ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2000, p. 688–692 Vol. 44, No. 3
0066-4804/00/$04.00+0

A Standardized Plaque Reduction Assay for Determination of Drug Susceptibilities of Cytomegalovirus Clinical Isolates

MARIE L. LANDRY,[1]* SYLVIA STANAT,[2] KAREN BIRON,[2] DONALD BRAMBILLA,[3] WILLIAM BRITT,[4] JANET JOKELA,[5] SUNWEN CHOU,[6] W. LAWRENCE DREW,[7] ALEJO ERICE,[8] BRUCE GILLIAM,[9] NELL LURAIN,[10] JODY MANISCHEWITZ,[11] RICHARD MINER,[7] MOSTAFA NOKTA,[12] PATRICIA REICHELDERFER,[13] STEPHEN SPECTOR,[14] ADRIANA WEINBERG,[15] BELINDA YEN-LIEBERMAN,[16] CLYDE CRUMPACKER,[5] AND THE AIDS CLINICAL TRIALS GROUP CMV RESISTANCE WORKING GROUP

Yale University, New Haven, Connecticut[1]; Glaxo Wellcome, Research Triangle Park, North Carolina[2]; New England Research Institute, Watertown, Massachusetts[3]; University of Alabama, Birmingham, Alabama[4]; University of Oregon, Portland, Oregon[6]; University of California, San Francisco, California[7]; University of Minnesota, Minneapolis, Minnesota[8]; University of North Carolina, Chapel Hill, North Carolina[9]; Rush Medical College, Chicago, Illinois[10]; National Institutes of Health and Food and Drug Administration,[11] and Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health,[13] Bethesda, Maryland; University of Texas, Galveston, Texas[12]; University of California, San Diego, California[14]; University of Colorado, Denver, Colorado[15]; Cleveland Clinic Foundation, Cleveland, Ohio[16]; and Harvard University, Boston, Massachusetts[5]

Received 22 July 1999/Returned for modification 22 September 1999/Accepted 29 November 1999

Twelve laboratories collaborated in formulating and testing a standardized plaque reduction assay for cytomegalovirus (CMV) cell-associated clinical isolates. Four characterized and plaque-purified CMV strains, as well as six coded clinical isolates obtained after antiviral therapy, were distributed and tested. Good agreement was obtained for four of the clinical isolates, but a broad distribution of results was obtained for two isolates. Analysis of these results indicates the problems associated with clinical isolates, including the large genetic variability and the highly cell-associated phenotype. This collaborative effort, by addressing these problems, represents a significant step toward the development of a standardized assay.

Cytomegalovirus (CMV) is a major opportunistic pathogen in immunocompromised hosts. Long-term therapy with ganciclovir (GCV) and foscarnet (PFA) has been associated with development of clinical resistance and progression of disease (3, 4, 9, 10). Standardized laboratory methods to rapidly and accurately determine the susceptibility of CMV isolates to antiviral drugs are needed. The inherent difficulties in working with CMV include the slow growth of the virus and the fact that CMV clinical isolates are strongly cell associated. Multiple passages in culture are typically required to generate sufficient extracellular virus for titration and testing. Standard susceptibility assays require the inhibition of viral replication in the presence of serial concentrations of drug. The slow growth of CMV has limited the usefulness of standard assays in patient management and in guiding clinical trials. Thus, the ultimate goal is to develop a rapid method that can be used directly on clinical samples to detect resistance mutations (1, 11). As a first step toward this goal, 12 laboratories in the CMV Resistance Working Group of the AIDS Clinical Trials Group (ACTG) have collaborated in formulating and testing a standardized plaque reduction assay to use as a "gold standard." In the first phase of this process, four characterized plaque-purified CMV strains (two laboratory strains and two clinical isolates) were distributed and tested in 12 laboratories using variations of a plaque reduction assay. In the second phase, a consensus assay was used to retest these strains, as well as six additional coded clinical isolates. The inhibition curves were calculated by computer modeling using a PROC NLIN program of SAS. The results are presented in this report.

* Corresponding author. Mailing address: Department of Laboratory Medicine, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06520-8035. Phone: (203) 688-3475. Fax: (203) 688-8177. E-mail: marie.landry@yale.edu.

MATERIALS AND METHODS

Cells and drugs. In phase 1, human diploid fibroblasts from a variety of sources, commercial and in-house, were used. In phase 2, MRHF (human foreskin) cell cultures (Biowhittaker, Walkersville, Md.) were used by all of the laboratories. GCV was provided by Syntex-Roche, and PFA was provided by Astra Pharmaceuticals. The drugs were prepared from common lots by the Viral Quality Assurance Laboratory of the ACTG at Rush-Presbyterian-St. Luke's Medical Center. GCV (4.5 mM) and PFA (20 mM) stocks were filter sterilized, shipped on dry ice to participating laboratories, and kept at −70°C until they were used. At the time of overlay preparation, the drugs were thawed in a 37°C water bath with intermittent shaking to ensure complete dissolution.

Virus strains. CMV strain AD169 and a laboratory-generated GCV-resistant mutant, XbaF, with a deletion in the UL97 gene (12), were plaque purified and provided by Karen Biron and Sylvia Stanat of Burroughs Wellcome Co. (Research Triangle Park, N.C.). A GCV-sensitive pretreatment (C9208) and GCV-resistant late-treatment (C9209) pair of clinical isolates were provided by Alejo Erice (University of Minnesota) (1); both were plaque purified three times prior to distribution. In phase 2, in addition to the above CMV strains, six recent clinical isolates (5, 7) were provided by Richard Miner and W. Lawrence Drew (Mt. Zion Hospital, San Francisco, Calif.) and Alejo Erice to the ACTG Viral Quality Assurance Laboratory, where they were grown, aliquoted and shipped under code names (CMV 1 to 6) on dry ice to participating laboratories. Clinical information, responses to therapy, original susceptibility test results, and mutation analyses for the eight clinical isolates are presented in Table 1.

Virus stock preparation and titration. Cell-free virus stocks (AD169 and XbaF) were titered by serial 10-fold dilution in tissue culture plates containing human fibroblast monolayers overlaid with agarose. After incubation for 7 to 10 days, the cultures were fixed and plaques were enumerated. PFU per milliliter were determined. The other CMV strains tested were cell-associated clinical isolates. For these, the virus was quantitated by estimating the number of plaque-forming cells (PFC) present in an infected culture (11). When viral cytopathic effects (CPE) involved approximately 70 to 80% of the monolayer, the cells were trypsinized and resuspended in minimal essential medium (MEM) with 8 to 10% fetal bovine serum (FBS), and an aliquot was counted in a hemacytometer. The number of PFC was then determined by multiplying the number of cells in the suspension by the percentage estimated to be infected, as evidenced by CPE. For the consensus assay, the cell concentration was adjusted to 400 PFC/ml in 2%

TABLE 1. Clinical histories, original IC_{50}s, and mutation analyses of clinical CMV isolates tested[a]

Strain	Code name	Clinical history	Original IC_{50} for GCV (μM)[b]	Original IC_{50} for PFA (μM)[b]	UL97 mutation	POL mutation
C9208	C9208	Pretreatment bone marrow isolate from patient with CLL	1.7	170	wt	wt
C9209	C9209	Blood isolate from patient mentioned above with CLL after 18 months of repeated GCV therapy	7.0	220	V460	
F9089	CMV 1	Progression of CMV retinitis on intravenous GCV	8.5	210	V594, S595	
K8313	CMV 2	Progression of CMV retinitis on intravenous GCV	8.0	206	I460	
MR11979	CMV 3	Developed CMV retinitis after 10 months of oral GCV prophylaxis; intravenous GCV and PFA failed after 5 months of therapy	>50	870	W603	C412, M802
V15885	CMV 4	Pretreatment isolate from BAL from patient with AIDS	1.2	128.46	wt	wt
V379354	CMV 5	Pretreatment blood isolate from patient with CLL	3.42	96.54	wt	wt
V917401	CMV 6	Blood isolate from patient with CLL mentioned above after 469 days of GCV and 98 days of PFA therapy; patient had persistent viremia and colitis	43.06	179.91	V460	A522, A841

[a] POL, polymerase gene; CLL, chronic lymphocytic leukemia; wt, wild type; BAL, bronchoalveolar lavage.
[b] Hybridization assay for C9208, C9209, and CMV 4 to 6 (1, 5, 7); plaque reduction assay for CMV 1 to 3 (3).

MEM to provide an inoculum dose of 60 to 80 PFC/0.2 ml. The plates were inoculated on the same day when possible. Alternatively, the infected cell suspension was centrifuged at low speed ($250 \times g$ for 10 min) and the cell pellet was resuspended in cryoprotective medium (20% FBS–10% dimethyl sulfoxide–MEM). The cell suspensions were frozen slowly at a titer of 4×10^5 PFC/ml and stored at −80°C until use, when they were thawed quickly and diluted in MEM with 2% FBS to obtain a concentration of 400 PFC/ml.

Consensus plaque reduction assay. In phase 1, several variations of a plaque reduction assay were used (3, 4, 11). In phase 2, the consensus assay described below was used by all of the laboratories. The consensus plaque reduction assay was the result of modifications following the first phase of the study.

MRHF cells, used at population-doubling levels of 26 to 30, in 24-well plates were inoculated when the cells were just confluent. Each well was inoculated with 0.2 ml of virus suspension containing 40 to 80 PFC of cell-associated virus. For cell-associated viruses, the medium was not aspirated prior to inoculation. After adsorption for 90 min at 37°C, the medium was carefully aspirated and the wells were overlaid with 1.5 ml of 0.4% agarose containing the appropriate concentration of the antiviral drug; three wells were used per drug concentration. For the overlay, a 0.8% stock of SeaPlaque agarose (FMC Bioproducts, Rockland, Maine) was mixed with an equal volume of 2× concentrations of drug in 2× MEM with 10% FBS and antibiotics. The following final drug concentrations were used in the consensus assay: GCV, 0, 1.5, 3, 6, 12, 24, 48, and 96 μM, and PFA, 0, 25, 50, 100, 200, 400, 800, and 1,600 μM. The final concentration of the overlay was 0.4% agarose in MEM with 5% FBS. The plates were incubated for 7 days at 37°C in a 5% CO_2 incubator or until there were at least 40 plaques in the control wells. Monolayers were fixed in 10% formalin in phosphate-buffered saline and then stained with 0.8% crystal violet in 50% ethanol. The plaques were counted microscopically at low power.

Statistical methods. Resistance was assessed by calculating a 50% inhibitory concentration (IC_{50}) for each assay in each laboratory. First, plaque ratios were formed by dividing the plaque counts at the positive drug doses in an assay by the mean of the plaque counts for the three controls in the same assay. The IC_{50} for each assay was then estimated from the set of ratios by fitting the median effect equation (2): $R = 1/1 + (IC_{50}/d)m$ where d is the drug dose ($d > 0$), IC_{50} is the 50% infectious dose, and m is a parameter. Curve fitting was done by the PROC NLIN program of SAS. A few values of R were greater than 1.0, but no adjustment was made for these in the analysis. Values above 1.0 occurred mainly at the lowest doses of each drug. IC_{50}s were not calculated for assays that did not reach endpoints. An assay was considered to have failed to reach an endpoint if R was >0.50 for all replicates at all drug concentrations. In this case, the IC_{50} was defined to be greater than the maximum dose used in the assay. The minimum, median, and maximum IC_{50}s across the 12 laboratories were calculated for each strain exposed to each drug. Robust standard deviations were also calculated from the 25th and 75th percentiles (Q1 and Q3, respectively) of the IC_{50}s for each combination of strain and drug. Assuming that the IC_{50}s are normally distributed, the standard deviation is approximately (Q3 − Q1)/1.33. A standard deviation was not calculated if Q3 was greater than the maximum dose of drug in the assay.

Definition of sensitive and resistant IC_{50}s. Sensitive, indeterminate, and resistant ranges for GCV and PFA were tentatively adopted from previous work as follows: for GCV, an IC_{50} of ≤6 μM was considered sensitive, >6 to 12 μM was considered indeterminate, and >12 μM was considered resistant; for PFA, an IC_{50} of <400 μM was considered sensitive and ≥400 μM was considered resistant (3, 8, 11). There was no indeterminate range for PFA.

RESULTS

Comparison of initial and ACTG consensus plaque reduction assays using plaque-purified viruses. In phase 1, several different plaque reduction assays were used by participating laboratories. In phase 2, only the consensus assay was used. In both phases, two cell-free CMV laboratory strains (AD169 and XbaF) and two cell-associated clinical isolates (C9208 and C9209) were tested against both GCV (Table 2) and PFA (Table 3). For a valid comparison, the results were included only when laboratories submitted results for a given CMV strain in both phases 1 and 2 of the study. When more than one result was provided, only the first was used for this comparison. In some cases, indicated in the table as not available (NA), the software for estimating IC_{50} failed to converge to a solution.

(i) GCV results. For GCV, the number of laboratories successfully completing assays for the cell-free laboratory strains (AD169 and XbaF) was greater than the number completing results for the cell-associated clinical isolates (C9208 and C9209). The coefficient of variation (CV) was similar in both phases for the cell-free laboratory strains and was not consistently lower with the ACTG consensus assay for the clinical isolates (Table 2).

The majority of the laboratories successfully distinguished sensitive (AD169 and C9208) and resistant (XbaF and C9209) strains. However, some results in both phases 1 and 2 did not fall into the anticipated range. For AD169, two results in phase 1 and three in phase 2 were outside of the expected sensitive range of ≤6.0 μM. For C9208 (phase 1) and C9209 (phase 2), single outlier results significantly impacted the CV.

(ii) PFA results. The results for PFA are shown in Table 3. In phase 1, all results were in the sensitive range for PFA ($IC_{50} < 400$ μM). However, in phase 2, one of seven results for C9208 and four of seven results for C9209 were in the resistant range. Likewise, some of the CVs were heavily influenced by one or two large IC_{50}s (e.g., C9208 and C9209 in phase 2). Thus, both the IC_{50}s and the CVs were consistently higher for phase 2 than for phase 1.

An additional finding noted by the participating laboratories was that plaque counts from wells containing the antiviral drug at the lowest concentrations frequently exceeded plaque counts in control wells without the drug when the CMV strain tested was resistant to the drug tested.

Intralaboratory reproducibility of consensus assay IC_{50} results for laboratory strain AD169. Clinical isolates were gen-

TABLE 2. Interlaboratory variability in GCV IC_{50} determinations by initial (phase 1) and ACTG consensus (phase 2) plaque reduction assays using plaque-purified viruses

Laboratory	GCV IC_{50} (μM) for:							
	AD169[a]		XbaF[a]		C9208[b]		C9209[b]	
	Phase 1	Phase 2	Phase 1	Phase 2	Phase 1	Phase 2	Phase 1	Phase 2
A	5.62	3.17						
B	5.49	1.86	86.91	>96	0.66	1.22	79.39	69.09
C	9.04	3.67	25.43	>96	1.63	9.81	NA	>96
D	1.33	10.99	48.66	NA[c]				
E	2.34	0.29	14.14	9.30	22.11	1.98	>96	64.24
F	1.02	3.18	5.42	16.03	0.40	0.14	67.68	>96
G	3.87	3.87						
H	1.20	1.74	17.86	19.16			96.40	28.25
I	2.70	1.40	37.20	28.69	5.92	4.37	>100	>96
J	2.20	9.57	28.48	43.94	3.60	7.72	>96	6.79
K	9.58	6.07	29.04	26.11			NA	
Total no. of assays	11	11	9	8	6	6	6	7
Mean IC_{50}	4.04	4.16	30.56	41.90	5.72	4.21	89.25	65.19
CV[d]	75.5	81.6	81.2	83.3	145	91.6	14.4	54.7
No. (%) resistant	0 (0)	0 (0)	8 (84)	8 (100)	1 (17)	0 (0)	6 (100)	6 (86)

[a] Cell-free laboratory strains.
[b] Cell-associated clinical isolates.
[c] NA, IC_{50} could not be determined due to inconsistent results.
[d] CV determined as (standard deviation/mean) × 100%.

erally tested only once; however, six laboratories tested the control strain, AD169, from two to four times during phase 2. Thus, the reproducibility of the IC_{50} results within each of these laboratories using the ACTG consensus assay was examined for AD169 (Table 4).

(i) GCV. AD169 was tested against GCV 19 times in six laboratories. Fifteen of the 19 results were in the sensitive range; however, 3 results were in the indeterminate range and 1 was resistant. Two laboratories (A and B) reported one of three IC_{50} results above 6.0 μM (9.73 and 78.49 μM), and one laboratory (K) had two of four results slightly above the sensitive range (6.07 and 6.69 μM).

(ii) PFA. Sixteen IC_{50} results from six laboratories were available for the susceptibility of AD169 to PFA. Of these, 15 results ranged from 39 to 184 μM, within the sensitive range, but 1 result (657.9 μM) was in the resistant range. Notably, this single resistant result for PFA was from the same test run and laboratory (B) which found an IC_{50} for GCV of 78.49 μM for AD169. These extreme outliers may represent errors in drug preparation.

With the exception of laboratory B, the CVs within each laboratory for testing AD169 for the consensus assay were lower than the CVs between laboratories (81.6% for GCV and 48.3% for PFA).

TABLE 3. Interlaboratory variability in PFA IC_{50} determinations by initial (phase 1) and ACTG consensus (phase 2) plaque reduction assays using plaque-purified viruses

Laboratory	PFA IC_{50} (μM) for:							
	AD169[a]		XbaF[a]		C9208[b]		C9209[b]	
	Phase 1	Phase 2	Phase 1	Phase 2	Phase 1	Phase 2	Phase 1	Phase 2
A	112.06	86.54						
B	59.69	184.22	117.46	244.42	115.51	63.29	116.67	545.94
C	65.69	43.90	116.49	243.09	NA[c]	437.56	222.42	>1,600
D								
E	48.15	43.54	54.96	35.20	56.56	52.53	190.69	690.59
F	50.09	70.81			219.24	140.7	319.91	336.21
G	83.32	77.51						
H	53.79	118.87	64.08	87.05			69.13	120.44
I	50.99	76.74	98.30	139.49	229.83	210.65	274.31	445.97
J	35.37	73.19	30.52	119.44	110.13	197.02	150.80	200.1
K	56.51	71.28	28.93	71.49				
Total no. of assays	10	10	7	7	5	6	7	7
Mean IC_{50}	61.57	84.66	72.96	134.31	146.25	183.62	191.99	562.75
CV[d]	35.3	48.3	52.1	61.0	51.4	76.6	45.9	88.4
No. (%) resistant	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (17)	0 (0)	4 (57)

[a] Cell-free laboratory strains.
[b] Cell-associated clinical isolates.
[c] NA, IC_{50} could not be determined due to inconsistent results.
[d] CV determined as (standard deviation/mean) × 100%.

TABLE 4. Intralaboratory variation in IC_{50} for laboratory strain AD169 using the ACTG consensus assay

Laboratory	GCV				PFA			
	n^a	IC_{50} (μM)	Mean (μM)	CV[b]	n	IC_{50} (μM)	Mean (μM)	CV
A	3	4.40	5.76	60.5	3	69.66	83.80	15.5
		3.17				86.55		
		9.73				95.19		
B	3	1.86	27.9	156.8	3	184.22	338.67	81.7
		78.49				657.39		
		3.44				173.86		
C	4	3.67	3.85	20.5	2	43.90	57.95	34.3
		4.37				72.00		
		2.82						
		4.56						
F	2	3.18	2.71	24.5	2	70.81	55.15	40.2
		2.24				39.48		
I	3	1.40	3.14	48.5	3	76.74	98.42	19.3
		4.24				111.87		
		2.68				106.63		
K	4	5.63	5.39	28.5	3	71.28	85.87	31.9
		6.07				68.87		
		3.18				117.45		
		6.69						

[a] Number of assays performed in each laboratory.
[b] CV determined as (standard deviation/mean) × 100%.

Use of the ACTG consensus plaque reduction assay for testing CMV clinical isolates. In phase 2, in addition to the four plaque-purified strains tested in phase 1 (Tables 2, 3, and 4), six recent CMV clinical isolates (CMV 1 to 6) were distributed to 12 laboratories and tested using the consensus protocol. These isolates were not plaque purified.

(i) GCV. The distribution of results for the GCV IC_{50} obtained by participating laboratories using the consensus protocol is presented in Table 5. The results for clinical isolates CMV 1 to 6 showed strong agreement in detecting the two highly resistant isolates, CMV 3 and CMV 6. For two isolates, CMV 2 and 5, there was consensus for an IC_{50} of <6.0. However, for two isolates, CMV 1 and CMV 4, there was a broad distribution of results.

(ii) PFA. For PFA (Table 6), all isolates tested had at least one assay result in the resistant range (>400 μM). However, only CMV 3 had a majority of test results in the resistant range. CMV 3 was obtained from a patient for whom PFA and GCV therapy for CMV retinitis had failed and who had mutations in both the UL97 and polymerase genes (Table 1). CMV 6 was also isolated from a patient who failed to respond to PFA, yet only 3 of 11 test results were in the resistant range. In fact, the mutations identified in the polymerase gene for CMV 6 have not been shown to confer PFA resistance.

TABLE 5. Frequency distributions of GCV IC_{50}s for recent clinical isolates using the ACTG consensus protocol

GCV IC_{50} (μM)	No. of assays with IC_{50} at each GCV concn					
	CMV 1[a]	CMV 2	CMV 3	CMV 4	CMV 5	CMV 6
≤1.5		2		1	2	
>1.5–3.0		3		2		
>3.0–6.0	2	2	1	1	7	
>6.0–12	2	2		2		
>12–24	2			2	2	
>24–48	2			2	1	1
>48–96	4		1	1		3
>96		1	10	1		8
Total no. of assays	12	10[b]	12	12	12	12
Median IC_{50}	22.94	2.97	>96	11.31	4.13	>96
No. (%) resistant	8 (67)	1 (10)	11 (92)	6 (50)	3 (25)	12 (100)

[a] CMV 1 to 6 were not plaque purified.
[b] Data not submitted by two laboratories.

TABLE 6. Frequency distributions of PFA IC_{50}s for recent clinical isolates using the ACTG consensus protocol

PFA IC_{50} (μM)	No. of assays with IC_{50} at each PFA concn					
	CMV 1[a]	CMV 2	CMV 3	CMV 4	CMV 5	CMV 6
<25				2		
>25–50	1	2			1	
>50–100	1	1		1	3	
>100–200	2	2	1	4	6	3
>200–400	4	4	2	4	1	5
>400–800	2	1	2			1
>800–1,600			2		1	2
>1,600			4	1		
Total no. of assays	10[b]	10[b]	11[c]	12	12	11[c]
Median IC_{50}	227.65	200.14	855.85	184.84	117.19	325.18
No. (%) resistant	2 (20)	1 (10)	8 (73)	1 (8)	1 (8)	3 (27)

[a] CMV 1 to 6 were not plaque purified.
[b] Data not submitted by two laboratories.
[c] IC_{50} could not be determined for one data set due to inconsistent results.

Impact of challenge dose on consensus assay IC_{50} results. To assess the impact of the challenge dose on the results, the reproducibility and accuracy of the challenge dose was examined for each of the six clinical isolates, CMV 1 to 6, for each of the assays shown in Tables 5 and 6. The target of a mean of 40 to 80 plaques per well was achieved in 86 of 138 assays (62%). The range of mean challenge doses was 22 to 233 plaques per well. Only 2 of 12 laboratories had challenge doses for all 12 assays within the range; 5 of 12 laboratories had 80% or more of the assays in the range. The coefficient of variation among triplicate wells within an assay ranged from 1 to 25.3%; however, the CV for 80% of the assays was <15%.

No correlation was found between high or low challenge dose and a greater incidence of sensitive or resistant IC_{50} results compared with challenge doses in the target range (data not shown).

DISCUSSION

Plaque reduction has been considered the gold standard for antiviral susceptibility testing (13) and is used successfully for rapidly growing, cell-free clinical isolates of herpes simplex virus. CMV presents a more difficult problem because isolates are slow growing and cell associated. Furthermore, plaques are more difficult to discern in the presence of drugs.

The work presented here represents the first attempt at developing a consensus plaque reduction assay for CMV drug susceptibility testing. Such an assay is essential for multicenter trials and to bring standardization to the field.

What further distinguishes this work is our attempt to do a plaque reduction assay using cell-associated virus, based on the method first reported by Stanat et al. (11). Standard CMV plaque reduction assays described in the past have required multiple serial passages of a clinical isolate in cell culture to produce sufficient cell-free virus, followed by virus titration to determine the challenge dose before the actual susceptibility testing is performed. In the assay presented here, the virus challenge dose consists of PFC, estimated by microscopic assessment of the CPE, thus avoiding serial passage and titration.

Such an assay can save weeks to months and provide results in a more clinically relevant time frame.

In general, strong agreement among laboratories was demonstrated for laboratory strains AD169 and XbaF and for the plaque-purified clinical isolates C9208 and C9209. However, plaque purification is a lengthy and time-consuming process and does not give an accurate representation of the population of viruses in the patient. Clinical isolates from patients obtained after antiviral treatment frequently consist of a mixture of viruses with different susceptibilities (6). This may in part explain the variability in results obtained for CMV strains 1 to 6; these strains were not plaque purified prior to distribution and were further passaged in each laboratory, which may have led to some divergence of resistance properties. However, CMV strains 4 and 5 were pretreatment isolates and were wild-type viruses by mutation analysis; nevertheless, 25 to 50% of the laboratories found them resistant to GCV. In contrast, both CMV strains 3 and 6 were obtained from patients for whom PFA therapy failed, and both had polymerase mutations; however, only CMV strain 3 was found to be PFA resistant by a majority of participating laboratories, using an IC_{50} of >400 μM to define resistance. The finding of PFA resistance for C9209 in phase two by four of seven laboratories also remained unexplained; there was no history of PFA therapy, no polymerase mutations were detected, and all seven laboratories reported results in the sensitive range in phase 1.

The occurrence of outliers, which could sometimes be quite extreme, was of concern. The frequency of outlier results was greater for some laboratories but did not appear to be related to a laboratory's prior experience with plaque reduction assays. It should be noted, however, that even laboratories that were experienced in plaque reduction were not experienced with the consensus assay. Outlier results also did not correlate with virus inocula that were higher or lower than the target of 40 to 80 plaques per well. Furthermore, outlier results for AD169 did not correlate with outlier results in concurrently tested clinical isolates.

The finding of increased plaque counts in wells with low doses of antiviral drugs was noted especially with resistant CMV strains. The reasons for this phenomenon are not known but may include a stimulatory effect on host cells or the virus or a simple measurement error.

The difficulty many of the laboratories experienced in achieving a challenge dose in the target range reflects the difficulty of determining a challenge dose by visual assessment of the CPE (i.e., PFC). This would most certainly improve with experience. The reproducibility of plaque counts among triplicate wells of an assay, however, was very good and comparable to that achieved with cell-free virus (13).

This collaborative effort represents a first step and highlights a number of issues for study in the future. Many previous publications on CMV susceptibility testing have focused on cell-free virus, on laboratory-passaged virus, or on sensitive virus strains. We have established a consensus assay, which is already in use for testing clinical isolates obtained from clinical trials. Improvements in the assay currently under evaluation include a reduction in the number of drug concentrations tested, the use of an overlay medium without agarose, direct inoculation of CMV-infected cells into overlay medium without adsorption, and counting plaques microscopically in unfixed monolayers.

Of note, some clinical isolates may exhibit variability in antiviral susceptibility results, and these isolates may represent mixed populations. Further testing of these isolates by other methods, such as nucleic acid sequence analysis, may be necessary to explain this variability.

In contrast to the ACTG assay for antiviral susceptibility for human immunodeficiency virus type 1 in peripheral blood mononuclear cells, the ACTG standardized consensus methodology for CMV presented in this report did not result in a significant decrease in the CV. It is clear from this study that any laboratory wanting to establish a CMV plaque reduction assay for cell-associated CMV isolates will need to devote substantial effort over an extended period of time to developing and documenting the reproducibility of results. Establishing sharply delineated boundaries between sensitive and resistant strains may be difficult. Rather, testing paired pre- and posttreatment isolates to detect trends toward resistance may give more meaningful results.

ACKNOWLEDGMENTS

This study was supported in part by National Institutes of Health grants AI41690, AI38858, and AI32766 and contract NO-AI35172.

The AIDS Clinical Trials Group CMV Resistance Working Group also included David Ferguson, Yale University; Sarah Martin-Munley, Astra Pharmaceuticals; Michael Polis, National Institutes of Health; Richard Pollard, University of Texas; and Sharon Safrin, University of California—San Francisco.

REFERENCES

1. **Chou, S., A. Erice, M. C. Jordan, G. M. Vercellotti, K. R. Michels, C. L. Talarico, S. C. Stanat, and K. K. Biron.** 1995. Analysis of the UL97 phosphotransferase coding sequence in clinical cytomegalovirus isolates and identification of mutations conferring ganciclovir resistance. J. Infect. Dis. **171:**576–583.
2. **Chou, T. C.** 1991. The median-effect principle and the combination index for quantitation of synergism and antagonism, p. 61–102. *In* D. C. Rideout and T. C. Chou (ed.), Synergism and antagonism in chemotherapy. Academic Press, San Diego, Calif.
3. **Drew, W. L., R. C. Miner, D. F. Busch, S. E. Follansbee, J. Gullett, S. G. Mehalko, S. M. Gordon, W. F. Owen, Jr., T. R. Matthews, W. C. Buhles, and B. DeArmond.** 1991. Prevalence of resistance in patients receiving ganciclovir for serious cytomegalovirus infection. J. Infect. Dis. **163:**716–719.
4. **Erice, A., S. Chou, K. K. Biron, S. C. Stanat, H. H. Balfour, Jr., and M. C. Jordan.** 1989. Progressive disease due to ganciclovir resistant cytomegalovirus in immunocompromised patients. N. Engl. J. Med. **320:**289–293.
5. **Erice, A., C. Gil-Roda, J.-L. Perez, H. H. Balfour, Jr., K. J. Sannerud, M. N. Hanson, G. Boivin, and S. Chou.** 1997. Antiviral susceptibilities and analysis of UL97 and DNA polymerase sequences of clinical cytomegalovirus isolates from immunocompromised patients. J. Infect. Dis. **175:**1087–1092.
6. **Gerna, G., F. Baldanti, M. Zavattoni, A. Sarasini, E. Percivalle, and M. G. Revello.** 1992. Monitoring of ganciclovir sensitivity of multiple human cytomegalovirus strains coinfecting blood of an AIDS patient by an immediate-early antigen plaque assay. Antiviral Res. **19:**333–345.
7. **Hanson, M. N., L. C. Preheim, S. Chou, C. L. Talrico, K. K. Biron, and A. Erice.** 1995. Novel mutation in the UL97 gene of a clinical cytomegalovirus strain conferring resistance to ganciclovir. Antimicrob. Agents Chemother. **39:**1204–1205.
8. **Jacobson, M. A., W. L. Drew, J. Feinberg, J. J. O'Donnell, P. V. Whitmore, R. D. Miner, and D. Parenti.** 1991. Foscarnet therapy for ganciclovir-resistant cytomegalovirus retinitis in patients with AIDS. J. Infect. Dis. **163:**1348–1351.
9. **Knox, K. K., W. R. Drobyski, and D. R. Carrigan.** 1991. Cytomegalovirus isolate resistant to ganciclovir and foscarnet from a marrow transplant patient. Lancet **337:**1292–1293.
10. **Leport, C., S. Puget, J. M. Pepin, S. Levy, C. Perronne, F. Brun-Vezinet, and J. L. Vilde.** 1993. Cytomegalovirus resistant to foscarnet: clinicovirologic correlation in a patient with human immunodeficiency virus. J. Infect. Dis. **168:**1329–1330.
11. **Stanat, S. C., J. E. Reardon, A. Erice, M. C. Jordan, W. L. Drew, and K. K. Biron.** 1991. Ganciclovir-resistant cytomegalovirus clinical isolates: mode of resistance to ganciclovir. Antimicrob. Agents Chemother. **35:**2191–2197.
12. **Sullivan, V., C. L. Talarico, S. C. Stanat, M. Davis, D. M. Coen, and K. K. Biron.** 1992. A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells. Nature **358:**162–164.
13. **Wentworth, B. B., and L. French.** 1970. Plaque assay of cytomegalovirus strains of human origin. Proc. Soc. Exp. Biol. Med. **135:**253–258.