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Comparison between Viremia and Antigenemia for Detection of Cytomegalovirus in Blood

WIM VAN DER BIJ,[1]* JURJEN SCHIRM,[2] RUURD TORENSMA,[1]† WILLEM J. VAN SON,[3] ADAM M. TEGZESS,[3] AND T. HAUW THE[1]

Department of Clinical Immunology,[1] Regional Public Health Laboratory,[2] and Renal Transplantation Unit,[3] University Hospital Groningen, 9713 EZ Groningen, The Netherlands

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In a prospective study, 139 serial blood samples from 15 transplant recipients were assessed for the presence of cytomegalovirus (CMV) by virus isolation (CMV viremia) and by direct staining of CMV antigens (CMV Ag) in blood leukocytes (CMV antigenemia). CMV was isolated from 23 samples, whereas CMV Ag was detected in 44 specimens. All positive samples were from a total of nine patients who were diagnosed as having active CMV infections. In seven patients, active CMV infections were diagnosed by virus isolation from blood and urine and by a significant rise of CMV-specific antibodies. In these patients, 21 of the 23 blood samples which were positive for CMV by cell culture were also positive by direct CMV Ag detection. Moreover, CMV Ag were detected in 23 of the 116 culture-negative samples. Twenty of these samples were from the acute phase of infection in the same seven patients. The remaining three CMV Ag-positive specimens were from the other two patients, from whom CMV was not isolated but who had serological evidence of concomitant active CMV infections. These results suggest that direct detection of CMV Ag in peripheral blood leukocytes is as specific as and more sensitive than current isolation techniques. Furthermore, by its sensitivity and inherent rapidity the antigen detection test proved to be the earliest diagnostic marker of active CMV infection in eight of the nine patients. Finally, it was shown that monoclonal antibodies to CMV immediate early antigens are a prerequisite for demonstration of CMV antigenemia.

Active cytomegalovirus (CMV) infection is a major threat in newborns (1) and immunocompromised individuals, such as transplant recipients (12, 15) and patients with acquired immunodeficiency syndrome (10). Rapid diagnosis of active CMV infection is of great importance to avoid overtreatment with immunosuppressive drugs (in transplant recipients) and to guide antiviral therapy. For these reasons, rapid and sensitive virus detection methods have been developed, such as DNA probe techniques (21), CMV antigen (CMV Ag) detection in biopsies and bronchoalveolar lavage (4, 8, 11), and immunofluorescence (IF) techniques for rapid detection of CMV early antigens (EA) in cell culture within a few days after inoculation of clinical specimens (6, 7, 11, 14, 19, 20, 22).

Demonstration of CMV in blood samples is particularly important because CMV viremia is considered to be a marker of active infection and has been shown to correlate well with significant CMV disease (5, 18). Consequently, assays aiming at rapid detection of CMV viremia are clinically relevant. Large-scale studies on the detection of infectious CMV in blood by the early antigen IF technique (19, 22) showed its usefulness as a more rapid and sensitive method than the time-consuming conventional culture system.

In a recent report (24), we have described a method for the detection of CMV Ag in blood leukocytes (CMV antigenemia) with the aid of a mixture of monoclonal antibodies (MAbs) C-10, C-11, and C-12. In the present study, we focused on a comparison between virus isolation from and CMV Ag detection in blood specimens with respect to sensitivity and rapidity in 15 patients at risk for active CMV infection. In another comparative experiment, the usefulness of other MAbs for the demonstration of CMV antigenemia was studied.

MATERIALS AND METHODS

Clinical specimens. A total of 139 blood samples from 15 consecutive transplant recipients (11 renal, 2 hepatic, and 2 cardiac transplant patients) were studied prospectively for the presence of CMV viremia and CMV antigenemia during the first 3 months after transplantation. Samples were obtained from the same venipuncture and processed independently for virus isolation and antigen detection. Furthermore, urine specimens were tested for CMV, and sera were tested for CMV-specific antibodies. Samples for all diagnostic procedures were obtained weekly during the inpatient period and subsequent outpatient visits and, for most patients, every 2 weeks during the last weeks of the 3-month period. Active CMV infection was diagnosed by virus isolation from the blood (by one or both assays mentioned below) or a significant rise of CMV-specific antibodies (or both). CMV syndromes (2) were defined as these laboratory criteria combined with the occurrence of two or more of the following CMV-related manifestations: unexplained fever for 2 or more days, arthralgia, leukopenia (a leukocyte count of $<3 \times 10^9$/liter) or thrombocytopenia (a platelet count of $<100 \times 10^9$/liter), and liver enzyme rises (serum alanine aminotransferase activity of >50 U/ml).

MAbs. Three murine MAbs C-10, C-11, and C-12, have been prepared in our laboratory. These are antibodies to CMV immediate EA (IEA) (16) of 70 kilodaltons and react with CMV-infected fibroblasts throughout the infectious cycle and with the classical inclusion-bearing cytomegalic cells in organs with disseminated CMV. No positive reaction has been found in uninfected fibroblasts, herpes simplex virus type 1-infected Vero cells, herpes simplex virus type 2-

* Corresponding author.
† Present address: Department of Microbiology, State University of Utrecht, 3511 EG Utrecht, The Netherlands.

or varicella-zoster virus-infected fetal fibroblasts, or lymphoblastoid cell lines that express Epstein-Barr nuclear (Raji) or virus capsid (P3HR1) antigens.

CMV viremia. For detection of infectious CMV in blood, both conventional cell culture and detection of CMV Ag in cell culture by IF were performed as previously described (19). Briefly, buffy coats from 7 ml of heparinized blood were obtained by dextransedimentation, and the cells were washed twice and suspended in 1.5 ml of culture medium (Hanks minimal essential medium supplemented with nonessential amino acids, vitamins, and 10% fetal bovine serum). For conventional cell culture, human embryonic lung fibroblasts were grown in standard culture tubes, inoculated in duplicate with 0.5-ml portions of the buffy-coat suspension, and placed at 36°C. On the next day, inoculum cells were replaced by fresh culture medium. Subsequent refreshments were done weekly. The fibroblast monolayers were examined for a CMV-specific cytopathic effect three times weekly during the first 2 weeks and weekly during the next 4 weeks. For detection of CMV Ag in cell culture by IF, lung fibroblasts were grown on 12-mm-diameter round cover slips in flat-bottom tubes and inoculated in duplicate with 0.2-ml portions of the suspension of buffy coat cells. Inoculum cells were replaced by fresh culture medium on the next day. At 4 to 6 days after inoculation, the cover slips were washed twice in phosphate-buffered saline (PBS), dried, fixed in acetone (−20°C), and attached to microscope slides. The cover slips were stained by indirect IF with a mixture of the CMV-specific MAbs (C-10, C-11, and C-12) and fluorescein isothiocyanate-labeled rabbit anti-mouse immunoglobulins (Dako, Copenhagen, Denmark) as the second antibody.

Urine specimens (CMV viruria). Urine specimens diluted with virus transport medium were inoculated in duplicate in 0.5-ml portions and processed for detection of CMV Ag by IF (after incubation for 1 day and 3 to 7 days) and for conventional cell culture.

CMV antigenemia. CMV Ag staining was performed as follows (24). EDTA-treated blood (3 to 5 ml) was mixed with a 5% solution of dextran (250 kilodaltons) in PBS at a 4:1 ratio and allowed to settle at 37°C. After 15 min, the entire supernatant was harvested and centrifuged for 10 min at 300 × *g*. The pelleted cells were suspended in an NH_4Cl solution (NH_4Cl [8.3 g/liter], $KHCO_3$ [1.0 g/liter], EDTA [0.03 g/liter], pH 7.4) on iced water for 5 min to lyse the contaminating erythrocytes. After being washed twice in PBS, the erythrocytes were suspended in PBS and counted. Cytocentrifuge preparations were made with 100 μl of a suspension of 1.5×10^6 cells per ml centrifuged for 5 min at 600 to 800 rpm (Cytospin-2; Shandon-Elliot, Astmoor, United Kingdom). The slides were dried quickly with a cold blower and fixed with acetone (10 min at room temperature). The slides were then incubated in triplicate with 25 μl of a 1:10 diluted mixture of MAbs C-10, C-11, and C-12 or with PBS (as a negative control) for 30 min at room temperature. Subsequently, after being washed in PBS for 5 min, the slides were incubated with horseradish peroxidase-labeled rabbit anti-mouse immunoglobulin (Dako) diluted 1:20 with PBS with 1% human serum for 30 min room temperature. After being washed three times in PBS, the enzyme reaction was performed with a 3-amino-9-ethylcarbazole solution in 0.1 M acetate buffer (pH 4.9) for 10 min at room temperature (10 mg of 3-amino-9-ethylcarbazole [Sigma Chemical Co., St. Louis, Mo.; no. 5754] dissolved in 2.5 m of *N*,*N*-dimethylformamide and then filled up to 50 ml with acetate buffer, subsequently filtered, and supplemented with 50 μl of H_2O_2 [30%] per 50 ml of acetate buffer immediately before use).

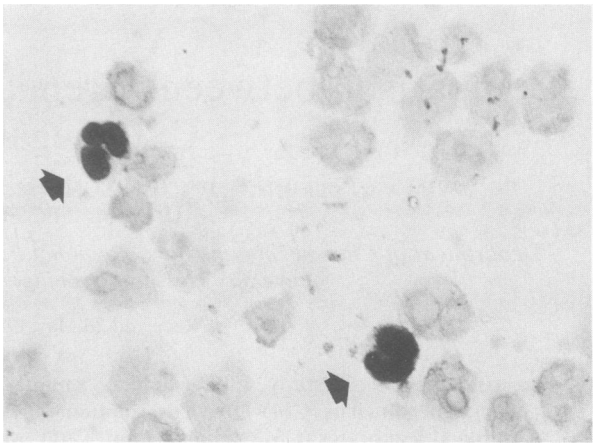

FIG. 1. Two CMV Ag^+ blood leukocytes (arrows) showing homogeneous nuclear staining.

The slides were then washed with acetate buffer for 5 min, rinsed with distilled water, mildly counterstained with hematoxylin (for about 1 min), carefully rinsed with tap water to remove excess staining solution, and mounted in glycerol-gelatin. Preparations known to contain CMV Ag^+ cells were used as positive controls. CMV Ag^+ cells were identified by characteristic (peri)nuclear staining (Fig. 1 and 2), mainly of polymorphonuclear leukocytes (24). The presence of endogenous peroxidase activity (in the cytoplasm of eosinophilic granulocytes [Fig. 2]) was generally a minor problem, largely depending on a correct pH (4.9) of the 3-amino-9-ethylcarbazole buffer. When still necessary, endogenous peroxidase activity can be eliminated by treatment of the slides with glucose-glucose oxidase (9) or H_2O_2-methanol after incubation and subsequent formaldehyde fixation of the MAb mixture (N. M. Jiwa, F. M. Van de Rijke, A. Molder, W. Van der Bij, T. H. The, P. H. Rothbarth, J. Velsing, M. Van der Ploeg, and A. K. Raap, submitted for publication).

In an additional experiment, the following CMV-specific MAbs (see Table 4) were also tested for the ability to detect CMV antigenemia: C-10, C-11, C-12, C-30, and C-32 (all produced in our laboratory; obtainable from MCA Develop-

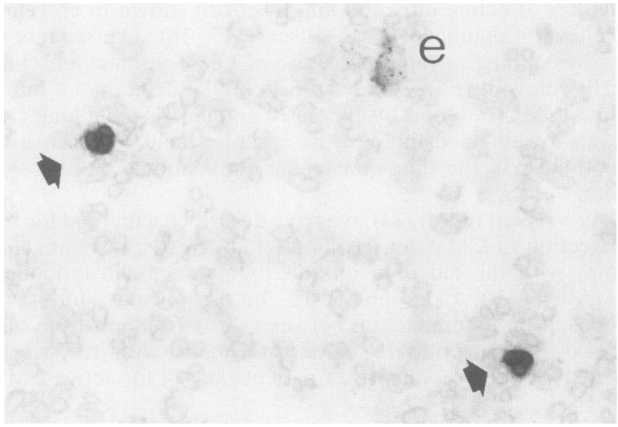

FIG. 2. Two CMV Ag^+ blood leukocytes (arrows) in which staining is most intense on the margin of the nucleus (perinuclear staining). e, Endogenous peroxidase activity.

TABLE 1. Detection of CMV by cell culture (CMV viremia) and CMV antigen detection in cytocentrifuged blood leukocytes (CMV antigenemia)

CMV antigenemia result	No. of CMV viremia results	
	Positive	Negative
Positive		
High (>10)[a]	15	6
Low (≤10)[a]	6	17
Negative	2	93

[a] Total number of CMV Ag^+ cells of triplicate cytospins.

ment BV, Groningen, The Netherlands), MAB810 (purchased from Chemicon, El Segundo, Calif.), 95/30 (kindly provided by S. Michelson, Paris, France), HCMV3 and HCMV19 (purchased from Bioscot, Edinburgh, United Kingdom), and MAb anti-EA (product 9121, purchased from Biotech, Rockville, Md.). Cytospin slides with high numbers of CMV Ag^+ cells (ranging from 30 to >1,000 positive cells per slide) were selected for staining with these MAbs. These slides were from nine patients (one newborn, four renal transplant recipients, one cardiac transplant recipient, one hepatic transplant recipient, and two patients with Wegener's granulomatosis) with virologically and serologically proven active CMV infections. Optimal dilutions (see Table 4) of the MAbs used were determined by application to AD169-infected human embryonic fibroblasts and CMV Ag^+ slides.

CMV serology. Levels of CMV-specific immunoglobulin G (IgG) and IgM antibodies were measured in duplicate by an enzyme-linked immunosorbent assay (13). Extinctions of a sample at several dilutions were compared with those of a reference serum pool. The antibody levels were expressed as percentages of that pool. Seroconversion or an increase of antibody levels exceeding the level of the previous serum by at least three times the standard deviation of that measurement was considered significant.

RESULTS

We assessed 139 blood samples from 15 transplant recipients prospectively for infectious CMV by cell culture (viremia) and for the presence of CMV antigens in blood leukocytes (antigenemia). CMV viremia was documented in 23 samples, and CMV antigenemia was found in 44 samples (Table 1).

Active CMV infection was present in 9 of the 15 patients (Table 2); 1 patient had a primary CMV infection, and 8 had secondary CMV infections. Most patients had minor clinical symptoms; a CMV syndrome occurred in two patients, but the outcome of the disease appeared to be uncomplicated in these patients. Active CMV infection was evidenced in seven of the nine patients both by recovery of CMV from blood and urine on one or more occasions and by a significant CMV antibody rise. CMV viremia was demonstrated in these patients by conventional cell culture or the IF assay for early antigen detection (or both methods), yielding 23 positive samples. CMV Ag^+ leukocytes were detected in 21 of these 23 samples. In addition, CMV Ag^+ leukocytes were demonstrated in 20 culture-negative blood samples in these seven patients. The remaining three CMV Ag-positive blood cell preparations were from two asymptomatic patients, from whom CMV could not be recovered but who displayed a significant rise of CMV-specific antibodies within 2 weeks after the CMV Ag^+ samples (patients 9 and 10). CMV Ag^+ cells were not detected in two culture-positive samples and in all samples of the remaining six transplant recipients without active CMV infection.

Furthermore, we compared virus isolation from the buffy coat and the cytospin technique with respect to the number of CMV Ag^+ cells (Table 1). Virus isolation by cell culture appeared to be associated with high numbers (>10 cells per measurement) of CMV Ag^+ cells and vice versa (chi square = 7.32, $P < 0.01$, df = 1).

CMV antigenemia could be demonstrated during a longer period than CMV viremia (5 ± 3 [mean ± the standard deviation] versus 3 ± 2 weeks, respectively). This is illus-

TABLE 2. Patient data and diagnostic results

Patient no.	Age (yr)	Sex and transplant type[a]	CMV serostatus[b]	CMV antigenemia		CMV viremia[e]	CMV viruria[e]	CMV ELISA[f]	CMV syndrome[g]
				Period[c]	Maximum[d]				
1	50	F, H	Seropositive	21–70	34	49 + 28	70 + 1	41	37–54
2	52	F, R	Seropositive	—[h]		—	—	—	—
3	22	M, H	Seronegative	—		—	—	—	—
4	60	F, R	Seropositive	35–91	69	28 + 6	56 + 1	48	—
5	40	M, C	Seropositive	19–82	72	34 + 6	34 + 1	41	—
6	25	F, R	Seronegative	—		—	—	—	—
7	59	F, R	Seronegative	—		—	—	—	—
8	48	M, R	Seropositive	—		—	—	—	—
9	60	F, R	Seropositive	32	1	—	—	38	—
10	63	M, R	Seropositive	53–59	5	—	—	66	—
11	52	F, R	Seronegative	—		—	—	—	—
12	50	F, R	Seropositive	25–46	40	32 + 6	52 + 1	37	—
13	46	M, R	Seropositive	21–50	245	28 + 6	35 + 1	40	—
14	29	M, R	Seropositive	28–55	242	49 + 6	35 + 7	40	38–52
15	31	M, C	Seronegative	21–52	305	35 + 4	35 + 7	35	—

[a] F, Female; M, male. H, R, and C refer to hepatic, renal, and cardiac transplant recipients.
[b] Determined immediately before transplantation.
[c] Period (days) between first and last positive CMV Ag detections.
[d] Total number of CMV Ag^+ cells of triplicate cytospins of maximum measurement.
[e] First positive measurement (day of sampling plus days required for processing of the sample).
[f] Significant increase of CMV-specific IgG or IgM (or both) antibodies (number of days after transplantation). ELISA, Enzyme-linked immunosorbent assay.
[g] Days after transplantation.
[h] —, CMV not detected, no antibody rise, or no CMV syndrome.

TABLE 3. Longitudinal course of CMV-related diagnostic parameters in patient 13[a]

Wk after transplantation	CMV antigenemia[b]	CMV viremia	CMV viruria	CMV ELISA[c]	
				IgG	IgM
1	−	−	−	55	<1
2	−	−	−	45	<1
3	1	−	−	44	<1
4	98*	+	−	29	<1
5	245*	+	+	72	<1
6	29*	−	+	≥500	14
7	3	−	+	≥1,000	10
8	−	−	+	≥1,300	10
9	−	−	+	≥1,100	12
10	−	−	+	≥1,300	18
12	−	−	+		

[a] See the text for details. +, CMV detected; −, CMV not detected.
[b] Total number of CMV Ag^+ cells of triplicate cytospins. *, Intermittent fever from days 29 to 41 after renal transplantation.
[c] Antibody levels are expressed as percentages of that of a reference serum pool. ELISA, Enzyme-linked immunosorbent assay.

trated by the longitudinal course of CMV viremia and CMV antigenemia in patient 13 (Table 3). Regarding the rapidity of the different methods, the following results were obtained. Buffy-coat specimens were identified as positive by conventional cell culture at a mean of 20 days (range, 7 to 42 days) after inoculation. The time required for EA detection in cell culture by IF averaged 4 to 6 days. The direct method of CMV Ag detection in blood leukocyte preparations usually yielded results within 3 to 5 h. The clinical relevance of these findings is illustrated by a chronologic comparison of the blood tests used for the nine patients with active CMV infections. Table 2 presents the number of days after transplantation when the first positive result was obtained by the different diagnostic techniques. CMV antigenemia was diagnosed from −1 to 56 days before CMV was demonstrated by cell culture of blood samples, from 8 to 50 days before virus isolation from urine, and from 6 to 22 days before a significant CMV antibody rise. Consequently, with one exception (patient 4), CMV Ag-positive cells were detectable before any other diagnostic result was obtained.

The ability of other MAbs (Table 4) to demonstrate CMV antigenemia was studied by using a panel of slides with proven CMV Ag positivity. It was found that CMV antigenemia could be demonstrated in all cases provided that MAbs to CMV IEA (16) were used. In our experience. MAbs MAB810 and HCMV19 generally displayed a lower staining intensity than did the other CMV IEA-specific MAbs. CMV EA (23)-specific MAbs appeared to be positive in a small number of the preparations, while CMV late antigen (LA) (23)-specific MAbs were not reactive.

TABLE 4. Usefulness of distinct CMV specific MAbs for demonstration of CMV antigenemia in nine patients with active CMV infections

MAb	Specificity[a]	Molecular mass (kDa)	Fluid and dilution used[b]	No. of patients with CMV antigenemia
C-10	IEA	70	Sup., 1:10	9
C-11	IEA	70	Sup., 1:10	9
C-12	IEA	70	Sup., 1:10	9
MAB810	IEA	68	Asc., 1:30	9
95/30	IEA	68	Asc., 1:100	9
HCMV19	IEA	66	Asc., 1:100	9
anti-EA	EA	ND[c]	Asc., 1:50	2
HCMV3	EA	ND	Asc., 1:50	1
C-30	LA	60	Sup., undiluted	0
C-32	LA	53	Sup., undiluted	0

[a] CMV-IEA was defined as described by Reddehase and Koszinowski (16), and CMV EA and LA were defined as described by The et al. (23).
[b] Sup., Hybridoma supernatant; Asc., murine ascites.
[c] ND, Not determined.

DISCUSSION

Isolation of CMV from blood specimens is considered to be a marker of active, clinically important CMV infection. However, because development of a cytopathic effect characteristic for CMV in the conventional culture technique is hampered by the slow replication of the virus, this method of virus isolation is of only limited value for the clinical management of immunocompromised patients. Recently, the time required for demonstration of infectious CMV in blood has been successfully reduced by using MAbs for detection of CMV EA in cell culture (14, 19). In the present study, we compared the detection of CMV in blood by cell culture with detection by a blood test based on direct detection of CMV antigens in blood leukocytes.

CMV Ag^+ blood leukocytes were detected in 44 samples, of which tissue culture proved to be positive in 21 cases. Twenty culture-negative samples were from seven patients from whom CMV was isolated both by a previous or subsequent blood sample by cell culture and by a urine sample, together with a significant CMV antibody rise. The remaining three samples were from two patients with serological evidence of active CMV infection. Thus, correct demonstration of CMV by antigen staining in blood leukocytes was supported by virus isolation from blood and urine or by confirmative serological tests during the same period (or by both methods). Since no CMV Ag^+ leukocytes were detected in only two culture-positive samples and the virus was not recovered by cell culture from 23 CMV Ag^+ samples, it appears that the technical sensitivity (i.e., the ability to detect CMV in CMV-positive blood samples) of direct CMV Ag detection is superior to that of virus isolation from blood. This is supported by the relationship between numbers of Ag^+ cells and virus isolation (Table 1). This higher sensitivity can be explained in a number of ways. Since CMV isolation depends on infectious CMV and, hence, on the functional integrity of the virus (and the fibroblast monolayer), it can be reasoned that virus isolation is sensitive to exterior influences, such as transport of the sample and toxicity by disrupted blood leukocytes (14, 19). This might explain the six culture-negative samples with high numbers of CMV Ag^+ cells (Table 1). If a virus replicative cycle is necessary for transfer of an infectious virus from blood cells to the fibroblast monolayer, another factor could be the abortive nature of CMV infection of blood leukocytes, as described for in vitro-infected blood leukocytes (3, 17) and as suggested by the $IEA^+/EA\pm/LA^-$ pattern of CMV-infected blood cells in vivo (Table 4). However, it is not clear whether antigen detection and virus isolation refer to the same type of blood leukocyte.

From a practical point of view, the cytospin technique has certain advantages over culture techniques. The test takes just a few milliliters of blood, is easy to perform, needs no culture facilities, and provides results with a negligible delay. Because of their characteristic staining, (even low numbers of) CMV Ag^+ cells are readily identifiable and allow a correct diagnosis. In the present report, the use of

MAbs to CMV IEA (16) is shown to be necessary for correct demonstration of CMV antigenemia.

The combination of rapidity and sensitivity results in early diagnosis (Tables 2 and 3) of active CMV infection (generally 1 week to several weeks before other diagnostic markers) and makes the cytospin technique relevant for the clinical management of immunosuppressed patients and for evaluative studies on the efficacy of antiviral drugs. Regarding the clinical sensitivity (i.e., the ability to diagnose an active CMV infection), the present results (9 of 9 patients) are comparable to those of a recent study (25 of 27 renal transplant patients) (24).

In summary this study suggests that detection of CMV Ag in blood leukocytes is as specific as and more rapid and sensitive than the virus isolation assays commonly used for demonstration of CMV in blood and thus for the diagnosis of active CMV infections.

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