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Monitoring of ganciclovir sensitivity of multiple human cytomegalovirus strains coinfecting blood of an AIDS patient by an immediate-early antigen plaque assay

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Summary

A plaque-reduction assay for chemosensitivity testing of human cytomegalovirus (HCMV) strains was developed based on early detection of viral plaques 96 h p.i. by a monoclonal antibody to the major immediate-early protein p72. Sequential HCMV isolates from an AIDS patient undergoing multiple courses of ganciclovir treatment during an 18-month follow-up were tested by the new assay, showing emergence of a ganciclovir-resistant strain. However, cloning of viral isolates and Southern blot hybridization analysis showed the simultaneous presence of three different HCMV strains in blood. Of these, the resistant strain was likely to be selected during prolonged maintenance antiviral treatment, emerging during full drug regimen, while the two sensitive strains reappeared in association with the resistant one following drug discontinuation. This finding was demonstrated by high levels of ID_{90} and ID_{99} in sequential mixed viral populations. The new plaque assay leads to reduction in time needed for chemosensitivity testing and permits rapid tracing of drug-resistant strains in a mixed viral population.

Human cytomegalovirus; Immediate-early antigen; Plaque assay; Ganciclovir-resistance; Acquired immunodeficiency syndrome

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Introduction

Prolonged antiviral treatment is often required for correct clinical management of human cytomegalovirus (HCMV) systemic infections in AIDS patients (Gerna et al., 1990a). Thus far, ganciclovir is the antiviral drug most widely used for treatment of HCMV infections and maintenance ganciclovir therapy represents the clinical and biological basis for the increasing emergence of ganciclovir-resistant strains in AIDS patients (Erice et al., 1989). Since resistance is progressive, it is important that rapid assays for chemosensitivity testing be available for timely discovery of initial in vitro resistance so that adequate measures for effective control of HCMV infection in vivo can be adopted. Methods currently in use are plaque-reduction assays based on a solid overlay and development of plaques which are stained with crystal violet 8–10 days after inoculation for visual counting (Biron et al., 1985).

In the present study, a new HCMV immediate-early antigen (IEA) plaque assay for detection of ganciclovir-resistant strains was developed. The assay is based on the observation that HCMV virions can undergo a complete replication cycle in the presence of non-inhibitory doses of antiviral drugs, and then spread to contiguous cells. Ninety-six h p.i. IEA plaques were evidenced by immunocytochemical staining. The assay was applied to chemosensitivity testing of sequential HCMV isolates from blood of an AIDS patient undergoing multiple courses of ganciclovir treatment and carrying in blood a mixture of three different HCMV strains (two sensitive and one resistant to ganciclovir). Determination of ganciclovir ID_{90} and ID_{99} allowed detection of the resistant strain in the mixed viral population of sequential HCMV isolates in the absence of clonal purification of single strains.

Materials and Methods

Plaque and chemosensitivity assays

Following initial experiments with AD169 HCMV reference strain, wild (cell-free) low-passage HCMV strains diluted to contain 100 PFU in 0.4 ml were centrifuged for 45 min at 700 × *g* onto confluent human embryonic fibroblast cell monolayers (Gleaves et al., 1984) grown in 24-well cell culture plates (Shigeta et al., 1991). Viral inoculum was then removed and cells washed. Subsequently, serial concentrations of ganciclovir in 1.0 ml of minimum essential medium supplemented with 2% fetal calf serum were added (3 replicate cell cultures/each drug concentration). Virus controls were performed in parallel in absence of ganciclovir. Following incubation at 37°C in a 5% CO_2 humidified atmosphere for 96 h, cell monolayers were fixed with methanol-acetone and plaques, not yet microscopically detectable, were evidenced by the immunoperoxidase technique, using a monoclonal antibody reactive to the HCMV major IE protein p72 (Gerna et al., 1990b). Two incubation times, each

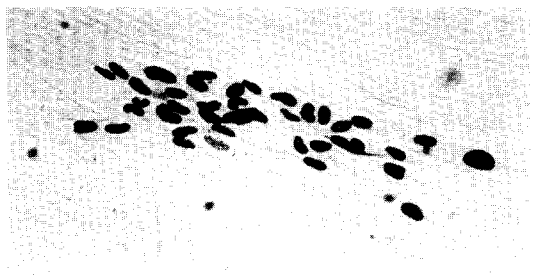

Fig. 1. Human cytomegalovirus immediate-early antigen plaque, as detected by light microscopy 96 h post-inoculation, using a monoclonal antibody to the major immediate-early protein p72. Immunoperoxidase technique. Magnification 400×.

of 30 min, were used for mouse monoclonal antibody and anti-mouse Ig peroxidase-conjugate, followed by incubation with substrate-chromogen solution (diaminobenzidine in H_2O_2 citrate buffer). Plaques (Fig. 1) were microscopically counted and the mean plaque counts for each drug concentration and each strain was expressed as percent of the mean plaque counts of virus controls. Percent values were plotted against drug concentrations and 50%, 90% and 99% inhibitory doses (ID_{50}, ID_{90} and ID_{99}) were determined by interpolation from the dose–effect curve. For comparison, conventional agarose plaque assay was performed, as reported (Biron et al., 1985).

In addition, a late antigen (LA)-plaque assay was performed at 96 h p.i. using trypsinized infected cells as inoculum (Dankner et al., 1990). Again, results were compared with those of a conventional agarose plaque assay using infected cells, which was completed 8 days p.i. The immunostaining procedure for LA-plaque assay was as above, except for monoclonal antibody which was directed to a late viral protein, as reported (Gerna et al., 1990b).

Isolation and cloning of sequential HCMV isolates

Twelve sequential HCMV isolates (11 from blood and one from pharynx) were recovered from an AIDS patient on human embryonic lung fibroblast cell cultures 1–3 weeks after inoculation of 2×10^5 polymorphonuclear leukocytes/tube culture. Virus isolates were identified by the immunoperoxidase or immunofluorescence technique as reported (Gerna et al., 1990b). Following 5–6 passages, 3 virus isolates (VR 2684, VR 3248 and VR 3480) were plaque-purified. Clonally purified strains were then propagated for 5 additional passages and tested for chemosensitivity to ganciclovir as well as for restriction enzyme DNA patterns by Southern blot hybridization analysis (see below).

Ganciclovir treatment

The patient was treated with 3 courses (each lasting 14 days) of ganciclovir at a full dosage (10 mg/kg/day) every other month between October 1988 and March 1989. Then, maintenance treatment (5 mg/kg/day) was performed till July 1989. Following 4 months of ganciclovir discontinuation, antiviral treatment was reinstated in November 1989 till May 1990 by alternating courses of treatment at a full dosage to intervals when the drug was administered at half a dosage (5 mg/kg/day) or at a dosage of 5 mg/kg/3 days/week.

Southern blot analysis

Following extraction according to a reported procedure (Chandler et al., 1986), DNA from viral isolates was submitted to digestion using 4 different restriction enzymes (*Eco*RI, *Hin*dIII, *Pst*I, and *Bgl*II). Digested DNA was electrophoresed on 0.7% agarose gel and then transferred onto nylon membranes (Gene Screen Plus, NEN, Boston, MA, USA). Comparison of HCMV DNA patterns was done by Southern blot hybridization (Chandler et al., 1986), using 4 (pCM1015, pCM1049, pCM1058, pCM1075) ^{32}P-labeled subgenomic fragments cloned in cosmids and spanning almost the entire UL fragment of HCMV genome (Fleckenstein et al., 1982). Subsequent rehybridizations of the same nylon membranes with different probes were carried out following removal of radiolabeled probes according to a reported procedure (Sambrook et al., 1989), which was slightly modified. In detail, after autoradiography, probes were removed from nylon membranes by immersion in 1 liter of 0.01 × SSC containing 0.01% SDS for 2 h at 75°C. Membranes were then rinsed briefly with 0.1 × SSC at room temperature. Most of the liquid was removed by placing membranes on a pad of paper towels. The damp membranes were then put between two sheets of Saran Wrap and applied to X-ray film to check that all of the probe had been removed. Then, membranes were stored frozen until needed.

Results

IEA plaque assay and chemosensitivity testing

Ganciclovir ID_{50}, ID_{90} and ID_{99} obtained by the IEA-plaque-reduction assay were compared on 5 HCMV-sensitive strains with the relevant ID_s calculated by using an 8-day conventional plaque assay. Results were not significantly different ($P > 0.5$). Similarly, 3 ganciclovir-resistant isolates gave comparable results (data not shown). In addition, for the same isolates ID_{50} and ID_{90} were determined by using trypsinized infected cells in both a 96-h LA-plaque-reduction assay and an 8-day conventional plaque assay. Again, results were not significantly different from those obtained when using cell-free virus

($P > 0.5$). IEA plaque assay was selected as the most suitable test for a rapid microscopic reading of results. LA-plaque assay could present problems in reading of results when infected cells were not satisfactorily dispersed as single cells during the trypsinization procedure. In addition, staining of agarose plaques by methylene blue or crystal violet could raise problems of false plaque counting when fibroblast cells were overcrowded in some areas of the cell monolayer.

In reconstruction experiments it was shown that the IEA plaque-reduction assay was able to detect up to 1% ganciclovir-resistant strain in a virus mixture containing 99% sensitive strain (increased ID_{99}).

Ganciclovir sensitivity of sequential isolates

The newly developed plaque-reduction assay was used to test 12 sequential HCMV isolates from an AIDS patient with HCMV disease receiving multiple courses of ganciclovir treatment at a full or reduced dosage during an 18-month follow-up. Monitoring of HCMV infection in blood was performed by sequential determination and quantitation of HCMV viremia (Gerna et al., 1990b), antigenemia (Revello et al., 1989) and DNAemia by the polymerase chain reaction (Gerna et al., 1990a). As shown in Table 1, during the first year of follow-up (October 1988–October 1989), when patient underwent multiple courses of ganciclovir treatment at a full or half a dosage with excellent clinical response, HCMV VR 2684 and VR 3248 appeared as sensitive as reference strain AD169 (ID_{50}, 3.4 μM; ID_{90}, 9.7 μM; and ID_{99}, 20.6 μM). Subsequently, between November 1989 and January 1990, following a course of full ganciclovir treatment during the first two weeks of November, which was only partially effective on HCMV retinitis relapse, the drug was administered at a maintenance dosage of 5 mg/kg/3 days/week. ID_{50}, ID_{90} and ID_{99} of blood isolates 3304, 3336 and 3372 recovered during that period were 5.0–6.0 μM, 11.9–17.5 μM and 25.0–30.0 μM respectively, thus showing a normal sensitivity to ganciclovir of the relevant strains. It is noteworthy that VR 3371 from pharynx showed a level of sensitivity comparable to that of VR 3372 recovered from a blood sample collected the same day (Table 1). In the same period, HCMV disease progressed as shown by continuous fever and worsening of bilateral HCMV retinitis associated to repeated virus isolation from blood, whereas in vitro resistance was not detected. This finding could be due either to insufficient ganciclovir administration, which allowed or only partially blocked replication of sensitive strains during that period or to initial emergence of a resistant HCMV strain or both. However, when drug dosage (February–March 1990) was raised to 5 mg/kg/day, HCMV VR 3450 (March 1990) was moderately resistant (ID_{50}, 18.7 μM; ID_{90}, 46.2 μM; and ID_{99}, 88.0 μM), while, at a full dosage of ganciclovir (10 mg/kg/day), VR 3480 (April 1990) appeared highly resistant (ID_{50} 42.5 μM; ID_{90}, 90.0 μM; and ID_{99}, 170.0 μM). Following discontinuation of ganciclovir treatment between May and October 1990, the new HCMV isolates 3513, 3560, 3615 and 3732 were apparently sensitive (ID_{50},

TABLE 1

Comparative ganciclovir sensitivity of sequential HCMV isolates recovered during follow-up of an AIDS patient

VR, date, site	Isolate cloning[a]	Antigenemia/ Viremia[b]	Ganciclovir				Presence of individual strains[c]
			Dosage	ID_{50} (μM)	ID_{90} (μM)	ID_{99} (μM)	
2684, 6 Oct 1988,	C	NA	NA	<1.5	6.25	21.8	A
blood	U	300/200	no	<1.5	6.25	21.8	A+C
3248, 10 Oct 1989,	C	NA	NA	2.96	5.77	30.0	B
blood	U	500/410	no	ND	ND	ND	A+B+C
3304, 28 Nov 1989, blood	U	2/3	5 mg/kg/3d/wk	5.46	17.5	30.0	A+B+C
3336, 19 Dec 1989, blood	U	14/1	5 mg/kg/3d/wk	5.92	17.5	25.0	A+B+C
3371, 13 Jan 1990, pharynx	U	NA	5 mg/kg/3d/wk	2.85	9.06	25.0	A+B+C
3372, 13 Jan 1990, blood	U	ND/ND	5 mg/kg/3d/wk	6.08	11.87	25.0	A+B+C
3450, 15 Mar 1990, blood	U	460/148	5 mg/kg/d	18.75	46.25	88.0	C
3480, 2 Apr 1990,	C	NA	NA	42.50	90.0	350.0	C
blood	U	1,000/150	10 mg/kg/d	40.0	82.5	170.0	C
3513, 7 May 1990, blood	U	500/150	no	7.2	20.62	95.0	A+B+C
3560, 4 Jun 1990, blood	U	950/46	no	8.75	67.5	188.0	A+B+C
3615, 13 Jul 1990, blood	U	800/600	no	6.00	37.5	167.0	A+B+C
3732, 15 Oct 1990, blood	U	1,000/1,000	no	7.2	42.5	150.0	A+B+C
AD169	NA	NA	NA	3.43	9.7	20.6	NA

C, cloned viral strain; U, uncloned HCMV isolate; NA, not applicable; ND, not done; ID_{50}, ID_{90} and ID_{99} represent 50%, 90%, and 99% inhibitory doses.

[a]Cloning was done by limiting dilution.

[b]Number of polymorphonuclear leukocytes positive for antigenemia/viremia, according to a reported method (Gerna et al. 1990b).

[c]A, B, and C indicate the 3 cloned HCMV strains which were shown to be variably represented in most isolates and, thus, to coinfect blood.

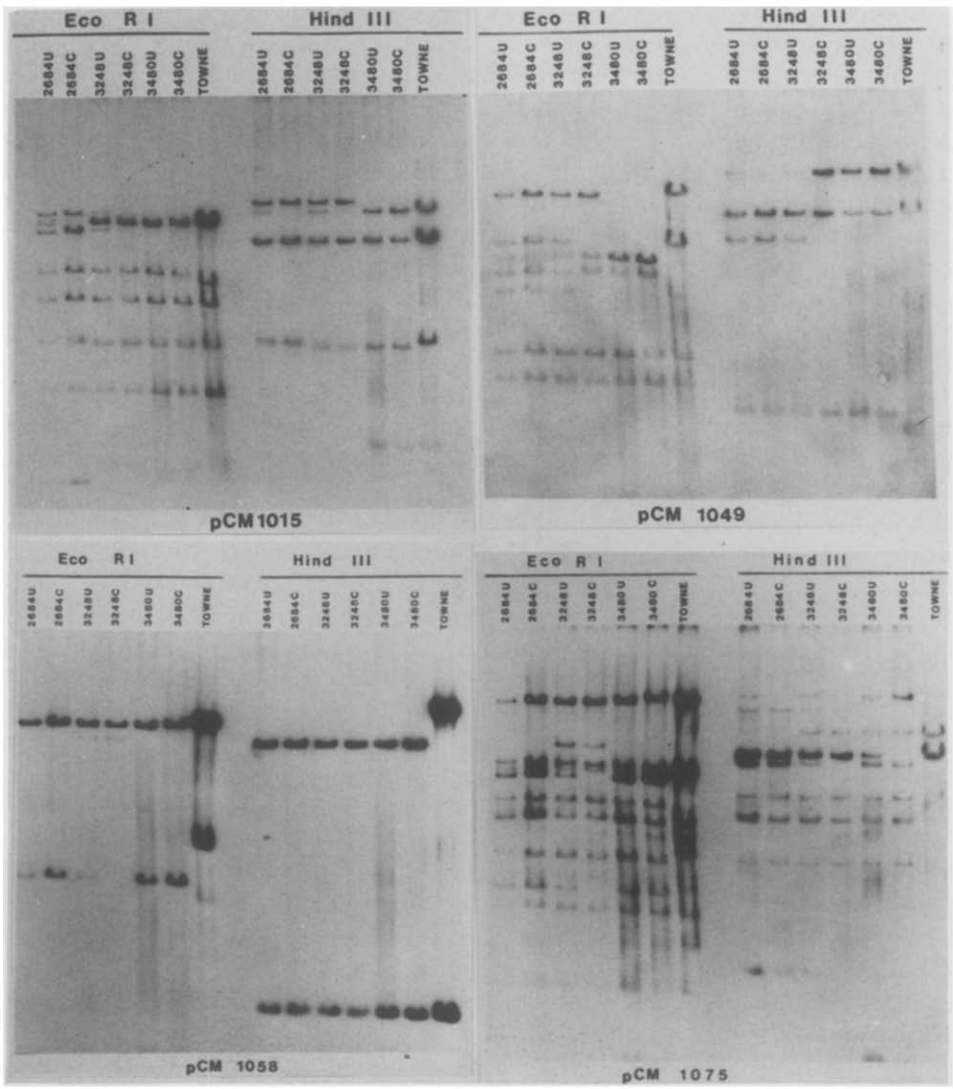

Fig. 2. Southern blot analysis of 3 sequential HCMV isolates (U) and the three relevant clonally purified strains (2684C = strain A; 3248C = strain B; 3480C = strain C), following digestion of viral DNA with *Eco*RI and *Hin*dIII. Subgenomic DNA fragment pCM1015, pCM1049, pCM1058 and pCM1075 cloned in cosmids (Fleckenstein et al., 1982) were labeled with ^{32}P. Comparative analysis of DNA patterns shows that the 3 cloned strains are different from each other and are represented at a variable proportion in different isolates. Each of the two combinations pCM1049-*Eco*RI and pCM1075-*Hin*dIII differentiates all 3 viral clones. In addition, other combinations confirm that the 3 clonally purified viral populations are true different strains.

6.0–8.7 μM), yet showing increased ID_{90} (20.6–67.5 μM) and ID_{99} (95.0–188.0 μM).

Southern blot analysis

Comparison of DNA profiles of sequential isolates suggested that multiple HCMV strains were present in most, if not all, isolates recovered from the patient since the beginning of follow-up. Initially, the 3 most represented strains were clonally purified from 3 different viral isolates (Table 1). In detail, the first strain, referred to as strain A in Table 1, was cloned from the initial HCMV isolate 2684 and shown to be sensitive to ganciclovir, while the second strain, referred to as strain B in Table 1, was cloned from isolate 3248 (recovered about a year later) and shown to be sensitive as well. The third strain, referred to as strain C in Table 1, was cloned from isolate 3480 and shown to be highly ganciclovir-resistant.

The comparison of restriction enzyme DNA patterns of the three cloned viral strains and the relevant sequential isolates showed that major differences in DNA patterns were given by *Eco*RI and *Hin*dIII. By using these two enzymes in combination with the 4 probes, it was concluded that the 3 cloned strains were true different strains (combinations pCM1049-*Eco*RI and pCM1075-*Hin*dIII) which were differently represented in different isolates, although all three were present in most of them (Table 1). In detail, in VR 2684 strain B does not appear to be represented (pCM1058-*Eco*RI, pCM1075-*Eco*RI) (Fig. 2), while both strain A (pCM1015-*Eco*RI, pCM1049-*Eco*RI and -*Hin*dIII, and pCM1075-*Hin*dIII) and strain C (pCM1015-*Hin*dIII, pCM1075-*Hin*dIII) appear to be present. In addition, VR 3248 contains a mixture of A (pCM1015-*Eco*RI, pCM1049-*Hin*dIII, pCM1075-*Hin*dIII), B (pCM 1075-*Eco*RI, pCM 1058-*Eco*RI) and C (pCM1015-*Hin*dIII and pCM 1075-*Hin*dIII). Finally, VR 3480 does not contain either A or B, as shown by the analysis of multiple combinations probe-restriction enzymes. The presence of A, B, and C in most viral isolates intermediate between VR 2684 and VR 3480 is shown in Fig. 4, where VR 3371 (from pharynx) shows a DNA profile similar to VR 3372 (from blood collected the same day) suggesting the simultaneous presence of the 3 cloned strains, although at a different proportion. In particular, strain C was shown to be already present in the first isolate (VR 2684) (Fig. 2) and was consistently found in all viral isolates recovered during the follow-up period (Table 1). Nevertheless, strain C conferred overt resistance only to the two intermediate isolates (VR 3450 and VR 3480) recovered during daily ganciclovir treatment at a full or half a dosage. Since DNA profile of VR 3480 (combination pCM 1075-*Hin*dIII, Fig. 2) could not be explained on the basis of a mixture of strains A, B and C and clonally purified strain 3480C (strain C) had a DNA pattern different from that of the relevant isolate, further extensive cloning of VR 3480 by plaque purification was performed. At least 4 DNA patterns were identified. However, they were interpreted as variants of C, since they were differentiated in only one (Fig. 3, pCM1075-*Hin*dIII) out of 8 combinations of the 4 probes with *Eco*RI and *Hin*dIII. In Fig. 3, three of the eight combinations tested are reported.

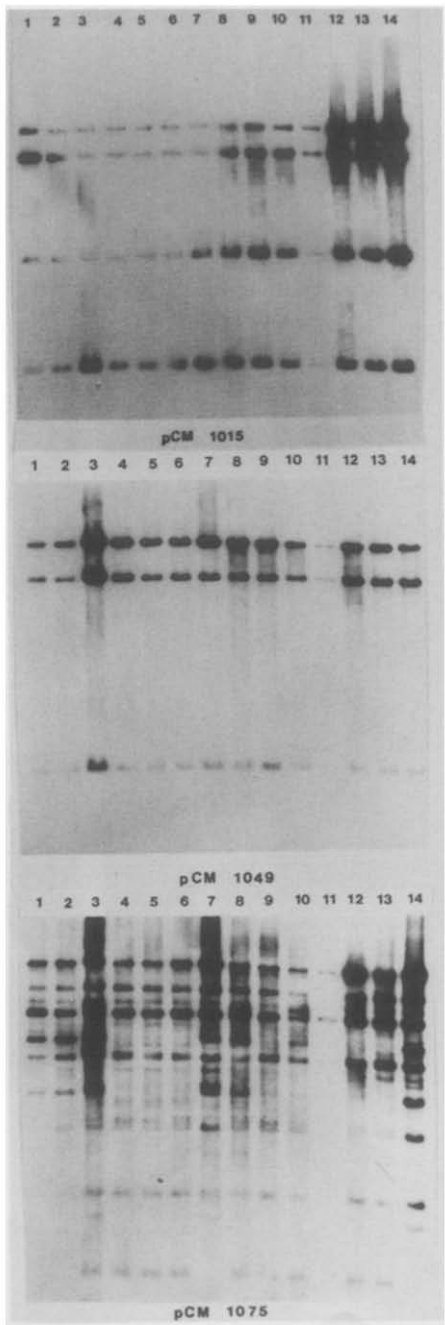

Fig. 3. Southern blot analysis of 14 clones from VR 3480, following digestion of viral DNA with *Hin*dIII. Four different DNA patterns are seen with pCM1075. The other two combinations shown (along with the remaining combinations of probes with *Eco*RI and *Hin*dIII) document the absence in VR 3480 (strain C) of either 2684C (strain A) or 3248C (strain B) strains, suggesting that the 4 DNA patterns represent variants of the same strain.

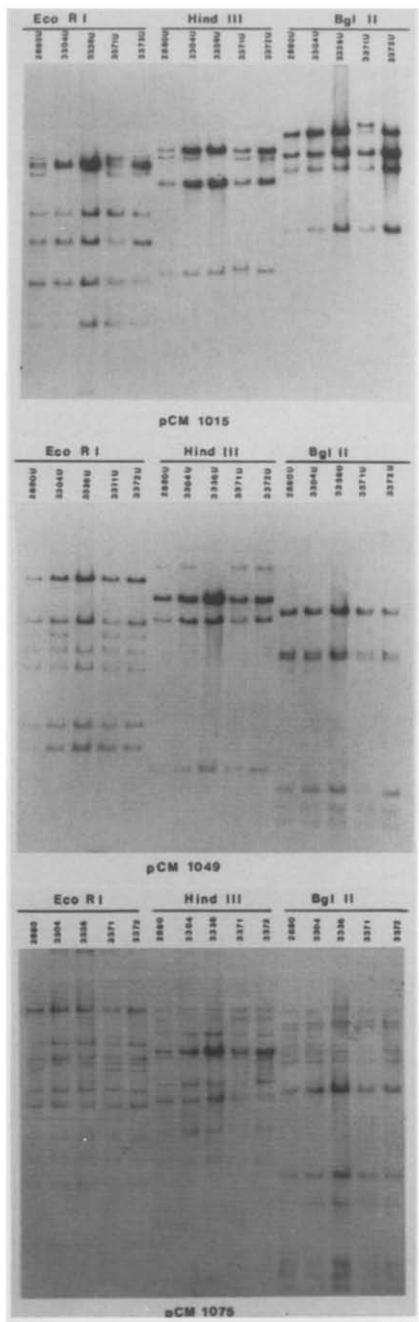

Fig. 4. Southern blot analysis of 5 uncloned (U) viral isolates, intermediate between VR 2684 and VR 3480, following digestion of viral DNA with *Eco*RI, *Hin*dIII and *Bgl*II. The comparative analysis of the combinations shown (along with the remaining combinations not shown) documents the presence in all isolates of the 3 cloned strains reported in Fig. 2, although at a different proportion.

Discussion

This paper reports monitoring of ganciclovir sensitivity of sequential HCMV isolates from an AIDS patient undergoing follow-up for more than 18 months. Firstly, an immediate-early antigen plaque-reduction assay was developed. Secondly, restriction-enzyme DNA analysis of multiple isolates by Southern blot showed the presence of multiple DNA patterns, which could be attributed to 3 different strains (one showing a high level of ganciclovir-resistance) as well as to some variants.

The IEA-plaque assay was selected as the most suitable among different plaque assays tested. Although requiring use of cell-free virus, following multiple passage propagation of viral isolates, it was considered to give clearcut results and to avoid problems in microscopic reading of results. In conventional plaque assays following 10–14 days incubation, non-immunologic staining of plaques may create problems in differentiating true viral plaques from false plaques due to uninfected cell packing. On the other hand, use of infected cells as inoculum may raise some bias in reading of results both in the LA and in the conventional plaque assays, whenever small clumps of infected cells are present in the inoculum. In this case, apparently high values of ID_{90} and ID_{99} could alter chemosensitivity testing results, suggesting unneeded changes in antiviral treatment. Recently introduced assays for a more precise quantitation of antiviral activity appear promising. However, DNA–DNA hybridization assay requires use of radiolabeled probes (Dankner et al., 1990) and ELISA systems appear, in our hands, very difficult to calibrate (Tatarowicz et al., 1991).

Isolation of multiple strains from sites other than blood has been reported in AIDS patients (Drew et al., 1984; Spector et al.,1984). However, we are not aware of reports describing the presence of multiple HCMV strains in blood. Coinfection of blood by multiple HCMV strains for a prolonged period of time, as here described, represents a finding of some interest, with special respect to the presence of a ganciclovir-resistant strain in a viral mixture containing two additional ganciclovir-sensitive strains.

Whether strain C present in the first isolate 2684 was ganciclovir-sensitive and then became resistant following multiple courses of ganciclovir treatment remains to be determined on multiple viral clones from sequential isolates. At the moment, however, this hypothesis appears reasonable, given ganciclovir sensitivity of first viral isolates. In addition, whether strain C present in the last viral isolates following VR 3480 shows the same level of resistance of strain C cloned from VR 3480, although suggested by high levels of ID_{90} and ID_{99} of these isolates, it has yet to be determined. If this assumption is demonstrated, then the most plausible interpretation for the apparent decrease in resistance of uncloned HCMV isolates following VR 3480 would be that the 2 sensitive strains A and B reemerged due to suspension of antiviral treatment. This hypothesis appears to be supported by the reduced level of ganciclovir sensitivity of late isolates, presumably due to the presence of resistant strain C

in combination with sensitive strains A and B, as shown by high levels of ID_{99} (95.0–188.0 μM).

The difference in DNA restriction patterns between cloned and uncloned VR 3480 was further investigated by examining by Southern blot multiple viral clones. It was found that, while with the combination pCM1075-*Hin*dIII, 4 different DNA patterns were detected, with all the other combinations, DNA patterns were entirely overlapping. Thus, it was concluded that, while strains A, B, and C were true different strains since giving variations in DNA patterns of a number of combinations, variations in clones of VR 3480 were apparently due to the presence of variants within a single major viral population. All variants showed a comparable level of resistance as compared to VR 3480 (data not shown). Whether different variants in VR 3480 were induced by ganciclovir treatment has not yet been determined.

Changes in the relative proportion of different strains present in a mixed viral isolate may have been determined by sequential passages in cell cultures. Thus, a useful correlation between the in vivo and in vitro conditions appears difficult to establish. Such a type of correlation could be better defined by performing chemosensitivity assays directly on viral populations from blood leukocytes on primary isolation. In such a case a single drug dose corresponding to peak serum levels achievable in humans (20 μM) could be used (Talenti and Smith, 1989).

Our data showing simultaneous presence of multiple HCMV strains in blood for a prolonged period of time suggest that chemosensitivity testing of uncloned HCMV isolates (particulary those recovered following long-term maintenance antiviral drug regimens) may provide misleading results in terms of drug sensitivity in these patients, when only ID_{50} is determined. In such cases, determination of ID_{90} and ID_{99} might help in predicting presence of a resistant strain.

In conclusion, we believe that the IEA-plaque-reduction assay is a valuable tool in defining sensitivity of viral isolates to antiviral drugs. In addition, in AIDS patients multiple HCMV strains may coinfect blood and in a mixed viral population sensitive strains may coexist with resistant ones. Evaluation of the efficacy of antiviral treatment must take into account this possibility.

Acknowledgements

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References

Biron, K.K., Stanat, S.C., Sorrell, J.B., Fife, J.A., Keller, P.M., Lambe, C.U. and Nelson, D.J. (1985) Metabolic activation of the nucleoside analog 9-{[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl}guanine in human diploid fibroblasts infected with human cytomegalovirus. Proc. Natl. Acad. Sci. USA 82, 2473–2477.

Chandler, S.H. and Mc Dougall, J.K. (1986) Comparison of restriction site polymorphisms among clinical isolates and laboratory strains of human cytomegalovirus. J. Gen. Virol. 67, 2179–2192.

Dankner, W.M., Scholl, D., Stanat, S.C., Martin, M., Sonke, R.L. and Spector, S.A. (1990). Rapid antiviral DNA-DNA hybridization assay for human cytomegalovirus. J. Virol. Methods 28, 293–298.

Drew, W.L., Sweet, E.S., Miner, R.C. and Mocarski, E.S. (1984) Multiple infections by cytomegalovirus in patients with acquired immunodeficiency syndrome: documentation by Southern blot hybridization. J. Infect. Dis. 150, 952–953.

Erice, A., Sunwen, C., Biron, K.K., Stanat, S.C., Balfour, H.H. and Jordan, M.C. (1989) Progressive disease due to ganciclovir-resistant cytomegalovirus in immunocompromised patients. N. Engl. J. Med. 320, 289–293.

Fleckenstein, B., Müller, L. and Collins, J. (1982) Cloning of the complete human cytomegalovirus genome in cosmids. Gene 18, 39–46.

Gerna, G., Parea, M., Percivalle, E., Zipeto, D., Silini, E., Barbarini, G. and Milanesi, G. (199Oa) Human cytomegalovirus viremia in HIV-1 seropositive patients at various clinical stages of infection. AIDS 4, 1027–1031.

Gerna, G., Revello, M.G., Percivalle, E., Zavattoni, M., Parea, M. and Battaglia, M. (1990b) Quantification of human cytomegalovirus viremia by using monoclonal antibodies to different viral proteins. J. Clin. Microbiol. 28, 2681–2688.

Gleaves, C.A., Smith, T.F., Shuster, E.A. and Pearson, G.R. (1984) Rapid detection of cytomegalovirus in MRC-5 cells inoculated with urine specimens by using low-speed centrifugation and monoclonal antibody to an early antigen. J. Clin. Microbiol. 19, 917–919.

Revello, M.G., Percivalle, E., Zavattoni, M., Parea, M., Grossi, P. and Gerna, G. (1989) Detection of human cytomegalovirus immediate-early antigen in leukocytes as a marker of viremia in immunocompromised patients. J. Med. Virol. 29, 88–93.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning. A laboratory manual, 2nd Edn, Vol. 2, p. 9.58. Cold Spring Harbor Laboratory Press, New York.

Shigeta, S., Konno, K., Baba, M., Yokota, T. and De Clercq, E. (1991) Comparative inhibitory effects of nucleoside analogues on different clinical isolates of human cytomegalovirus in vitro. J. Infect. Dis. 163, 270–275.

Spector, S.A., Hirata, K.K. and Neuman, T.R. (1984) Identification of multiple cytomegalovirus strains in homosexual men with acquired immunodeficiency syndrome. J. Infect. Dis. 150, 953–956.

Tatarowicz, W.A., Lurain, N.S. and Thompson, K.D. (1991) In situ ELISA for the evaluation of antiviral compounds effective against human cytomegalovirus. J. Virol. Methods 35, 207–215.

Talenti, A. and Smith, T.F. (1989) Screening with a shell vial assay for antiviral activity against cytomegalovirus. Diagn. Microbiol. Infect. Dis. 12, 5–8.