglio Nazionale delle Ricerche, Progetto Finalizzato Biotecnologie e Biostrumentazione.

We thank Linda D'Arrigo for revision of the English and Gabriella Garbagnoli and Teresa Pollini for technical assistance.

REFERENCES

1. **Bein, G., A. Bitsch, J. Hoyer, and H. Kirchner.** 1991. The detection of human cytomegalovirus immediate early antigen in peripheral blood leukocytes. J. Immunol. Methods **137:**175–180.
2. **Gerna, G., M. Parea, E. Percivalle, D. Zipeto, E. Silini, G. Barbarini, and G. Milanesi.** 1990. Human cytomegalovirus viremia in HIV-1 seropositive patients at various clinical stages of infection. AIDS **4:**1027–1031.
3. **Gerna, G., E. Percivalle, F. Morini, and M. G. Revello.** Unpublished data.
4. **Gerna, G., M. G. Revello, E. Percivalle, M. Zavattoni, M. Parea, and M. Battaglia.** 1990. Quantification of human cytomegalovirus viremia by using monoclonal antibodies to different viral proteins. J. Clin. Microbiol. **28:**2681–2688.
5. **Gerna, G., A. Sarasini, A. Di Matteo, M. Parea, M. Torsellini, and M. Battaglia.** 1989. Rapid detection of human rotavirus strains in stools by single-sandwich enzyme-linked immunosorbent assay systems using monoclonal antibodies. J. Virol. Methods **24:**43–56.
6. **Gerna, G., D. Zipeto, M. Parea, M. G. Revello, E. Silini, E. Percivalle, M. Zavattoni, and G. Milanesi.** 1991. Monitoring of human cytomegalovirus infections and ganciclovir treatment in heart transplant recipients by determination of viremia, antigenemia and DNAemia. J. Infect. Dis. **164:**488–498.
7. **Martin, D. C., D. A. Katzenstein, G. S. M. Yu, and M. C. Jordan.** 1984. Cytomegalovirus viremia detected by molecular hybridization and electron microscopy. Ann. Intern. Med. **100:** 222–225.
8. **Miller, H., E. Rossier, R. Milk, and C. Thomas.** 1991. Prospec-

tive study of cytomegalovirus antigenemia in allograft recipients. J. Clin. Microbiol. **29:**1054–1055.

9. **Randall, R. E., and N. Dinwoodie.** 1986. Intranuclear localization of herpes simplex virus immediate-early and delayed-early proteins: evidence that ICP4 is associated with progeny virus DNA. J. Gen. Virol. **67:**2163–2177.
10. **Revello, M. G., E. Percivalle, A. Di Matteo, F. Morini, and G. Gerna.** 1992. Nuclear expression of the lower matrix protein of human cytomegalovirus in peripheral blood leukocytes of immunocompromised viremic patients. J. Gen. Virol. **73:**437–442.
11. **Revello, M. G., E. Percivalle, M. Zannino, V. Rossi, and G. Gerna.** 1991. Development and evaluation of a capture ELISA method for detection of IgM antibody to the human cytomegalovirus major DNA binding protein. J. Virol. Methods **35:**315–329.
12. **Revello, M. G., E. Percivalle, M. Zavattoni, M. Parea, P. Grossi, and G. Gerna.** 1989. Detection of human cytomegalovirus immediate-early antigen in leucocytes as a marker of viremia in immunocompromised patients. J. Med. Virol. **29:**88–93.
13. **Revello, M. G., M. Zavattoni, E. Percivalle, P. Grossi, and G. Gerna.** 1989. Correlation between immunofluorescent detection of human cytomegalovirus immediate-early antigens in polymorphonuclear leukocytes and viremia. J. Infect. Dis. **160:**159–160.
14. **Spector, S. A.** Personal communication.
15. **Van der Bij, W., J. Schirm, R. Torensma, J. Van Son, A. M. Tegzess, and T. H. The.** 1988. Comparison between viremia and antigenemia for detection of cytomegalovirus in blood. J. Clin. Microbiol. **26:**2531–2535.
16. **Van der Bij, W., R. Torensma, W. J. Van Son, J. Anema, J. Schirm, A. M. Tegzess, and T. H. The.** 1988. Rapid immunodiagnosis of active cytomegalovirus infection by monoclonal antibody staining of blood leukocytes. J. Med. Virol. **25:**179–188.
17. **Wunderli, W., M. K. Kägi, E. Gruter, and J. D. Auracher.** 1989. Detection of cytomegalovirus in peripheral leukocytes by different methods. J. Clin. Microbiol. **27:**1916–1917.
18. **Zipeto, D., E. Silini, M. Parea, R. Maccario, G. Peri, E. Percivalle, G. Milanesi, M. G. Revello, and G. Gerna.** 1991. Abstr. 3rd Int. Cytomegalovirus Workshop, p. 107.