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Detection of Human Cytomegalovirus Immediate Early Antigen in Leukocytes as a Marker of Viremia in Immunocompromised Patients

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Peripheral blood polymorphonuclear (PMN) cells from 35 immunocompromised patients (22 heart transplant recipients and 13 AIDS patients) and four normal subjects were tested for the presence of human cytomegalovirus (HCMV) immediate early antigen (IEA) (antigenemia) by indirect immunofluorescence (IFA) and IEA-specific monoclonal antibodies (MAb). PMN samples were tested in parallel for HCMV isolation (viremia) by using MAb to viral early antigens (EA) and the IFA technique 24–48 hr after inoculation onto human fibroblast monolayers. HCMV was isolated from 26 of 83 PMN samples examined: of these, 25 were also positive for HCMV IEA (96% sensitivity). Seven additional PMN samples negative for viral isolation resulted IEA-positive (87.7% specificity). Six of the seven discordant samples were taken from four patients during ganciclovir treatment. The transitory dissociation between positive HCMV antigenemia and negative viremia during antiviral treatment was followed, at the end of the therapy, either by virus clearance and disappearance of IEA-positive PMNs (one patient) or by reappearance of viremia (three patients). Among concordant positive samples, a significant correlation was observed between the number of IEA-positive PMN leukocytes and EA-positive nuclei of infected fibroblasts, when the same number of PMNs were used for both tests.

KEY WORDS: early antigens, immunofluorescence, ganciclovir

INTRODUCTION

Human cytomegalovirus (HCMV) infection in transplant recipients or AIDS patients is usually severe, when not life-threatening [Winston et al., 1979; Peterson et al., 1980; Macher et al., 1983]. Antiviral treatment (ganciclovir or DHPG) is now available for therapeutic use [Collaborative Study, 1986]. Nevertheless, since ganciclovir is not free from side effects, its use requires virological diagnosis of HCMV infection, and, in addition, rapid techniques for HCMV detection are needed so that specific chemotherapy can be started promptly. Monoclonal antibodies (MAb) to HCMV proteins synthesized early during the replicative cycle have allowed a new approach to early identification of viral isolates [Griffiths et al., 1984; Shuster et al., 1985; Stirk and Griffiths, 1987].

Recently, the detection of HCMV immediate early antigen (IEA) in the nuclei of peripheral blood polymorphonuclear (PMN) leukocytes by the immunoperoxidase (IPA) technique has been reported as a new, rapid, and specific technique for the diagnosis of HCMV infection [van der Bij et al., 1988a,b]. However, while attempting to confirm these results by using the IPA technique, we often encountered problems in the interpretation of test results because of non-specific staining. Thus, we used as an alternative the indirect immunofluorescence technique (IFA), which gave, as a rule, clear-cut results. Our study shows 90% agreement between detection of HCMV IEA-positive PMNs (HCMV antigenemia) by IFA and virus isolation from peripheral blood PMNs (HCMV viremia) in transplanted as well as in AIDS patients. Interestingly, during the course of ganciclovir therapy, a temporary dissociation between HCMV antigenemia and viremia was observed with persistence of IEA-positive PMNs in the absence of HCMV isolation. HCMV IEA detection in PMN cells by IFA appears a sensitive and specific method for both diagnostic purposes and monitoring of antiviral treatment.

MATERIALS AND METHODS

HCMV IEA Detection in PMNs

HCMV IEA detection in peripheral blood PMNs was done according to a reported procedure [van der Bij et

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al., 1988b] with some important modifications. PMNs, separated by sedimentation on a 6% dextran solution, were centrifuged at 1,000 rpm for 10 min at room temperature, suspended in minimum essential medium supplemented with 2% fetal calf serum, and counted. Aliquots of 2×10^5 PMNs in 0.2 ml were then inoculated onto each of two human embryonic lung fibroblast (HELF) monolayers for HCMV isolation (see below). The remaining PMNs were centrifuged again, washed with phosphate-buffered saline (PBS), resuspended in PBS, and centrifuged onto glass slides (2×10^5 cells in 100 μl/slide) using a cytocentrifuge (Cytospin-2, Shandon, UK). PMN spots were then fixed for 5 min in cold methanol-acetone (2:1), air-dried, and either stained immediately or stored at −80°C. In the staining procedure, a pool of monoclonal antibodies (MAb) to HCMV IEA (provided initially by Dr. T.H. Thé, and subsequently obtained from Biotest AG, Dreieich, FRG) was incubated for 30 min at 37°C. Slides were then rinsed with PBS and reacted with fluorescein-conjugated goat anti-mouse Ig (Cappel, Cooper Biomedical, Malvern, PA) diluted in PBS with 0.0005% Evans Blue counterstain for an additional 30 min. Slides were then rinsed, mounted, and observed under a fluorescence microscope. The number of HCMV IEA-positive cells was counted and recorded.

HCMV Isolation and Identification

HELF monolayers grown in shell vials were inoculated with PMN cells and centrifuged at room temperature for 45 min at 1,700 rpm for early identification of HCMV isolates. In parallel, conventional isolation and identification procedures were carried out using HELF culture tubes (see below). Early identification of HCMV isolates was accomplished by IFA 24–48 hr after inoculation of PMN samples, using a commercial Mab to HCMV early antigen (EA) (Biotech Research Inc., Rockville, MD). HCMV EA-positive HELF nuclei were counted and the number recorded. Inoculated HELF tubes were checked every other day for appearance of cytopathic effect (cpe). Virus isolates were then identified by using an HCMV-specific guinea pig immune serum and the indirect immunoperoxidase (IPA) technique [Gerna et al., 1976]. In addition, IPA was performed blindly in HELF tubes with no apparent cpe 10 days after inoculation of PMN samples. If negative, cultures were blindly passaged after 10 additional days.

Patients Examined

Twenty-two heart transplant recipients, 13 patients with AIDS, and four immunocompetent individuals (all but one admitted to the University Hospital of Pavia, Italy) were examined for HCMV isolation and IEA detection in PMN leukocytes. A total of 83 PMN samples were tested for both HCMV antigenemia and viremia: 64 were multiple samples from 16 patients, whereas the remaining 19 belonged to as many patients and were examined only once for determination of both parameters. In addition, all patients included in this study were routinely examined for HCMV infection by culturing clinical specimens (blood, urine, and throat washing) for HCMV isolation. Furthermore, the determination of virus-specific antibody of the IgG and IgM classes was performed on sequential serum samples, using indirect and capture ELISA methods, respectively [Schmitz et al., 1980]. Ganciclovir was administered by intravenous infusion at a dosage 10 mg/kg/day for 14 days, unless otherwise indicated (Table I).

RESULTS

HCMV IEA-Positive PMNs Detection by IFA

In preliminary experiments, the IPA technique for the detection of IEA-positive PMN leukocytes was performed according to the original report [van der Bij et al., 1988b]. However, non-specific staining still present after treatment for the removal of endogenous peroxidase activity hindered a clear-cut identification of IEA-positive PMN cells. In order to avoid such a problem, we used IFA. With this technique, a dramatic improvement in the quality of staining was achieved. HCMV IEA-positive nuclei of PMNs were easily detected in the absence of any non-specific staining. Positive nuclei were either evenly fluorescent or more markedly stained along the edges (Fig. 1). Both nuclear and perinuclear staining patterns could be observed in the same preparation. The ratio of IEA-positive:IEA-negative PMNs was consistently lower than 1:100.

Correlation Between HCMV Antigenemia and Viremia

As reported in Table II, HCMV was isolated and identified by EA detection in HELF cultures in 26 out of the 83 PMN samples examined. Virus was also isolated from 25 of these 26 samples by conventional isolation and identification procedures. HCMV IEA were detected in 25 of the culture-positive PMN leukocyte preparations (96% sensitivity). In the discordant sample, only two fibroblast cells showed nuclear positivity for HCMV EA and virus was not isolated by conventional procedures, whereas PMN spots were consistently negative for HCMV IEA on repeated testing. In the group of 57 PMN samples negative for HCMV isolation, 50 resulted equally negative for IEA detection in PMNs, but seven samples showed HCMV IEA positivity in the absence of virus isolation (87.7% specificity). Six of these seven discordant samples were taken from four patients during ganciclovir treatment (see below). The last discordant sample contained only two IEA-positive PMN cells and belonged to a heart transplant recipient admitted to another hospital. Inappropriate handling of this sample might have affected virus isolation. Among the concordant samples, six of the 25 HCMV-positive and four of the 50 HCMV-negative PMN preparations were taken during antiviral treatment.

TABLE I. HCMV Viremia and Antigenemia in Seven Patients Treated With DHPG

Patient (underlying disease)	Days after transplantation or AIDS diagnosis	HCMV (no. positive cells)		Days after initiation of therapy[a]	Outcome of HCMV infection in blood
		Viremia	Antigenemia		
B.G.B.	51	13	35	−8	
(heart	61	4	2	2	Cleared
transplant)	69	0	0	10	
	126	0	0	67	
G.A.	46	>50	ND[b]	−8	
(heart	55	>50	>50	1	Cleared
transplant)	63	0	0	9	
	97	0	0	43	
R. A.	277	4	ND	−16	
(heart	296	0	0	3	Cleared
transplant)	389	0	0	96	
R.S.	34	34	ND	−5	
(heart	40	>50	7	1[c]	
transplant)	57	40	7	18	
	77	30	25	38	Not cleared
	99	0	2	60[d]	
	152	7	1	113	
S.M.	128	>50	ND	−21	
(AIDS)	150	>50	>50	1	
	157	15	>50	8	
	197	30	>50	48	Not cleared
	205	0	>50	56[e]	
	211	0	7	62	
	231	>50	6	82	
B.C.	242	15	ND	−4	
(AIDS)	251	0	8	5	
	257	0	5	11	Not cleared
	305	>50	30	59	
S.G.	57	>50	25	−5	
(heart	69	0	2	7	Cleared
transplant)	96	0	0	34	

[a]Standard DHPG dosage was 10 mg/kg/day for 14 days.
[b]ND, not done.
[c]DHPG was administered for 24 days at 5 mg/kg/day.
[d]Eleventh day of therapy (second course of DHPG at standard dosage).
[e]Sixth day of therapy (second course of DHPG at 20 mg/kg/day for 14 days).

HCMV Viremia and Antigenemia During Ganciclovir Therapy

In Table I, clinical and virological findings obtained from sequential blood samples from the seven patients examined during ganciclovir therapy are reported. Of these patients, five were heart transplant recipients (four with a primary and one with a recurrent HCMV infection) and two were AIDS patients with a recurrent infection. Among them, three gave consistently concordant results with regard to antigenemia and viremia, whereas the remaining four exhibited discordant results in one or two sequential samples. Of the first three patients, two (B.G.B., G.A.) were positive for both parameters on the 2nd and 1st day of therapy, respectively, but in both cases, 1 week later, HCMV was neither detected nor isolated. The third patient (R.A.) was already negative for both HCMV antigenemia and viremia after 3 days of therapy. This patient was the only heart transplant recipient in this study undergoing a recurrent HCMV infection and receiving antiviral treatment.

Of the four patients with discordant results, two (R.S., heart transplanted, and S.M., AIDS patient) received two courses of ganciclovir (25 and 35 days apart, respectively), the second one at double dosage, because of persistence of viremia (and fever). In both cases, antigenemia and viremia were positive during the first course of therapy, whereas during the second one, viremia was no longer detected but HCMV antigenemia was still present. After completion of the second course of therapy, HCMV was isolated again from the blood of both patients. Similarly, the third patient (B.C.) failed to clear the virus from the blood since HCMV was not isolated during ganciclovir treatment, but IEA-positive PMN cells were consistently present. In this patient, dissociation of the two parameters disappeared at the end of therapy with reappearance of HCMV. On the other hand, in the fourth patient (S.G.) showing per-

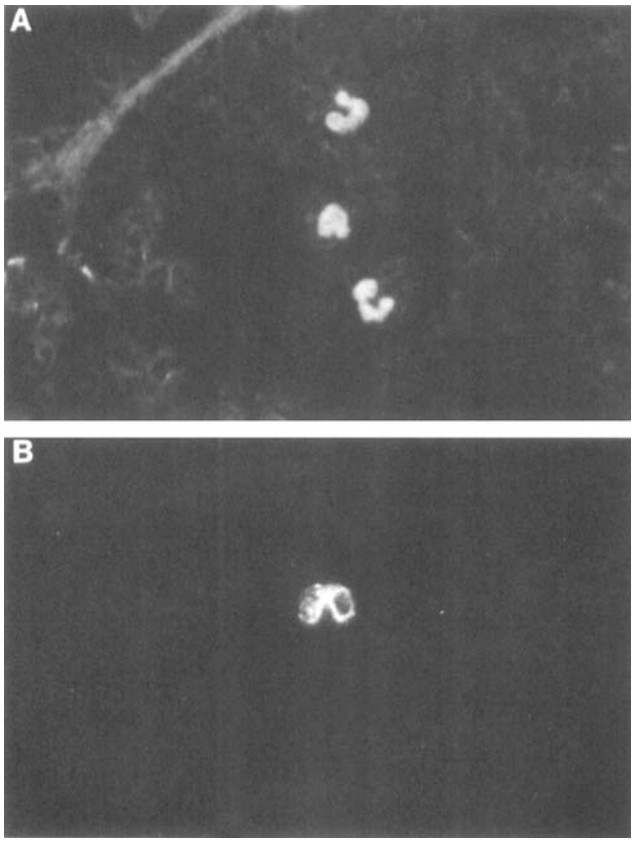

Fig. 1. HCMV immediate early antigens (IEA) in polymorphonuclear leukocytes stained by the indirect immunofluorescence technique using MAb to IEA. **A:** Three fluorescent nuclei evenly stained; **B:** a single nucleus markedly stained along the nuclear membrane.

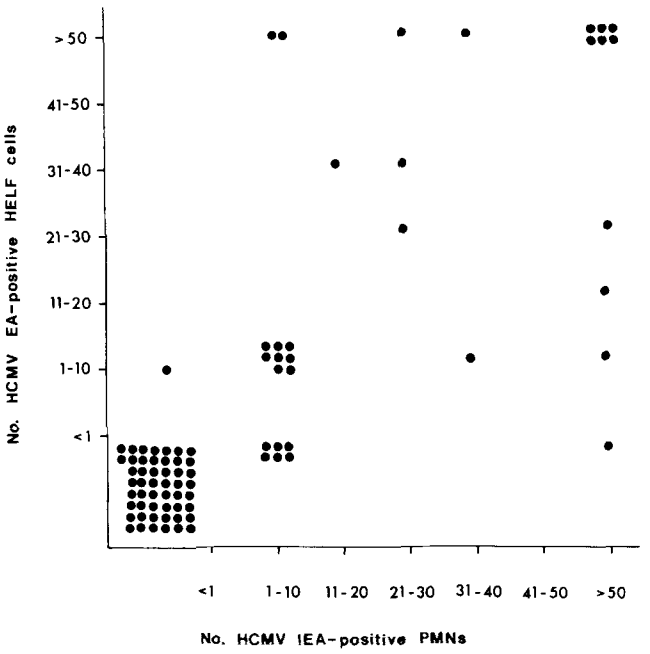

Fig. 2. Correlation between number of HCMV EA-positive HELF cells/culture and number of HCMV immediate EA-positive PMN leukocytes. The same number of PMN cells (2×10^5) was used for both tests. Apart from discrepant results, correlation was shown to be statistically significant in 19 of 25 PMN samples positive for both parameters and taken in the absence of ganciclovir treatment ($r = .70$).

TABLE II. Comparison Between Detection of HCMV IEA in PMN Cells and Early Identification of HCMV Isolates by EA Detection in HELF Cultures

Culture EA	PMNs IEA Positive	PMNs IEA Negative	Total
Positive	25	1[a]	26
Negative	7[b]	50	57
Total	32	51	83

[a]Only two HELF cells were positive for EA.
[b]Six samples were examined during DHPG treatment. In the additional sample, only two PMN cells were positive for IEA.

sistence of antigenemia and absence of virus isolation after 7 days of therapy, IEA-positive PMNs were not detected (and HCMV was not isolated) at a subsequent control. The number of HCMV IEA-positive cells was low (less than ten) in five of the six discordant blood samples, whereas in one sample, belonging to patient S.M., and examined after 6 days of therapy during the second course of DHPG, more than 50 IEA-positive PMN cells were counted.

Correlation Between the Number of HCMV EA-Positive HELF Cells and IEA-Positive PMN Leukocytes

Since the same number of PMN cells was used for both preparation of cell spots and inoculation onto HELF monolayers, a comparison was attempted between the number of HCMV EA-positive HELF cells as detected 24–48 hr after inoculation of PMNs and the number of IEA-positive leukocytes on cytospin preparations. PMN slides and inoculated coverslips were blindly scored by two different observers. The level of correlation detected is shown in Figure 2. It is interesting to note that, apart from the eight discordant results (six related to ganciclovir treatment, and two with only two cells positive for either IEA or EA), the correlation is statistically significant for the 19 PMN samples taken in the absence of ganciclovir therapy and found positive for both IEA and EA ($r = .70$). Six samples positive for both parameters but taken during antiviral treatment were excluded from the comparison.

DISCUSSION

As mentioned above, by using MAb to HCMV EA, it is now possible to shorten considerably the time required for the identification of HCMV isolates. The validity of this approach has been confirmed in this study, where 25 of 26 PMN samples positive for EA in cell cultures were also positive for virus isolation by con-

ventional procedures. However, recent reports [van der Bij et al., 1988a,b] describing rapid detection of HCMV IEA directly in peripheral blood PMN cells appear to provide the most rapid method so far proposed for the diagnosis of HCMV infections. In order to evaluate this entirely new approach, we planned this study, which was carried out by using the same MAbs of the original report and by performing in parallel HCMV IEA detection and HCMV isolation from PMNs. Using the IFA instead of the IPA technique, IEA-positive cells were unequivocally detected in the absence of any background staining. In addition, fluorescence was consistently so typical in its nuclear and/or perinuclear pattern that detection of even one single positive cell was just a matter of a careful examination of the slide.

In our study, HCMV IEA detection in PMNs and early identification of HCMV isolates using the "shell vial" technique and an EA-specific MAb showed 90% agreement. HCMV was isolated from 25 of 32 IEA-positive PMN samples (78%), whereas, according to van der Bij et al. data [1988a], viremia was detected only in 52% of HCMV IEA-positive samples (23/44). Such a discrepancy may be explained by the following: 1) in our study, the same number of PMN cells was used for cytospin preparations and cell culture inoculation; 2) inoculated shell vials were routinely centrifuged, and it has been reported that a higher efficiency of virus isolation follows centrifugation [Gleaves et al., 1984]; and 3) fibroblast monolayers inoculated with leukocytes deteriorate very easily, and thus, in our experience, it is better to look for HCMV EA 24–48 hr after inoculation rather than waiting for 4–5 days [as reported by van der Bij et al., 1988a]. Indeed, the correlation observed between HCMV IEA-positive PMNs and EA-positive fibroblasts, even in the presence of a very low number of positive cells, substantiate the hypothesis that antigenemia is a marker of viremia and that cocultivation of HCMV IEA-positive cells results in active viral replication and production of new viral progeny.

Nevertheless, since our study was not prospectively designed, we cannot confirm, as reported by van der Bij et al. [1988a], that early in the phase of HCMV infection a dissociation between positive antigenemia and absence of virus isolation may occur. However, the same type of dissociation was observed in the present study during ganciclovir therapy. In four viremic patients treated with DHPG, HCMV was not isolated, but IEA-positive PMNs were detected in sequential blood samples taken during antiviral treatment. Even though it is possible that the virus was not isolated for technical reasons, this seems unlikely in view of the good correlation found in this study between the two parameters.

By analyzing the results reported here, one may conclude that during intravenous ganciclovir treatment viremia is the first to disappear (hence, the dissociation). The finding that such a dissociation was not detected in three patients might be because of inadequate sequential sampling. However, persistence of HCMV antigenemia up to and beyond the end of therapy may indicate, as it did in three of our patients, uneffectiveness of DHPG treatment and reappearance of viremia. A more prolonged follow-up study is required before a final conclusion can be drawn. In addition, it would also be of interest to correlate viremia and antigenemia with the presence of recently reported mRNA specific for HCMV immediate early genes in peripheral blood leukocytes [Stockl et al., 1988] in order to get a more complete picture of the still puzzling aspects of HCMV infection in blood.

In conclusion, detection of IEA-positive PMNs appears to be a substantial addition to methods presently available for the rapid diagnosis of HCMV infection and monitoring of the efficacy of ganciclovir treatment in immunocompromised patients. However, IFA should replace IPA for IEA test performance.

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