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Rapid Screening for Resistance to Ganciclovir and Foscarnet of Primary Isolates of Human Cytomegalovirus from Culture-Positive Blood Samples

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A rapid screening assay for the detection of resistance to ganciclovir and foscarnet of primary isolates of human cytomegalovirus from culture-positive blood samples was developed by using single doses of both drugs and an immediate-early antigen plaque reduction assay. Results of the rapid assay with peripheral blood leukocytes as the viral inoculum were compared with those of a conventional assay with cell-free virus from the relevant viral isolates. Both assays gave overlapping results. The rapid assay offers the following major advantages: (i) it provides results within 4 to 6 days, (ii) it can be performed with peripheral blood leukocytes, and (iii) it reliably detects drug-resistant human cytomegalovirus strains.

Disseminated human cytomegalovirus (HCMV) infections of immunocompromised hosts represent the clinical basis for antiviral treatment with HCMV-specific antiviral drugs (ganciclovir or foscarnet). The extensive use of antiviral agents has caused the progressive emergence of drug-resistant HCMV strains. While ganciclovir-resistant HCMV strains have been reported since 1989 (5–7), foscarnet-resistant strains have rarely been reported (11). However, in the last few years a double resistance to both ganciclovir and foscarnet has been described (10, 15).

The currently available antiviral susceptibility assays, such as plaque reduction assay (PRA) (1), DNA hybridization assay (3), or in situ enzyme-linked immunosorbent assay (14), require HCMV isolation and multiple passages in cell cultures to obtain cell-free virus. This approach requires 4 to 6 weeks before chemosensitivity testing results are achieved. Recently, a rapid assay performed directly on primary isolates from clinical specimens was reported (12). However, the assay was suitable only for body fluids such as urine, amniotic fluid, or bronchoalveolar lavage fluid, not for blood specimens.

In this report we describe a rapid assay for the direct determination of resistance to ganciclovir and foscarnet of HCMV strains on primary isolation from peripheral blood leukocytes (PBLs). The assay is based on an immediate-early antigen (IEA) PRA, and its results were compared with those of the same assay (herein referred to as the conventional assay) performed with cell-free virus from the relevant isolates.

In the conventional assay, percent plaque reductions were plotted against serial twofold drug concentrations (1.56 to 50.0 μM for ganciclovir and 26.0 to 830.0 μM for foscarnet), and the 50, 90, and 99% inhibitory doses (ID_{50}, ID_{90}, and ID_{99}s, respectively) were interpolated from the dose-effect curve (7). The antiviral drug susceptibility of a series of control HCMV strains was determined by using 10 HCMV strains for ganciclovir and 35 HCMV strains for foscarnet. All of these strains were recovered from immunocompromised patients prior to any specific anti-HCMV treatment. Mean ID_{50}s, ID_{90}s, and ID_{99}s for ganciclovir and foscarnet were calculated (Table 1) and were used to determine the susceptibility indices (SIs) of the test strains. The SIs for 50, 90, and 99% IDs of isolates (SI_{50}s, SI_{90}s, and SI_{99}s, respectively) were determined by dividing the ID_{50}s (or ID_{90}s or ID_{99}s) for the test isolates by the mean ID_{50} (or ID_{90} or ID_{99}) for the relevant control strains. In the conventional assay an SI_{50} of <2.9 for both ganciclovir and foscarnet was considered to define an HCMV strain that was susceptible to both drugs, whereas an SI_{50} of 3.0 to 3.9 defined a strain with decreased susceptibility to either drug, and an SI_{50} of ≥ 4.0 indicated a strain resistant to either drug.

While in the conventional assay the titer of cell-free virus was determined in a 4-day IEA plaque assay (7), in the rapid assay the amount of infectious virus present in a PBL sample was determined in the 20-h shell vial assay as routinely performed for the quantification of viremia (8). This assay was based on a previous finding showing that an infected leukocyte was able to infect a single cultured fibroblast (8). Thus, when an approximate range of 15 to 250 leukocyte infectious units (LIUs) was detected in the blood sample of a patient, on the following day a new blood sample was drawn from the same patient and the IEA-PRA was performed with a number of PBLs containing 15 to 150 LIUs for each test replicate. The assay could be performed either in shell vials or in 24-well cell culture plates. However, use of shell vials appeared preferable because some damage occurred in the cell monolayers on 24-well plates during centrifugation of the PBL inoculum at $700 \times g$ for 45 min. Following removal of the viral inoculum, cells were washed and single concentrations (arbitrarily selected in order to give an SI_{50} of >5.0 for strains resistant to either drug in the rapid assay) of either ganciclovir (20.0 μM) or foscarnet (415.0 μM) in 1.0 ml of minimum essential medium supplemented with 2% fetal calf serum were added (three replicate cell cultures for each drug concentration). Following incubation at 37°C in a humidified atmosphere with 5% CO_2 for about 90 h, one coverslip was fixed with methanol-acetone and plaques were evidenced by the immunoperoxidase technique by using a monoclonal antibody to the HCMV major immediate-early protein p72 (8). If plaques were detected, the remaining two cell monolayers were also fixed. If not, the incubation time was prolonged for 2 additional days. Plaques

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were counted microscopically, and the mean count for each drug concentration was expressed as a percent reduction in the mean plaque counts of virus controls. A plaque reduction of < 50% showed that the ID_{50} for the HCMV strain under study was > 20.0 μM for ganciclovir and > 415.0 μM for foscarnet. Thus, a strain was considered to be resistant to ganciclovir when the SI_{50} (calculated by using the mean ID_{50} for control strains reported in Table 1) was >6.9 and resistant to foscarnet when the SI_{50} was >5.9. In the rapid assay, because of the unavailability of large amounts of PBLs from immunocompromised patients, the use of single drug concentrations was mandatory. For the same reason, a precise cutoff for the rapid assay (with multiple drug concentrations) could not be determined.

Twelve blood samples were drawn from as many immunocompromised patients (nine patients with AIDS and three transplant recipients) when quantitative levels of HCMV viremia (8) and antigenemia (7) were in the range of 14 to 260 and 69 to 1,000, respectively (Table 2). Of the nine HCMV strains recovered from AIDS patients, five (PBL samples 29457, 31255, 31780, 31302, and 31730; Table 2) were collected several months after the initiation of specific antiviral treatment with either ganciclovir alone (specimens 31255, 31780, and 31730) or sequential or combined courses of ganciclovir and foscarnet (specimens 29457 and 31302).

When reading the results of both the conventional and the rapid PRAs, a plaque was considered to consist of a group of at least 15 contiguous IEA-positive fibroblast nuclei (Fig. 1A). Test results were considered reliable with a minimal mean plaque number of ≥10. In addition, in the rapid assay virus titration in LIUs and the various plaquing capacities of different HCMV strains did not hamper the feasibility of the assay when the viremia level was in the range of 15 to 20 (8).

Comparative results obtained in parallel by the rapid and the conventional assays are reported in Table 2. All chemosensitivity findings were in agreement between the two assays. Of the 12 isolates tested, 7 showed susceptibility to ganciclovir and foscarnet comparable to that of control strains, whereas of the remaining 5 isolates, 2 were ganciclovir resistant, 2 showed a clear-cut resistance to both drugs, and 1 (sample 31255) displayed a decreased susceptibility to ganciclovir. Such a decreased susceptibility detected by the rapid assay in sample 31255 became overt ganciclovir resistance in sample 31780, which was taken from the same patient after a 2-month follow-up period.

An interesting finding observed during the performance of both the rapid and the conventional assays was that the time required for plaque formation increased to 6 days when HCMV isolates showing double resistance to ganciclovir and foscarnet were recovered, whereas only single infected cells were observed with the same strains after a 4-day incubation period (Fig. 1B). In other words, foscarnet resistance (associated with ganciclovir resistance) appeared to be characterized by a delay in IEA plaque formation.

The rapid IEA-PRA described herein appears to be a highly reliable assay since its results have consistently been confirmed by the conventional assay. With respect to the rapid test previously developed for determination of HCMV susceptibility to ganciclovir directly from clinical specimens (12), our assay offers some major advantages. First, IEA-PRA appears to be more reliable and easier to perform than the late antigen-expressing cell reduction assay. In addition, microscopic reading of plaques is much quicker and less cumbersome than counting of single infected cells (12). In our assay PBL clumps did not interfere with the test results since only single infected cells were detected in the 20-h shell vial titration assay and this was due to the low ratio of infected PBLs to uninfected PBLs

TABLE 1. Antiviral susceptibility testing of two series of HCMV strains recovered from immunocompromised patients who had not previously been treated with either antiviral drug[a]

No. of HCMV strains	Ganciclovir		Foscarnet	
	ID_{50} (μM)	ID_{90} (μM)	ID_{50} (μM)	ID_{90} (μM)
10 VRs	2.9 ± 2.1 (<1.56–7.0)	8.3 ± 3.1 (3.5–12.0)	22.4 ± 12.0 (5.0–50.0)	69.9 ± 28.2 (<26.0–111.0)
35 VRs				143.2 ± 38.0 (50.0–240.0)

[a] Values are means ± standard deviations (ranges). VR, virus recovery.

TABLE 2. Results of rapid and conventional IEA-PRAs on 12 HCMV strains from PBL samples drawn from immunocompromised viremic patients

PBL sample no., cause of immunosuppression[a]	pp65Ag[b]	Viremia[c]	Rapid assay on cultured PBL samples				Conventional assay on viral isolates					
			Ganciclovir		Foscarnet		Ganciclovir			Foscarnet		
			ID_{50} (μM [% PR])[d]	SI_{50}[e]	ID_{50} (μM [% PR])	SI_{50}	ID_{50} (μM [SI_{50}])	ID_{90} (μM [SI_{90}])	ID_{99} (μM [SI_{99}])	ID_{50} (μM [SI_{50}])	ID_{90} (μM [SI_{90}])	ID_{99} (μM [SI_{99}])
25976, HLT	125	14	<20.0 (100)	<6.9	<415.0 (100)	<5.9	2.0 (0.7)	4.7 (0.6)	10.0 (0.4)	25.0 (0.4)	103.0 (0.7)	160.0 (0.7)
26038, AIDS	69	19	<20.0 (100)	<6.9	<415.0 (100)	<5.9	1.5 (0.5)	3.5 (0.4)	5.0 (0.2)	75.0 (1.1)	130.0 (0.9)	190.0 (0.9)
26082, AIDS	1,000	260	<20.0 (97.7)	<6.9	<415.0 (96.5)	<5.9	3.1 (1.1)	10.0 (1.2)	22.0 (1.0)	67.0 (0.9)	144.0 (1.0)	201.0 (0.9)
26144, HT	800	200	<20.0 (90.0)	<6.9	<415.0 (92.9)	<5.9	2.1 (0.7)	7.5 (0.9)	25.0 (1.1)	60.0 (0.8)	103.0 (0.7)	190.0 (0.9)
29457, AIDS	350	24	**>20.0 (26.7)**[f]	**>6.9**	**>415.0 (0)**	**>5.9**	**50.0 (17.2)**	**93.0 (11.2)**	**200.0 (8.9)**	**740.0 (10.5)**	**1,330.0 (9.3)**	**1,590.0 (7.4)**
29856, AIDS	80	40	<20.0 (90.0)	<6.9	<415.0 (86.7)	<5.9	1.5 (0.5)	7.0 (0.8)	25.0 (1.1)	111.0 (1.6)	177.0 (1.2)	201.0 (0.9)
30778, SLT	280	53	<20.0 (94.3)	<6.9	<415.0 (98.1)	<5.9	2.0 (0.7)	11.0 (1.3)	25.0 (1.1)	85.0 (1.2)	207.0 (1.4)	300.0 (1.4)
31255, AIDS	400	70	<20.0 (64.3)	<6.9	<415.0 (98.6)	<5.9	10.0 (3.4)	23.0 (2.8)	46.0 (2.1)	ND[g]	ND	ND
31780, AIDS	800	40	**>20.0 (38.3)**	**>6.9**	<415.0 (100.0)	<5.9	**23.0 (7.4)**	**34.0 (4.1)**	50.0 (2.2)	ND	ND	ND
31302, AIDS	1,000	97	**>20.0 (14.3)**	**>6.9**	**>415.0 (0)**	**>5.9**	**46.0 (15.9)**	**97.0 (11.7)**	**200.0 (8.9)**	**498.0 (7.1)**	**797.0 (5.6)**	**1,594.0 (7.4)**
31730, AIDS	135	42	**>20.0 (48.1)**	**>6.9**	<415.0 (100.0)	<5.9	**25.0 (8.6)**	**93.0 (11.2)**	**180.0 (8.0)**	ND	ND	ND
32925, AIDS	600	38	<20.0 (100.0)	<6.9	<415.0 (100.0)	<5.9	3.0 (1.0)	10.3 (1.2)	21.0 (0.9)	88.0 (1.2)	185.0 (1.3)	348.0 (1.6)

[a] HLT, HT, SLT, heart-lung, heart, and single lung transplant, respectively.
[b] Number of pp65-positive/2×10^5 PBLs examined.
[c] Number of infected fibroblast nuclei in a shell vial culture inoculated with 2×10^5 PBLs.
[d] PR, plaque reduction.
[e] SI_{50} (or SI_{90} or SI_{99}) = ID_{50} (or ID_{90} or ID_{99}) for the relevant isolate/mean ID_{50} (or ID_{90} or ID_{99}) for a series of HCMV strains from untreated patients (see Table 1).
[f] IDs and SIs values indicating resistance are reported in boldface type.
[g] ND, not done.

(mostly >1:1,000). In addition, nonspecific staining of PBL clumps adhering to the cell monolayer could be readily distinguished from true IEA plaques. Second, since the presence of HCMV in peripheral blood is often associated with HCMV disease in immunocompromised patients (9), the documented validity of the rapid assay for determination of the drug susceptibilities of leukocyte-transmitted HCMV strains justifies its use for the adoption of therapeutic decisions. The clinical significance of the results provided by the direct determination of the drug susceptibilities of HCMV strains infecting body fluids (12) remains to be investigated. In fact, it is well known that different HCMV strains may infect different body sites (13), and thus, the results of chemosensitivity testing of HCMV strains recovered from body fluids may not be valid for viral strains circulating in blood. Third, detection by the rapid assay of HCMV strains showing either ganciclovir resistance or resistance to both drugs represents a major advantage in the management of HCMV infections. Direct rapid testing of drug-resistant HCMV strains from clinical specimens was not reported previously (12). Finally, the detection by the rapid assay (later confirmed by the conventional assay) of blood isolates showing double resistance to ganciclovir and foscarnet prospectively points to an increasing risk of the emergence of double resistance in immunocompromised patients undergoing multiple courses of sequential or combined (2, 4) treatment with both anti-HCMV drugs.

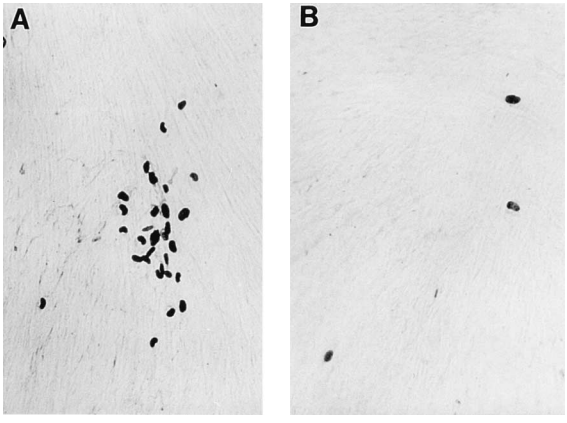

FIG. 1. Virus controls of HCMV strains with different susceptibilities to antiviral drugs in the rapid assay. (A) Typical morphology of an HCMV IEA plaque as detected by the immunoperoxidase technique 96 h after inoculation of a drug-susceptible (or a ganciclovir-resistant) HCMV strain. (B) Only single infected fibroblast nuclei were detected 96 h after inoculation of an HCMV strain resistant to both drugs. A plaque size comparable to that reported in panel A was observed at 144 h with strains resistant to both ganciclovir and foscarnet. Similar results were obtained when the viral inoculum was represented either by cell-free virus in the conventional assay or by leukocyte-associated virus in the rapid assay.

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