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Flow Cytometric Analysis of Herpes Simplex Virus Type 1 Susceptibility to Acyclovir, Ganciclovir, and Foscarnet†

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We established a quantitative flow cytometric method for determination of herpes simplex virus type 1 (HSV-1) susceptibility to acyclovir (ACV), ganciclovir, and foscarnet in vitro. Susceptibility was defined in terms of the drug concentration which reduced the number of cells expressing HSV-1 glycoprotein C (gpC) with a fluorescence intensity of $\geq 10^2$ by 50% (IC_{50}). Flow cytometry allowed us to use a high (1.0) as well as a low (0.005) multiplicity of infection, and determination of the IC_{50} was possible after one or more viral replicative cycles. IC_{50}s were dependent on virus input and on time postinfection. In mixture experiments, 1 to 2% resistant viruses added to a sensitive strain could be detected. The results obtained by flow cytometry showed a good qualitative correlation with those achieved by cytopathic effect inhibitory assay. However, flow cytometry might detect more quantitative differences in drug susceptibility, especially among resistant strains, as confirmed also by determination of intracellular drug phosphorylation. The mean IC_{50}s for ACV-sensitive strains were 0.45 to 1.47 μM, and those for ACV-resistant strains were between 140 and 3,134 μM. Flow cytometric analysis was fast and accurate, automatizable, and highly reproducible. Flow cytometry may be a more powerful tool than standard cytopathic effect-based assays and could have advantages for the detection of low levels of drug resistance or mixtures of sensitive and resistant virus strains.

For many years, herpes simplex virus (HSV) diseases have been successfully treated with acyclovir (ACV). After long-term treatment of immunocompromised patients with ACV, the emergence of drug-resistant virus variants has been observed, and there has been an increasing number of ACV treatment failures which were associated with ACV-resistant viruses in these patients (3). The initial phosphorylation of virus-selective nucleoside analogs to the monophosphate is done by the HSV-encoded thymidine kinase (TK). Cellular enzymes convert the nucleoside analog monophosphate to its triphosphate, which is a potent inhibitor of the HSV-encoded DNA polymerase. In contrast to the nucleoside analogs, foscarnet (PFA) inhibits viral DNA polymerase directly without a requirement for previous activation.

Concerning HSV, the crucial question whether the susceptibility to a drug in vitro predicts the success or failure of clinical therapy was answered by Safrin et al. (16), whose study strengthened the notion that HSV drug resistance in vitro is indeed clinically relevant, thereby emphasizing the importance of the determination of antiviral resistance. The procedures used for the testing of viral susceptibility include plaque reduction assay (PRA) (12, 20), dye uptake assay (8, 9), virus yield reduction assay (1, 13, 17), enzyme-linked immunosorbent assay (14, 19), and nucleic acid hybridization (4, 5). An assay should be rapid, quantitative, highly standardized, easy to perform, and inexpensive and should have no limitations in virus input. From the therapeutic standpoint, it would be very desirable to detect evolving resistance in a patient instead of detecting the already-established resistant virus strain. Furthermore, the mechanisms and kinetics of selection of resistant virus populations in vivo are not fully understood.

The widely used standard biological tests are limited by the fact that high multiplicities of infection (MOI) (>0.1 PFU per cell) cannot be used, which does not allow analysis of all virus subpopulations present in an isolate. In addition, several cycles of virus replication are needed in vitro. Flow cytometry has been extensively used for basic, clinical, and applied research (for a review, see reference 10). This technique has already been used to study the effects of antiviral drugs on HSV infection. One study examined the effectiveness of ACV, phosphonoacetic acid, and a xanthate derivative by determining the effect of infection in cell cultures on the binding of propidium iodide to cellular DNA (15), and another monitored the effect of ACV and interferons, using polyclonal antibodies for detection of viral antigens in infected cells (18).

We established and evaluated for the first time a quantitative flow cytometric method for testing of HSV type 1 (HSV-1) susceptibility to different antiviral drugs, based on the measurement of HSV-1 glycoprotein C (gpC) expression in infected cells.

MATERIALS AND METHODS

Viruses and cells. J. P. Kruppenbacher (Universität zu Köln, Cologne, Germany) kindly provided the ACV-sensitive HSV-1 laboratory strain K1. The ACV-resistant K1r strain was selected out of the sensitive strain K1 after 10 passages in the presence of increasing concentrations of ACV (from 6 to 100 μM) in vitro. The clinical HSV-1 strains were isolated from immunocompromised patients (AIDS and bone marrow transplant patients).

For all experiments except the cytopathic effect (CPE) inhibitory assay, human foreskin fibroblasts (HFF) between passages 11 and 20 were used. HFF were grown in minimal essential medium (MEM) (Gibco, Eggenstein, Germany) supplemented with 2 mM glutamine, 0.1 mg of streptomycin per ml, 0.1 mg of penicillin per ml, 1% nonessential amino acids (Seromed, Berlin, Germany), and 10% fetal calf serum (FCS) (Gibco). Vero cells obtained from American Type Culture Collection were grown in Dulbecco's modified Eagle medium (Gibco) supplemented with glutamine, antibiotics, and FCS as described above for MEM and used for the CPE inhibitory assay.

143B TK-deficient cells were grown as described for HFF and infected for analysis of intracellular nucleoside anabolism.

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† T.M. dedicates this work to his teacher Professor Hans J. Eggers on the occasion of his 70th birthday.

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Antiviral drugs. ACV (Zovirax) was supplied by Wellcome, Burgwedel, Germany. Ganciclovir (GCV) was purchased as Cymeven (Syntex, Aachen, Germany), and PFA was purchased as Foscavir (Astra Chemical, Wedel/Holstein, Germany). Stock solutions prepared according to the manufacturers' instructions were stored at −20°C before being used. Further dilutions on the day of use were done with complete MEM.

Antibodies. One human immune serum against HSV-1 was used as the primary antibody, and peroxidase-conjugated $F(ab')_2$ fragments of goat anti human immunoglobulin G (Dianova, Hamburg, Germany) were used as the secondary antibody for determination of virus titers. Fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibody (MicroTrak; Syva Company, Palo Alto, Calif.) to HSV-1 gpC was used for flow cytometric analysis of viral drug susceptibility and for virus typing. As a negative control, we used FITC-conjugated mouse immunoglobulin G (Immunotech, Hamburg, Germany).

Peroxidase staining for determination of virus titer. HFF monolayers in 96-well plates were overlaid with 100 μl of virus dilutions (dilutions from 10^{-1} to 10^{-8}). After adsorption for 60 min at 37°C and 5% CO_2, the medium was removed and replaced with 100 μl of fresh complete MEM. After overnight incubation (18 to 20 h), peroxidase staining was performed. After fixation of cells with methanol for 15 min at −20°C, the monolayers were incubated for 15 min with phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (this PBS was also used for dilution of antibodies) at 37°C and 5% CO_2 (all further incubations were done under these conditions). Immune serum against HSV-1 (dilution of 1:20) was added. After 45 min of incubation, the monolayers were washed with PBS three times and secondary antibodies were added. After 45 min, the monolayers were washed three times with PBS and substrate (3-amino-9-ethylcarbazole) (Sigma, Deisenhofen, Germany) was added. Plaques were counted under a light microscope.

Standard flow cytometric assay for testing of susceptibility of HSV-1 strains to antiviral drugs. Standard infection of HFF was performed in six-well plates by mixing 1 ml of complete MEM containing 4×10^5 cells with the same amount of HSV-1 PFU in 1 ml of MEM (MOI of 1). Two milliliters of antiviral drug dilutions (final concentration, 0.20 to 3,200 μM) was added to each well. Two wells were left drug free to allow maximal expression of HSV-1 gpC. Unless stated otherwise, the infection was stopped after 18 h (approximately one HSV replicative cycle), and fibroblasts were removed from the plates by mild trypsin treatment. Cells from two wells containing the same drug concentration were mixed together. After centrifugation, the fibroblasts were resuspended in fluorescence-activated cell sorter (FACS) buffer (PBS, 2% FCS, 0.1% sodium azide) and transferred to two Eppendorf tubes. The cells were fixed by incubation with 2% paraformaldehyde for 10 min at 4°C (all incubations and washing procedures with FACS buffer were done at 4°C). The cells were then washed twice and permeabilized with 0.1% saponin (Sigma) in FACS buffer for 30 min (cells were maintained in saponin throughout the staining procedure). After that, 2 to 6 μl (depending on the MOI) of FITC-conjugated antibody directed to gpC of HSV-1 was added (for the control, 1.5 μl of FITC-conjugated mouse immunoglobulin was added). After 30 min, the cells were washed twice and resuspended in 0.5 ml of FACS buffer. A total of 1.5×10^4 cells, or 1.5×10^5 cells after infection with a low MOI, were analyzed with a FACScan (Becton Dickinson) using CELLQuest software, version 1.1.1. Susceptibility to antiviral drugs was determined by calculation of the concentration of drug (micromolar) which reduced by 50% (IC_{50}) the number of infected cells (area under the curve [AUC]) expressing gpC with a fluorescence intensity (FI) of $\geq 10^2$. The IC_{50}s were calculated graphically by linear regression analysis (Charisma 4.0; Micrografx Inc.).

CPE inhibitory assay. The quantitative microtiter CPE reduction assay was performed as described by Kruppenbacher et al. (7). Briefly, 50 μl of drug solution (serial twofold dilutions to reach the final concentration), a constant amount of virus in a 50-μl volume (MOI, ~0.001), and 100 μl of Vero cell suspension (5×10^4/well) were distributed into 96-well plates. Development of CPE was monitored by light microscopy for 3 to 4 days and was recorded semiquantitatively. The sensitivity was expressed as an IC_{50}, which is the concentration of the drug (micromolar) reducing the viral CPE by 50%.

HPLC analysis of nucleoside anabolism in HSV-1-infected cells. [8-^{3}H]GCV (12.4 Ci/mmol) and [8-^{3}H]ACV (12.7 Ci/mmol) (Moravek; Brea) were used for analysis of nucleoside anabolism as described previously (11). 143B cells were infected at an MOI of 1, and 1 h postinfection, 5 μCi of high-performance liquid chromatography (HPLC)-purified radioactively labeled compound was added at a specific radioactivity of 100 mCi/mmol. After 20 h of incubation, cells were harvested and washed three times with PBS. They were resuspended in PBS and extracted with 0.5 M perchloric acid. The extracts were neutralized to pH 6.8 with 2.5 M KOH in 1.5 M KH_2PO_4 and centrifuged for 5 min at 14,000 × *g*, and the supernatants were used for HPLC. HPLC was performed on a Sephasil C_{18} reversed-phase column (250 by 4.6 mm; Pharmacia, Uppsala, Sweden) with 20 mM KH_2PO_4 (pH 6.0)–7.5% methanol as the mobile phase, using isocratic elution at a flow rate of 1.0 ml/min. Fractions containing the nucleoside phosphates (mono-, di-, and triphosphates) and the unphosphorylated compounds were collected according to their retention times as determined by external-standard runs. The radioactivity of each fraction was determined by liquid scintillation counting.

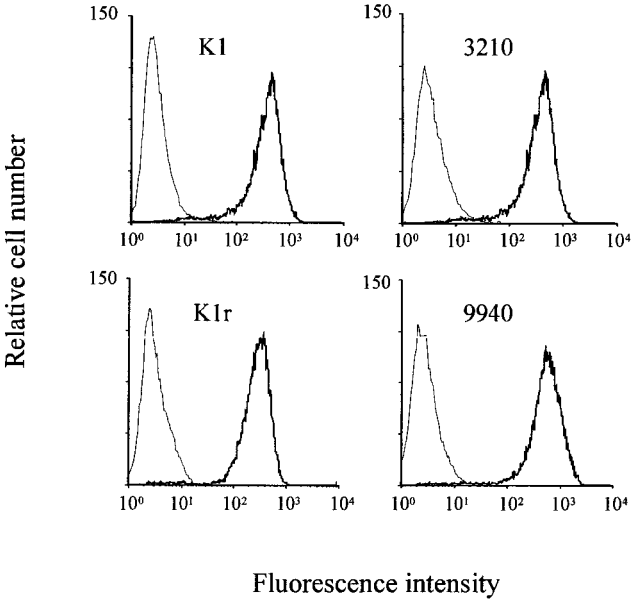

FIG. 1. Pattern of HSV-1 gpC expression (without drug). Results are shown for the negative control (infected cells treated with an appropriate control antibody) (thin lines) and infected cells stained with a monoclonal antibody to HSV-1 gpC (thick lines). Here we show histograms for two ACV-sensitive strains (K1 and 3210) and two ACV-resistant strains (K1r and 9940), all with the same pattern of gpC expression in HFF with an FI of $\geq 10^2$ 18 h postinfection at an MOI of 1.

RESULTS

Kinetics of HSV-1 gpC expression in HFF after infection. In order to define the optimum time postinfection for flow cytometric determination of viral drug susceptibility, we first determined the kinetics of HSV-1 gpC expression. HFF were infected with two HSV-1 laboratory strains at an MOI of 1, one of which was sensitive (K1) and the other of which was resistant (K1r) to ACV. The expression of viral gpC was monitored 18, 36, and 54 h postinfection. Since both laboratory strains showed a maximum gpC expression with an FI of $\geq 10^2$ already 18 h postinfection, we included 18 h postinfection in our standard protocol. Eleven HSV-1 strains (two laboratory strains and nine clinical isolates) were included, all of which showed identical patterns of gpC expression (Fig. 1).

Influence of antiviral drugs on gpC expression. In order to standardize our flow cytometric assay, we extensively studied the effect of MOI and the modulation of gpC expression by the different drugs. Experiments were done using a high MOI (1.0) and a low MOI (0.005), and accordingly we analyzed different numbers of cells (MOI of 1, 1.5×10^4 cells; MOI of 0.005, 1.5×10^5 cells). Results of an experiment using the sensitive laboratory strain K1 at an MOI of 1 are shown in Fig. 2A. As can be seen, with increasing drug concentrations, the number of cells expressing gpC with an FI of $\geq 10^2$ became smaller and the majority of cells shifted to the left in the range of an FI of $<10^2$. For standardized calculation of the IC_{50}, the AUC with an FI of $\geq 10^2$ measured for each drug concentration was overlaid and subtracted from the AUC of the untreated control, which represented 100% gpC expression. The differences were plotted against the drug concentration used (Fig. 3). As mentioned above, when using a very low MOI (0.005), we obviously had to increase the number of cells analyzed after single-cycle infection experiments (18 h) to find enough in-

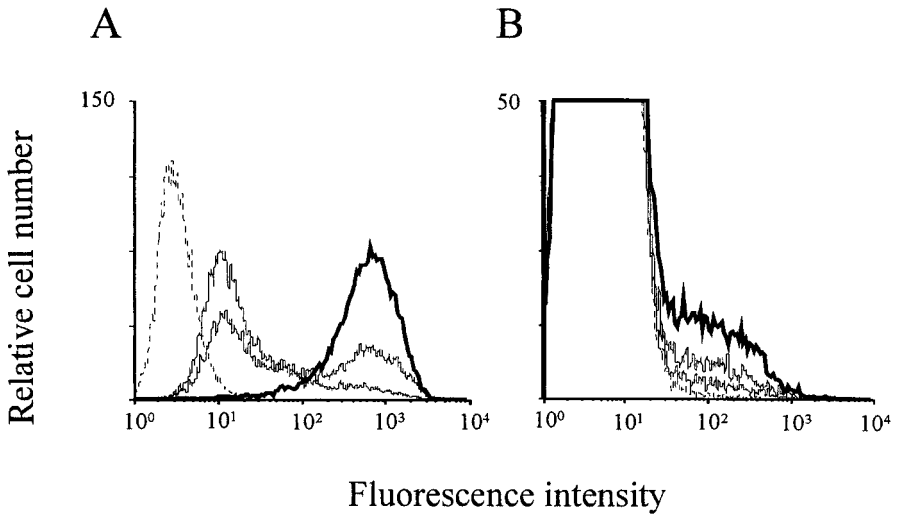

FIG. 2. Influence of ACV on HSV-1 gpC expression. HFF were infected with strain K1 at an MOI of 1 (A) or 0.005 (B), and expression of gpC was determined 18 h postinfection by flow cytometry with 1.5×10^4 (A) or 1.5×10^5 (B) cells. Maximum gpC expression with an FI of $\geq 10^2$ by infected HFF not treated with drug is shown (thick lines), and the histograms of intermediate gpC expression represent effects of ACV at 0.39 (upper thin line under thick line) and 0.78 (lower thin line under thick line) μM. With increasing drug concentrations, more and more cells are shifted from an FI of $\geq 10^2$ to an FI of $\leq 10^2$. Results for negative controls are also shown (dotted lines).

fected cells by FACS analysis. As is shown in Fig. 2B, the peak of the uninfected cells (FI between 10^0 and $10^{1.2}$) became very large, and the clear second peak of infected cells seen at an MOI of 1 became a shoulder on the first peak, but it was nevertheless possible to measure the number of cells expressing gpC with an FI of $\geq 10^2$ and the dose-dependent reduction by antiviral drugs. The histograms for all nucleoside analogs and for PFA were essentially the same also when we used different virus isolates. One advantage of the method was that the number of infected cells was determined exactly and automatically in each experiment, allowing calculation of the actual MOI. When an MOI of 0.005 was used, the infected cells determined by FACS analysis were between 0.3 and 1.0%. Summarizing the data presented, the flow cytometric analysis enabled us to test antiviral activity of substances in a single-cycle infection using viral MOI of 0.005 and 1. An MOI of 1 gave a clear separation between infected and uninfected cells, and we incorporated this MOI into our standard procedure for susceptibility determinations.

Drug susceptibilities of the viral strains studied. We chose six ACV-sensitive and five ACV-resistant HSV-1 strains which had been characterized by CPE inhibitory assay. All isolates were tested for susceptibility to ACV, GCV, and PFA by flow cytometry under our standard conditions (18 h postinfection;

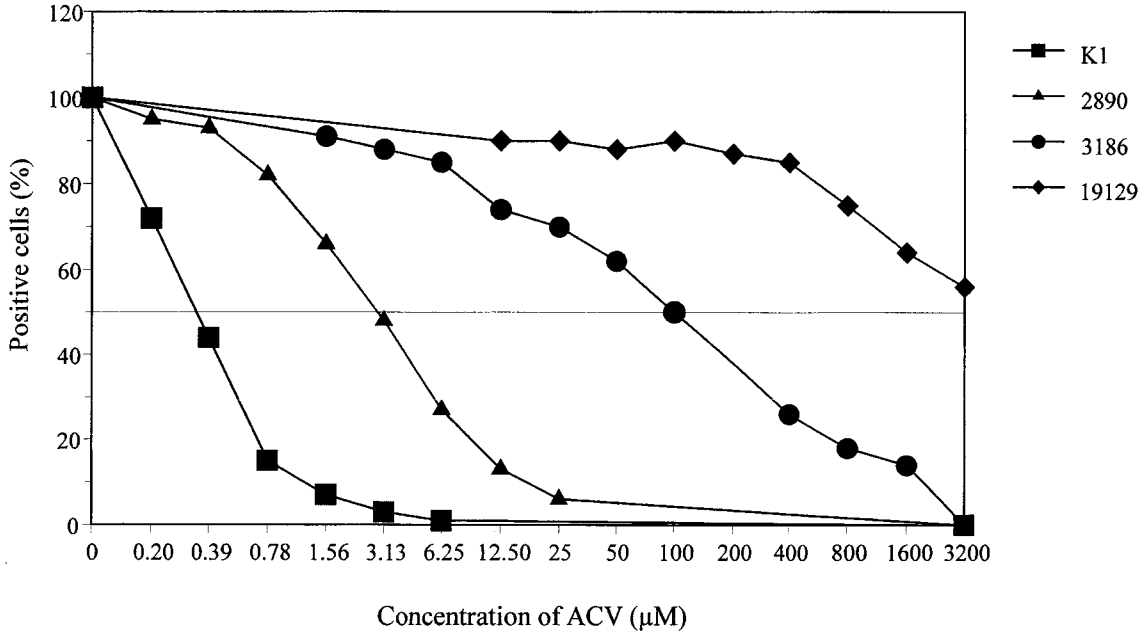

FIG. 3. Susceptibility of HSV-1 strains to ACV as determined by flow cytometry. HFF were infected at an MOI of 1 and assayed 18 h postinfection. Curves were obtained from one experiment using sensitive (K1 and 2890) and resistant (3186 and 19129) virus strains.

TABLE 1. Susceptibilities of all tested HSV-1 strains to nucleoside analogs and PFA determined by flow cytometry and by CPE inhibitory assay

HSV-1 strain	IC_{50} (μM) of[a]:					
	ACV		GCV		PFA	
	FC	CPE	FC	CPE	FC	CPE
K1	0.45 ± 0.13	0.37	0.23 ± 0.08	0.20	213 ± 11	65
3210	1.17 ± 0.68	0.36	0.36 ± 0.07	0.29	220 ± 30	ND[b]
2890	1.47 ± 0.25	0.32	1.28 ± 0.28	0.44	296 ± 50	ND
15882	1.09 ± 0.45	0.51	0.98 ± 0.18	ND	243 ± 11	ND
23237	1.37 ± 0.18	0.55	0.77 ± 0.15	ND	270 ± 26	ND
1439	1.12 ± 0.23	0.26	0.55 ± 0.18	ND	220 ± 26	ND
K1r	2,233 ± 991	320	467 ± 135	80	423 ± 48	60
19129	3,134 ± 116	107	2,100 ± 819	33	247 ± 134	29
3186	140 ± 53	77	3.37 ± 1.11	38	190 ± 36	160
9940	700 ± 229	102	120 ± 25	65	310 ± 78	27
8411	500 ± 123	162	202 ± 28	>100	320 ± 26	45

[a] IC_{50}s (means ± standard deviations [SD]) obtained 18 h postinfection at a MOI of 1 by flow cytometric analysis (FC) of at least three separate experiments or by standard CPE inhibitory assay.
[b] ND, not done.

MOI of 1). The mean IC_{50}s for the six ACV-sensitive strains were between 0.45 and 1.47 μM, and those for the five ACV-resistant strains were between 140 and 3,134 μM (Fig. 3 and Table 1).

The IC_{50}s of GCV for the ACV-sensitive strains were similar (mean IC_{50}s from 0.23 to 1.28 μM).

ACV-resistant strains were cross-resistant to GCV. Clinical isolate 3186, showing the lowest resistance to ACV and GCV as determined by cytometric assay, with values far lower than were seen for the other four resistant isolates, raised the question of an intermediate susceptibility. The mean GCV IC_{50} of 3.37 μM for this isolate was approximately five times higher than that for the sensitive strains tested. A standard CPE inhibitory assay had identified this isolate as resistant to GCV, and control experiments confirmed this result.

All strains sensitive to the nucleoside analogs were sensitive to PFA (mean IC_{50}s from 213 to 296 μM). PFA IC_{50}s for the resistant strains (mean IC_{50}s from 190 to 423 μM) were similar to those for the sensitive strains.

Flow cytometric results were compared with those achieved earlier by standard CPE inhibitory assay for diagnostic purposes. As shown in Table 1, the two methods gave identical qualitative results, and there is a good quantitative correlation between the two methods for the sensitive strains. The IC_{50}s for our resistant prototype strain K1r as well as strain 19129 were quantitatively much higher than those for the other three resistant strains. This difference was not detected by our standard biological assay. Summarizing these data, the range of IC_{50}s detected for ACV-resistant strains by flow cytometry (140 to 3,134 μM) was much wider than that detected by CPE inhibitory assay for the same isolates (77 to 320 μM).

Effect of MOI on determination of IC_{50}s for resistant strains by flow cytometry. One of our hypotheses was that by using higher virus concentrations, i.e., a more representative fraction of the respective virus strain in a test system, it might be possible to get more-differentiated information about drug sensitivity or resistance. The possibility of increasing the virus input in the biological assay is limited, but with flow cytometry this can be done without problems. We therefore wanted to clarify whether the difference in IC_{50}s observed when flow cytometric analysis and the CPE inhibitory assay were compared could be due to differences in virus input. HFF were infected at an MOI of 0.005 and 1, and the effects of ACV and PFA were measured 18 h postinfection (Table 2). In fact, with an MOI of 0.005, the results obtained by flow cytometry for both ACV and PFA were five to seven times lower than the results obtained with an MOI of 1. HSV-1 strain 19129 still proved to be more resistant than the other two strains. These results indicated that a dependence of the IC_{50}s on MOI which was more pronounced with resistant strains (Table 2) than with sensitive strains (Table 3, 18 h postinfection) could be detected after single-cycle infection.

Increase of IC_{50} for ACV-sensitive strains with a low MOI and prolonged incubation time. Up to this point, all tests were done with short infections. We now wanted to analyze whether multiple replication cycles in the presence of drug could change the IC_{50}. HFF were infected at an MOI of 0.005, and ACV IC_{50}s for sensitive strains were determined 18, 36, and 54 h postinfection (approximately after one, two, and three viral replicative cycles, respectively). Values obtained 54 h postinfection were 4 to 10 times higher than IC_{50}s determined 18 h postinfection (Table 3). Interestingly, strain 15882, which exhibited the largest increase in IC_{50} 54 h postinfection, was obtained from a bone marrow transplant patient receiving ACV therapy and from whom strain 19129, which showed the greatest resistance of all isolates tested, was isolated 82 days later. As control experiments, the same virus strains were tested under the same conditions but with an MOI of 1 (Table 3). Essentially no change of IC_{50}s (all values were within a twofold increase) was observed. Summarizing these data, we were able to demonstrate that the IC_{50}s were also dependent on the number of replicative cycles during drug treatment when a low MOI was used.

A small percentage of resistant viruses is capable of altering the susceptibility of a sensitive-strain population. Supposing that the effect of more replicative cycles under drug pressure could lead to selection of resistant viruses preexisting in the total virus population, we wanted to determine the sensitivity of our test system for detection of resistant virus added in small amounts. For this purpose, we infected HFF at an MOI of 0.01 with a mixture containing ACV-sensitive strain 15882 to which we added 2% or 5% ACV-resistant strain 19129. As shown in Fig. 4, addition of 2 and 5% resistant viruses to the sensitive HSV-1 strain was followed by a three- to fivefold increase in the IC_{50}. In other experiments, we were able to show that even 1% added resistant virus could be detected (data not shown).

Intracellular phosphorylation of antiviral compounds. The major cause of HSV resistance to nucleoside analogs is alterations in viral TK resulting in an impaired phosphorylation of these compounds. In a last series of experiments, we wanted to analyze whether the phosphorylation capacity of some of the strains used was related to the broader range of IC_{50}s deter-

TABLE 2. Effect of MOI on IC_{50}s for resistant HSV-1 strains

HSV-1 strain	IC_{50} (μM) of:					
	ACV			PFA		
	FC[a]	FC[b]	CPE[c]	FC[a]	FC[b]	CPE[c]
19129	3,134 ± 116	450 ± 132	107	247 ± 134	60 ± 26	29
9940	700 ± 229	115 ± 35	102	310 ± 78	63 ± 16	27
8411	500 ± 123	110 ± 36	162	320 ± 26	70 ± 17	45

[a] IC_{50}s (means ± SD) obtained by flow cytometric analysis (FC) of three separate experiments 18 h postinfection at an MOI of 1.
[b] IC_{50}s obtained as described in footnote *a* but at an MOI of 0.005.
[c] IC_{50}s obtained by standard CPE inhibitory assay.

TABLE 3. Influence of MOI and time postinfection on ACV IC_{50}s for sensitive HSV-1 strains

HSV-1 strain	IC_{50} (μM) with an MOI of[a]:					
	0.005			1		
	18 h	36 h	54 h	18 h	36 h	54 h
K1	0.49 ± 0.03	2.72 ± 0.18	4.17 ± 0.23	0.51 ± 0.04	0.55 ± 0.07	0.66 ± 0.15
3210	0.45 ± 0.02	1.95 ± 0.26	4.35 ± 0.31	1.05 ± 0.18	1.55 ± 0.24	1.90 ± 0.18
2890	0.67 ± 0.18	1.35 ± 0.31	3.73 ± 0.25	1.47 ± 0.08	1.70 ± 0.23	2.10 ± 0.26
15882	0.75 ± 0.13	3.15 ± 0.25	7.25 ± 0.73	1.60 ± 0.35	1.90 ± 0.25	2.35 ± 0.57
23237	0.95 ± 0.23	1.52 ± 0.28	6.90 ± 0.85	1.30 ± 0.23	ND[b]	2.05 ± 0.65
1439	0.85 ± 0.23	1.12 ± 0.26	3.40 ± 0.89	1.45 ± 0.13	ND	1.85 ± 0.56

[a] Values are means ± SD from three separate experiments determined at the indicated times postinfection.
[b] ND, not done.

mined by flow cytometry than by the biological assay. We therefore determined their intracellular drug anabolism. The results are summarized in Table 4. The data indicated an impaired phosphorylation of nucleoside analogs by HSV-encoded TK of the resistant isolates. The levels of total nucleoside phosphates (mono-, di-, and triphosphates) induced by clinical isolates 19129, 9940, and 8411 were comparable to those of the resistant laboratory strain K1r and close to the cellular background phosphorylation for ACV and GCV. Interestingly, isolate 3186, showing the lowest resistance to ACV among the resistant strains and still some sensitivity to GCV, exhibited an intermediate capacity to phosphorylate GCV which was impaired only three- to fourfold in comparison with that of the sensitive laboratory strain K1. By confirming the flow cytometric result, the phosphorylation results suggested that this method was able to discriminate an intermediate susceptibility. This was not possible by our standard biological assay.

DISCUSSION

One challenge imposed by the emergence of drug-resistant HSV strains is the development and implementation of assays to test the susceptibility of isolates to antiviral drugs for scientific and clinical purposes, since it has been shown that in vitro resistance is of clinical relevance (16). Among the assays for antiviral drug susceptibility, the PRA is generally considered to be a reference standard (2, 12, 20). In our laboratory, the CPE inhibitory assay (7), a standardized modification of the PRA, is routinely used. It is well-known that different resistant virus isolates exhibit various degrees of resistance which can be due to different mutations leading to resistance and/or due to different amounts of resistant virus subpopulations in the heterogeneous virus isolate. It is reasonable to assume that for both scientific and clinical reasons, differentiated information for individual virus strains is needed. From the clinical point of view, it would be useful to detect not only resistance but also emergence of resistance in a patient. In this study, we wanted to test the capability of flow cytometry for quantitative evaluation of HSV-1 susceptibility to antiviral drugs, since we expected that this method could give us additional information. In previously published studies, flow cytometry was used to determine the effect of infection in cell cultures on the binding of propidium iodide to cellular DNA (15) or to monitor the effect of ACV and interferons by using a polyclonal antibodies for detection of viral antigens in infected cells (18). These

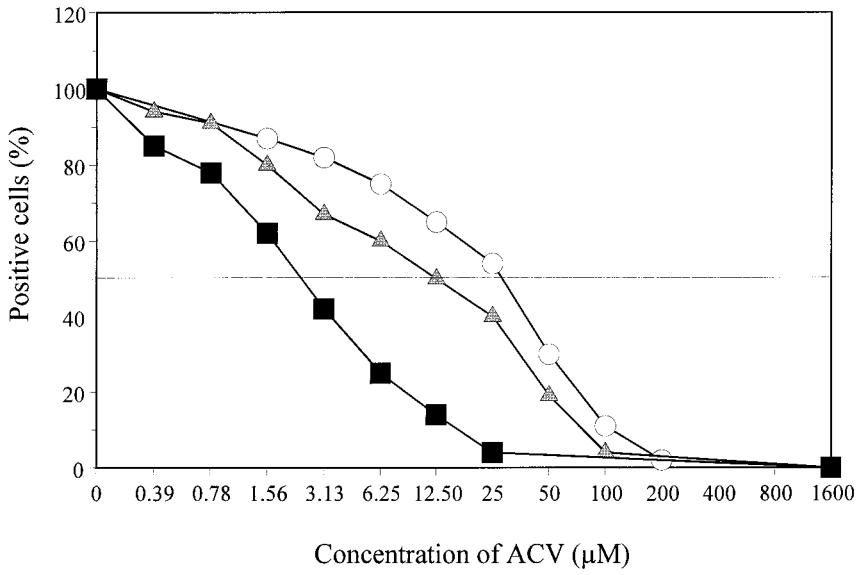

FIG. 4. Sensitivity of flow cytometry for detection of small amounts of resistant viruses within a total viral population. HFF were infected with ACV-sensitive HSV-1 strain 15882 to which we added 2 or 5% ACV-resistant strain 19129 (IC_{50} = 450 μM at an MOI of 0.005). The susceptibility of these mixtures was determined by flow cytometry 54 h postinfection at a total MOI of 0.01. The ACV IC_{50} for the sensitive strain (■) (IC_{50} = 3.95 μM) increased with the addition of 2% (▲) (IC_{50} = 12 μM) and 5% (○) (IC_{50} = 19 μM) resistant strain. IC_{50}s were calculated graphically by linear regression (not shown).

TABLE 4. Intracellular phosphorylation of antiviral compounds

HSV-1 strain	Phosphorylation level[a]	
	ACV	GCV
ACV sensitive		
K1	9	700
3210	11	640
2890	11	1,500
ACV resistant		
K1r	0.5	3
19129	0.5	1.5
3186	2.3	220
9940	0.5	2
8411	0.5	1
Control[b]	0.4	1.5

[a] The level of nucleoside phosphates (mono-, di-, and triphosphates) in infected TK-deficient cells was determined by HPLC and expressed in picomoles per 10^6 cells (means from two separate experiments).
[b] Mock-infected TK-deficient cells.

studies were done at later times postinfection, without determination of the MOI and not quantitatively. In contrast, we used a monoclonal antibody directed to HSV-1 gpC to quantitatively determine the IC_{50}. Antibodies to gpC were chosen since it is a true late protein, and we found a very good detection of expression of gpC by flow cytometry, as reported also by other researchers (6).

Concerning the FACS method, it must be mentioned that analysis of several thousand cells, providing quantitative data rapidly (1 min for measurement and an additional few minutes for data analysis) without need of a more subjective microscopic evaluation, is possible. Furthermore, the FACS method allows parameters for subsequent analyses to be set with ease and stored in a computer to be recalled when needed. Similarly, all original data and histograms from experiments are automatically documented.

Our method could be standardized for quantitative use, since the number of HSV-1 gpC-expressing cells with an FI of $\geq 10^2$ was correlated to the drug concentration. By flow cytometry, it was possible to use high and low MOI and to measure antiviral activity after one or more viral replicative cycles. Our standard flow cytometric assay provided us with results about viral drug susceptibility within 24 h. In contrast, the results from our CPE inhibitory assay can be obtained only after 3 to 4 days. In every FACS analysis, the number of total cells analyzed was exactly defined and the number of infected cells was determined accurately during the test, a fact that enabled us to control the actual MOI in each experiment. It is worth mentioning that the flow cytometric assay allowed not only discrimination between infected and uninfected cells but also discrimination of infected cells with a lower level of expression of gpC resulting from the drug effect. According to our experience, the biological assays cannot distinguish these cells.

When the results obtained by flow cytometry were compared with those achieved by CPE inhibitory assay, the qualitative correlations were nearly identical throughout all the experiments. On the other hand, when the data were compared quantitatively, obviously, significant and systematic differences existed. For the sensitive strains, the IC_{50}s of ACV and GCV obtained by flow cytometry were up to fourfold higher than those determined by biological assay. These differences were due mainly to the higher MOI utilized by our standard flow cytometry assay. Interestingly, this difference was not seen with our most sensitive, plaque-purified standard laboratory strain, K1. By measuring the IC_{50}s for the sensitive strains after several replicative cycles at a low MOI, we demonstrated a 4- to 10-fold increase in IC_{50}s. The highest increase (10-fold) was observed for strain 15882, isolated from a bone marrow transplant patient who was undergoing ACV therapy and from whom strain 19129, expressing the highest resistance among all strains tested, was isolated 82 days later, which might be attributed also to selection of resistant subpopulations within the virus strain. These findings suggest that by using flow cytometry for testing of drug susceptibility at different times postinfection, it might be possible to detect an evolving resistant viral subpopulation within phenotypically sensitive strains if drug concentrations which inhibit the sensitive virus population are used. Since we did not identify clones of resistant virus from these pools, the concept of resistant subpopulations remains a hypothesis which will need to be tested in future experiments.

The differences in IC_{50}s between the biological assay and the FACS analysis for the ACV-resistant strains were much greater (up to 70-fold), which could be attributed even more to the MOI used. The IC_{50}s for the different ACV-resistant strains obtained by FACS were much more wide-ranging than those obtained by the biological assay. One of the two strains exhibiting the greatest resistance to ACV was our highly resistant laboratory strain K1r, which was selected in vitro under drug suppression. Strain 3186, expressing the lowest resistance among all ACV-resistant strains, still exhibited some sensitivity to GCV measured by flow cytometry which was not detectable by biological assay. The flow cytometric result was confirmed by measuring the intracellular drug phosphorylation, which showed an intermediate phosphorylation capacity of this strain.

Moreover, the sensitivity of flow cytometry was confirmed by mixture experiments which showed that by adding a small amount of resistant virus to a sensitive strain, the drug susceptibility of this strain could be reduced.

The IC_{50}s of PFA were comparable for all strains tested.

In this study, we demonstrated the flow cytometric test as a suitable method for quantitative, fast, accurate, highly reproducible, and standardized analysis of the susceptibility of HSV-1 to antiviral drugs. Furthermore, flow cytometry provided more-differentiated results than biological assay for characterization of viral populations. The facts that the FACS system is not available everywhere and requires highly skilled workers may be the major obstruction for introducing this method in routine virology laboratories.

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