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Review

Antiviral drug susceptibility assays: going with the flow

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Abstract

This review describes the procedures for the use of fluorochrome labeled monoclonal antibodies and flow cytometry for the detection and quantification of virus infected cells. The application of this technology for (1) identifying virus infected cells in clinical specimens obtained from human cytomegalovirus (HCMV) and human immunodeficiency virus (HIV) infected individuals; (2) screening antiviral compounds active against HCMV, HDSV and HIV; and (3) performing drug susceptibility testing for HCMV, HSV and HIV clinical isolates are reviewed. The flow cytometry drug susceptibility assay is rapid, quantitative, and easily performed. It should be considered by anyone interested in performing drug susceptibility testing for any virus for which there are reliable monoclonal antibodies.

Keywords: Antiviral drugs; Drug susceptibility assays; Flow cytometry

1. Introduction

Flow cytometry is a rapid, quantitative method for the multiparametric measurement of fluorescent cells. In vitro studies employing the combination of fluorochrome labeled monoclonal or polyclonal antibodies directed against viral antigens and flow cytometry have been used to detect virus infected cells, to determine viral pathogenesis, to measure the effects of antiviral compounds on the synthesis of viral antigens and nucleic acids in virus infected cells, and, most recently, for drug susceptibility testing. In vivo studies employing this technology have been used for diagnosis of viral infections, to monitor disease progression, and to monitor the effect of antiviral therapy on viral burden. The identification and quantitation of fluorochrome labeled virus infected cells by flow cytometry is a very useful technique that is easily performed. The use of flow cytometry in virus research was last reviewed by this author in 1994 (McSharry, 1994). Since then the number of publications reporting on the use of flow cytometry for the detection of viruses in clinical samples, for studying in vitro and in vivo viral pathogenesis, and for studying the effects of antiviral drugs on virus replication has exploded. A comprehensive review of the literature is beyond the scope of this review. Therefore, this review will be limited to the use of flow cytometry for studies involving: (1) the direct detection of

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virus infected cells in clinical samples, (2) the effects of antiviral drugs on the synthesis of viral antigens and nucleic acids in virus infected cells, and (3) drug susceptibility testing. Furthermore the review will be limited to studies on cells infected with human cytomegalovirus (HCMV), herpes simplex viruses (HSV-1 and 2), and human immunodeficiency virus (HIV).

2. Flow cytometry

2.1. The analytical flow cytometer

Flow cytometry is the measurement of the physical and/or chemical properties of cells as they pass single file in a fluid stream through a measuring apparatus, the fluorescence-activated cell sorter (FACS). Fig. 1 illustrates the components of a modern analytical flow cytometer including the single argon ion laser and the rectangular flow cell (the small black square on the left center of the diagram) with the attached optical assembly including lenses, mirrors, filters, and photomultiplier tubes. All flow cytometers have four basic components: (1) a fluidic system to carry a suspension of single cells from the sample tube into a quartz flow cell containing a sheath fluid; (2) a focused light source, usually an argon ion laser with an excitation energy of 488 nm; (3) filters and photodetectors to collect the scattered light and the light emitted from fluores-

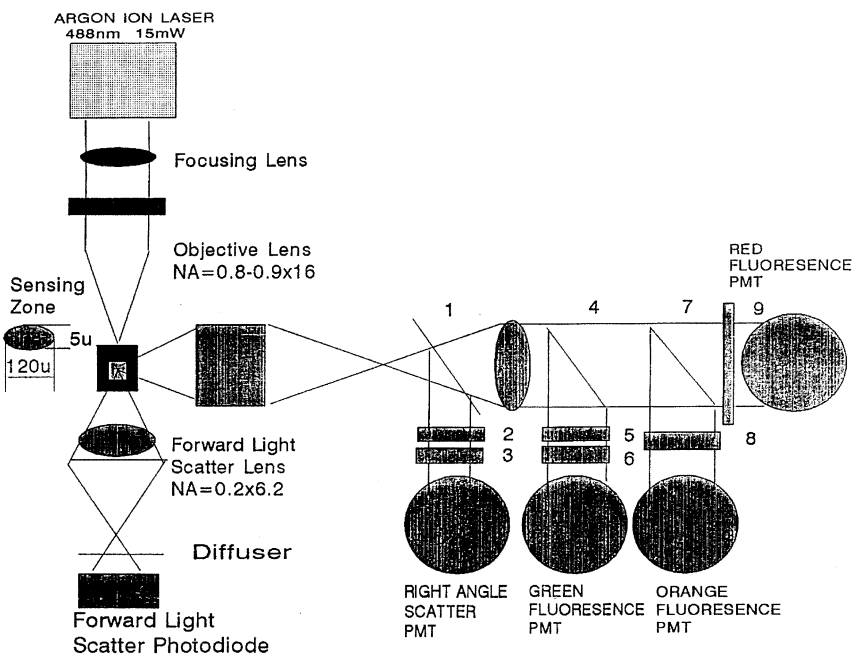


Fig. 1. Diagram of a flow cell with attached optical systems. As cells pass through the flow cell (the small black square box in the center left of the figure), a laser beam intersects the stream of cells and scatters light. Forward angle light scatter passes through the forward light scatter lens, and the energy is collected by the forward light scatter photodiode. Right angle light scatter passes through the objective lens, the beam splitter (1), laser line filter (2), and diffuser (3), and the energy is collected by the right angle light scatter photomultiplier tube (PMT). If the cells are labeled with fluorescent molecules, the laser will excite these molecules to emit light at higher energies. The energies of different wavelengths pass through the objective lens and various filters and are collected and amplified by the various photomultiplier tubes (green, orange or red PMTs). The amplified signals are converted into digital information and stored in a computer for further analysis. The numbers in the figure refer to the following: (1) beam splitter; (2) laser line filter, 396–496 nm band pass; (3) diffuser; (4) dichroic mirror 1, 570 nm long pass filter; (5) laser cut filter, 490 nm short cut; (6) green filter, 515–530 nm band pass; (7) dichroic mirror 2, 610 long pass; (8) orange filter, 565–592 nm band pass; and (9) red filter, 660 nm long pass (from McSharry (1994), with permission).

cently labeled cells and processors to convert these light signals to analogue electrical impulses and then to digital signals; and (4) computers to collect, store and analyze the data. The single cell suspensions to be analyzed are introduced into a stream of sheath fluid that keeps the cells flowing in single file at uniform speed through the center of the quartz flow cell. As each cell passes a point where the laser intersects the path of the fluid stream the laser light will be scattered. The scattered light is collected as forward-angle light scatter (FALS, FW-SC), which yields information on the number and size of the cells, and as right angle light scatter (RALS, RT-SC), which yields information on the granularity of the cells. If the cells passing through the laser beam are fluorescent the laser will excite the fluorescent molecules and each fluorochrome will emit energy at higher wavelengths characteristic of that fluorochrome. For example, fluorescein isothiocyanate (FITC) has a peak emission at 520 nm; phycoerythrin (PE) at 580 nm, propidium iodide (PI) at 580 and 620 nm, and 7-aminoactinomycin D (7-AAD) at 650 nm. The emitted energies of different wavelengths pass through filters (a green filter, 515–530 band pass; an orange filter, 565–592 nm band pass; and a red filter; 600 nm long pass) that allow light of only a particular wavelength to pass; then they are collected and amplified by the photomultiplier tubes. The amplified signals are converted into digital information that is stored in a computer for further analysis and printed in graph form as a hard copy.

Analytical flow cytometers use a single laser beam for the simultaneous quantitative measurement of cell number, cell size, cellular granularity, and up to three different fluorochromes. In addition to the number of fluorescently labeled cells, the amount of each fluorochrome associated with each cell can be measured by the flow cytometer. The ability of the flow cytometer to simultaneously measure multiple characteristics of a cell leads to the term multiparametric analysis. This type of analysis will be used to describe the virus cell interactions described in this review. Modern analytical flow cytometers with internal computers allow the operator to set parameters for various analyses with ease and to store these parameters to be recalled when needed for analysis of the cells using the same protocol. This feature saves tremendous amounts of time while performing different types of analyses during the workday. Some flow cytometers have more than one laser and can detect additional fluorescent molecules. Other flow cytometers have the ability to sort cells into separate populations on the basis of preselected parameters. For more detailed information on the mechanics and practical uses of a flow cytometer, the reader is referred to the following references (Watson, 1991; Shapiro, 1995; Robinson et al., 1999).

2.2. Detection of virus infected cell cultures by flow cytometry

This section describes the current procedures used in my laboratory to detect virus infected cells. It is divided into two parts, cell free virus and cell associated virus, because the procedures for infecting cell monolayers with these virus samples are very different.

2.2.1. Cell free virus

Remove the medium from the cell monolayers and add the cell free virus to the cell monolayers at a multiplicity of infection (MOI) of 0.01–10.0 PFU/cell. After a suitable adsorption period (1–2 h), unadsorbed virus is removed and virus growth medium is added to the flask. The infected cells are incubated at 37°C until at least half of the cells in the culture are synthesizing the antigen(s) of interest. Incubation times will depend on the time required to complete the replication cycle of the virus under study in that cell culture system and the MOI. For example, HSV type 1 has a replication cycle in MRC-5 cells of about 8 h. Therefore, after infection at low MOI (0.01 PFU/cell) and overnight incubation where multiple rounds of virus replication can occur a large proportion of the cells in the culture will be synthesizing all of the viral antigens. The replication cycle of HCMV in HFF cell monolayers is approximately 96 h. Therefore, after infection of cells at an MOI of 1–10 PFU/cell, most of the cells in the monolayer will be synthesizing the immediate early (IE), early and late antigens after 96 h of incubation. At lower MOI (0.01–0.1

PFU/cell), it will take at least 144 h of incubation for greater than 50% of the cells to be synthesizing all of these antigens.

2.2.2. Cell associated virus

Some viruses such as fresh HCMV clinical isolates remain cell associated when grown in cell culture. For infection of monolayers with cell associated virus, virus infected cells are added directly to the media overlaying the cell monolayers at an MOI of 0.001–0.1 infected cell per uninfected cell. The monolayers are incubated until approximately 50–100% of the cells in the monolayer show cytopathic effects (CPE). The HCMV replication cycle in MRC-5 cells is approximately 96 h. Therefore, it will take at least 96 h for one cycle of virus replication and somewhat longer for a significant number of cells to synthesize all of the viral antigens. In practice, at an MOI of 0.01 virus infected cells per cell, it takes at least 144 h for enough cells to synthesize the IE, early and late HCMV antigens. Some clinical isolates grow more slowly than others and will require more than 144 h to spread infection to at least 50% of the cells in the monolayer. Microscopic observation of the infected cell monolayers during the incubation period is useful for determining when at least 50% of the cells in the monolayer are infected. The virus infected cells used to initiate the infection are synthesizing these antigens and they will be detected in the flow cytometry assay. Therefore, it is important when using cell associated virus as the inoculum to keep the MOI low (0.001–0.01) to keep this background from interfering with the analysis.

2.3. Harvesting virus infected cells

The following procedure is used for harvesting cell culture monolayers infected with either cell free or cell associated virus. At the end of the incubation period, remove the medium, wash the cell monolayers twice in PBS without Ca^{2+} and Mg^{2+} and remove the cells from the flask with trypsin/EDTA. Treatment of the cell monolayer with trypsin/EDTA will destroy the proteins on the cell surface, precluding cell surface staining of trypsinized cells. However, the procedure does not permeabilize the plasma membrane. The trypsinized cells are resuspended in medium containing 10% serum and the cells are pelleted by low speed centrifugation. The cell pellets are washed twice with PBS without Ca^{2+} and Mg^{2+} by low speed centrifugation and resuspend in the residual PBS that remains in the tube after decanting the supernatant. This amount is approximately 0.2 ml when a 15 ml centrifuge tube is used to collect the cells. The resuspended pellets are incubated on ice for 1 h to disperse the cell pellet into a single cell suspension. The cells are permeabilized by the addition of nine volumes of ice cold absolute methanol to yield approximately 1×10^6 cells/ml. Place 1 ml of the cell suspension in microfuge tubes and store at -70°C until the permeabilized cells are ready to be treated with antibodies and fluorescent dyes.

Methanol puts holes in both the plasma membrane and the nuclear envelope to allow fluorochrome labeled antibodies and fluorescent DNA binding agents to enter these compartments and bind with specific antigens and nucleic acids. After incubation, unbound antigens and nucleic acid dyes can be easily washed out of the cell. Permeabilization of the cells with methanol has several advantages: (1) it works with cells derived from clinical specimens as well as cultured cells, (2) it is compatible with most antibodies that are used to identify antigens in virus infected cells and with dyes such as PI and 7-AAD that are used to label cellular DNA, (3) it destroys the infectivity of many viruses making for safer handling of methanol permeablilzed specimens; and (4) methanol treated cells can be stored at -70°C for long periods of time without the loss of the ability of cellular or viral antigens to react with antibodies or DNA staining reagents. Two major disadvantages of the methanol permeabilization procedure include the destruction of the light scatter properties of cells and the loss of the ability of some surface antigens to react with antibodies.

2.4. Antibody treatment for intracellular antigens and DNA staining with PI or 7-AAD

To identify the virus-infected cells, one or more viral specific antigens in the cytoplasm, and/or in the nucleus must be labeled directly or indirectly with fluorochrome labeled antibody to a viral antigen(s). For indirect immunofluorescence staining, an unlabeled antibody (monoclonal or polyclonal) is diluted in PBS containing 1% BSA to the appropriate concentration to stain the maximal number of virus infected cells without staining uninfected cells. This concentration must be empirically determined for each antibody by treating uninfected cells and virus infected cells with different dilutions of antibody followed by flow cytometric analysis. The dilution of antibody that detects the greatest number of virus infected cells and the lowest number of uninfected cells is the appropriate antibody concentration for use in the analyses. To ensure antibody excess, an antibody concentration that is twice the ideal concentration is usually used to treat the virus infected cells. To treat the permeabilized cells with the appropriately diluted antibody, remove the permeabilization material from the sample by low speed centrifugation, wash the cells once with PBS without Ca^{2+} and Mg^{2+}, resuspend the cells in 0.2 ml of the diluted antibody, and incubate at 37°C for 1 h. Many investigators incubate cells with antibody at 4°C with good results. After incubation, wash the antibody treated cells three times with PBS containing 1% BSA. Then add a fluorochrome labeled second antibody to the cells and incubate at 37°C for an additional 1 h. Wash the cells three times with PBS/Tween 20 to remove any unbound second antibody, add PI or 7-AAD to allow for identification of intact cells with a 2N or greater DNA content and analyze the cells for two color fluorescence by flow cytometry. For direct labeling, the cells are treated with fluorochrome labeled antibody (polyclonal or monoclonal) at the appropriate concentration in PBS with 1% BSA. After incubation at 37°C for 1 h, the cells are washed three times in PBS/Tween 20 to remove unbound antibodies, PI or 7-AAD is added to identify cells with a 2N or greater DNA content and the cells are analyze for two color fluorescence by flow cytometry. Since PI will bind to both RNA and DNA the cells must be treated with RNAse before the addition of PI to make the staining specific for DNA. RNA does not bind 7-AAD, therefore, RNAse treatment is not required when using 7-AAD.

2.5. Flow cytometric analysis of fluorescently labeled cells

Once the cells are treated with fluorochrome labeled antibodies and nucleic acid stains, the number of antigen positive cells can be measured by flow cytometry. The initial analysis will distinguish intact cells from debris. This is often performed by analysis of right angle light scatter (RT-SC) versus forward angle light scatter (FW-SC). Cells of the appropriate size and granularity are gated and debris that is usually smaller than intact cells is excluded from the analysis. A more precise way to distinguish intact cells from debris is the use of a fluorescent DNA stain such as 7-AAD or PI and forward angle light scatter. The 7-AAD or PI staining will identify cells on the basis of DNA content and forward angle light scatter will determine the cell size. Cell free nuclei that have the appropriate amount of DNA on the basis of 7-AAD staining are smaller than intact cells and are excluded from the analysis on the basis of forward angle light scatter. Using these two parameters to identify intact cells, it is possible to accurately distinguish cells from debris. After identifying the cells to be analyzed, a gate is drawn around that population and each cell in the population is further analyzed for cell associated fluorescence intensity. Controls should include uninfected cells treated with fluorochrome labeled antibodies and PI or 7-AAD and virus infected cells treated with fluorochrome labeled isotype control antibodies and PI or 7-AAD.

This introduction outlined the steps required to infect cells with viruses or virus infected cells and to prepare virus infected cells for analysis of the percentage of cells synthesizing viral antigens by flow cytometry. The remainder of the review will focus on applications of this technology for: (1) identifying virus-infected cells in clinical specimens from HCMV or HIV infected individuals;

(2) screening antiviral compounds active against HCMV, HSV and HIV; and (3) performing drug susceptibility testing for HCMV, HSV and HIV.

3. Human cytomegalovirus (HCMV)

3.1. Detection of HCMV infected cells in clinical samples

3.1.1. Bronchoalveolar lavage (BAL) specimens

Laboratory diagnosis of HCMV infection usually involves isolation of virus from blood, BAL, or urine by inoculation of cultured cells with the clinical specimen. This procedure can take up to 6 weeks before a positive culture is obtained (Boeckh and Boivin, 1998). To speed up this process, several laboratories have used indirect immunofluorescence with monoclonal antibodies to HCMV antigens and flow cytometry for the direct detection of HCMV infected cells in BAL or peripheral blood mononuclear cells (PBMCs). Cells in BAL specimens were treated with a murine monoclonal antibody to an early HCMV antigen followed by FITC-labeled goat antimouse antibody and the percentage of antigen positive cells was measured by flow cytometry (Elmendorf et al., 1988). The results showed that five of 10 BAL specimens contained antigen positive cells. Four of the antigen positive BAL specimens were also positive for HCMV by culture and one was negative by culture. One BAL sample was positive by culture and negative by the flow cytometry assay. The five antigen positive BAL specimens had between 8 and 39% of the cells positive for the HCMV antigen. The data suggest that if at least 10% of the cells in the BAL specimen were antigen positive by flow cytometry there was good correlation between the culture assay and the flow cytometry assay. The flow cytometry assay takes only a few hours from the time the clinical sample is collected to the production of a positive or negative result and it is not labor intensive. Thus, the direct detection of HCMV infected cells in BAL specimens by flow cytometry is much faster than isolation of virus in culture. These results suggest that monoclonal antibodies to HCMV antigens and flow cytometry could be used as a rapid technique for the laboratory diagnosis of HCMV pneumonia. The ability to have a rapid diagnostic technique for HCMV disease will enable the physician to treat patients with this disease in a more timely manner.

3.1.2. PBMCs

HCMV infected PBMCs obtained from kidney transplant patients were detected by indirect immunofluorescence using a monoclonal antibody to an HCMV IE antigen and the percent of antigen positive cells was measured by flow cytometry (McSharry, 1994). Patients with acute HCMV infection had between 0.6 and 8.1% of their PBMCs positive for the HCMV IE antigen. There was a high correlation between the percent of IE antigen positive PBMCs and isolation of the infectious virus from PBMCs in cell culture. Furthermore, when transplant patients with acute HCMV infection were treated with ganciclovir, the percentage of IE antigen positive PBMCs decreased. These results suggest that this assay could be used as a rapid diagnostic procedure for HCMV infection and to monitor HCMV infected patients on antiviral therapy.

The use of monoclonal antibodies and flow cytometry to detect HCMV infected cells in leukocytes obtained from transplant patients with acute HCMV infection was recently confirmed (Imbert-Marcille et al., 1997). These investigators used monoclonal antibodies to IE, pp65 or late HCMV antigens to detected antigen positive leukocytes. Using monoclonal antibodies to pp65 and flow cytometry they showed that leukocytes obtained from acutely infected transplant patients had between 0.04 and 1.15% of their leukocytes positive for the pp65 antigen. Leukocytes obtained from uninfected individuals had 0.01–0.08% of the leukocytes positive for this antigen. Monoclonal antibodies to IE and late antigens detected lower percentages of antigen positive leukocytes than monoclonal antibodies to the pp65 antigen.

The large discrepancy in the percentage of antigen positive cells between these two studies (McSharry, 1994; Imbert-Marcille et al., 1997) is most likely due to the different populations of cells that were analyzed and the different monoclonal anti-

bodies used in the two studies. In the former study only lymphocytes and monocytes were analyzed, whereas in the latter study the analysis included lymphoyctes, monocytes and granulocytes. Since granulocytes add many more cells to the population under study, the percentage of antigen positive cells should be lower. In addition, the use of monoclonal antibody to the pp65 antigen was unique to the latter study. Since the pp65 antigen is found in phagocytic cells in HCMV infected patients, a population that was not examined in the former study, a different percentage of antigen positive cells may be expected.

Taken together, these publications suggest that flow cytometry should be useful for detecting HCMV in BAL and peripheral blood specimens obtained from patients with acute HCMV disease. However, as presently performed, flow cytometry has not made its mark in this field. This could be due to a few technical problems. First, detection of antigen positive cells in blood only occurs during the acute phase of the disease. Once the virus has settled into organs such as the retina it is much more difficult to detect antigen positive blood cells by flow cytometry. Second, the detection system using only one monoclonal antibody at a time may not be ideal. Further study using combinations of antibodies to IE, early (pp65) and late antigens may make this a more useful assay, particularly for diagnosis of HCMV infection in BAL cells obtained from patients with acute HCMV pneumonia. Development of technologies combining in situ PCR and flow cytometry for detection of HCMV infected BAL cells and peripheral blood cells may also enhance the feasibility of this technology for the rapid diagnosis of HCMV infections (see below under Section 5).

3.2. Drug susceptibility assays for HCMV clinical isolates

In the last few years, flow cytometric techniques have been developed for drug susceptibility testing of HCMV laboratory strains and clinical isolates. These techniques are rapid, quantitative, objective, and easily performed. Furthermore, they yield IC_{50} values that are similar or identical to the more time consuming and labor intensive plaque reduction assays (PRA). This section will describe the use of fluorochrome labeled monoclonal antibodies to HCMV antigens and flow cytometry to measure the effect of antiviral drugs on the percentage of cells synthesizing these antigens. The ability to use the flow cytometry drug susceptibility assay to distinguish between drug sensitive and drug resistant HCMV clinical isolates will also be illustrated.

Ganciclovir is the current drug of choice for treatment of HCMV disease (Crumpacker, 1996). When therapy fails because of the selection of ganciclovir resistant mutants, foscarnet or cidofovir can be used for treatment of life threatening disease (Palestine et al., 1991; Polis et al., 1995). Long term administration of ganciclovir, foscarnet or cidofovir often leads to the selection of drug resistant mutants with changes in the nucleotide sequence of the UL97 gene for ganciclovir resistant mutants or in the viral DNA polymerase genes for mutants resistant to any of the three drugs (Field and Biron, 1994; Erice et al., 1997; Jabs et al., 1998). Treatment of HCMV infected immunocompromised patients with these drugs for as little as 3–9 months leads to the selection of drug resistant mutants in approximately 30% of patients (Jabs et al., 1998). Thus, there is a need for drug susceptibility testing of clinical isolates obtained from immunocompromised patients with HCMV disease.

3.2.1. Genotypic assays

Both genotypic and phenotypic methods are available for determining drug susceptibilities of HCMV clinical isolates (Cockerill, 1999; Erice, 1999). Genotypic assays that detect specific mutations known to be associated with resistance to a particular drug are rapid and often automated. However, they depend on prior knowledge of the specific nucleotide changes that lead to drug resistance or of the gene targeted by the antiviral agent. The variety of possible genetic changes both within the gene(s) targeted by the antiviral drug or within other viral genes that could lead to resistance precludes the availability of genetic probes for all possible mutations. In contrast, phenotypic assays determine drug susceptibilities

of viruses irrespective of genetic changes and may be more useful for detecting drug resistant viruses.

3.2.2. Phenotypic assays

3.2.2.1. PRA. The standard phenotypic assay for HCMV drug susceptibility testing is the PRA (Stanat et al., 1991). The assay involves isolation of infectious virus from clinical specimens by inoculation of cell cultures, the production of CPE characteristic of HCMV infection, and confirmation that the virus causing the infection is HCMV. Clinical isolates of HCMV remain cell associated when grown in cell culture. To produce cell free virus to perform the PRA, the cell associated virus is passed approximately 10 times. Once cell free virus is available, it is titered by a plaque assay. Then approximately 50 PFU of cell free virus are added to monolayers in a 24 well plate followed by an agarose overlay containing various concentrations of the drug under study. After 10–14 days of incubation, the agarose overlay is removed, the monolayers are stained and the plaques counted with the aid of a microscope. This process can take up to 3 months. However, the PRA is very accurate and yields IC_{50}, IC_{90} and even IC_{95} values for clinical isolates to various drugs. A more rapid PRA uses HCMV infected cells in place of cell free virus (Lurain et al., 1996). To perform the PRA on cell associated HCMV clinical isolates, medium containing various concentrations of antiviral drugs is placed in different wells of 24 well plates and then approximately 100 virus infected cells are added to each well. After 7 days of incubation at 37°C, the plaques are counted under a microscope. This assay can be completed within a few weeks after obtaining the clinical sample. In addition to saving time, the assay is performed on fresh clinical isolates that have not been repeatedly passed in culture. However, this assay must be read using a microscope making it very labor intensive and often subjective.

3.2.2.2. Dram vial assays. A number of other phenotypic assays for measuring the drug susceptibility of HCMV clinical isolates have been developed that rely on the detection of viral antigens or viral DNA that are produced during infection of cell cultures with HCMV clinical isolates. The assays involved inoculation of cell cultures with the patient's peripheral blood leukocytes in the absence and presence of inhibitory concentrations of antiviral drugs. After 4–6 days of incubation, infected cells were detected by treatment with a fluorochrome labeled monoclonal antibody to the HCMV IE or late antigens followed by fluorescence microscopy (Pepin et al., 1992; Gerna et al., 1995). These assay systems required less time than the PRA. However, they remain labor intensive, they do not lead to the determination of IC_{50} values, and they are only useful when a relatively large amount of virus infected cells is present in the clinical sample. The in situ DNA hybridization assay that measures virus replication by detection of de novo viral DNA synthesis has also been developed for drug susceptibility testing; however, it does not save time in sample preparation and is not very sensitive (Boeckh and Boivin, 1998; Erice, 1999).

3.2.2.3. Flow cytometry based drug susceptibility assays. Several recent publications have reported on the use of fluorochrome labeled monoclonal antibodies to HCMV antigens and flow cytometry for the phenotypic determination of the susceptibility of HCMV laboratory strains and clinical isolates to ganciclovir (Lipson et al., 1997; Kesson et al., 1998; McSharry et al., 1998a,b). The flow cytometry based drug susceptibility assay has several advantages over the currently used phenotypic assays. First, a large number of cells can be analyzed in less than 1 min allowing for the determination of a robust statistical difference between susceptible and resistant clinical isolates. Second, when virus infected cells are used as the inoculum, the time required to obtain enough infected cells for analysis is reduced to approximately 1 week after virus isolation. Third, this drug susceptibility assay requires only 72–96 h of incubation before analysis of the percentage of cells synthesizing IE antigens and only 144 h of incubation before analysis of the percentage of cells synthesizing late antigens. Fourth, since a large percentage of the cells in the clinical sample are analyzed, there is a better chance of detecting

small numbers of drug resistant mutants that may be present among the population of virus infected cells. Fifth, the IC_{50} values of HCMV clinical isolates for ganciclovir determined by the flow cytometry assay are similar or identical to those obtained with PRA and the CPE assays. Sixth, the assay of virus infected cells can be performed on fresh clinical isolates that have not been repeatedly passed in culture, thus avoiding the possibility of introducing mutations into the virus population that may influence the phenotype of the original clinical isolate. Thus, the flow cytometry based susceptibility assay for HCMV is rapid, quantitative, objective, potentially automatable, and easily performed.

Cell free virus. Three recent publications described the use of flow cytometry based drug susceptibility assays for cell free HCMV laboratory strains and clinical isolates (Lipson et al., 1997; Kesson et al., 1998; McSharry et al., 1998a). Monolayer cultures of MRC-5, lung, or HFF cells were infected with cell free HCMV at various MOIs in the presence of various concentrations of ganciclovir or foscarnet. After 4–10 days of incubation, the cells were harvested, permeabilized and treated with polyclonal or monoclonal antibodies to HCMV IE and/or late antigens. Flow cytometry was used to measure the percentage of antigen positive cells. The IC_{50} values were calculated from the reduction in the percentage of antigen positive cells at each drug concentration. Drug susceptible HCMV laboratory strains had IC_{50} values ranging from 1.5–20 μM ganciclovir and 77–140 μM foscarnet by the flow cytometry assay and 6–9 μM ganciclovir and 56 μM foscarnet by PRA. Clinical isolates had IC_{50} values of 1.5–25 μM ganciclovir and 80–140 μM foscarnet with the flow cytometry assay and 3–10 μM ganciclovir and 45–141 μM foscarnet with PRA. Resistant clinical isolates had IC_{50} values from 47 to >96 μM ganciclovir and >800 μM foscarnet by the flow cytometry assay and >96 μM ganciclovir and >800 μM foscarnet by PRA. These results showed that the flow cytometry drug susceptibility assay could determine IC_{50} values for both HCMV laboratory strains and clinical isolates. For the drug sensitive strains, the IC_{50} values for ganciclovir were approximately 2-fold higher by the flow cytometry assay than by PRA, but both assays clearly identified drug susceptible and drug resistant strains of HCMV. However, when the IC_{50} values obtained from the three independent studies are compared there is a wide variation in IC_{50} values for the same laboratory strain of HCMV. These variations in the IC_{50} values are probably due to differences in the MOI used by the different laboratories. Therefore, there is a need to standardize the assay with regard to cell lines, MOI, time of incubation post infection, and the antibodies used to detect the HCMV infected cells. This standardization is being undertaken in the author's laboratory.

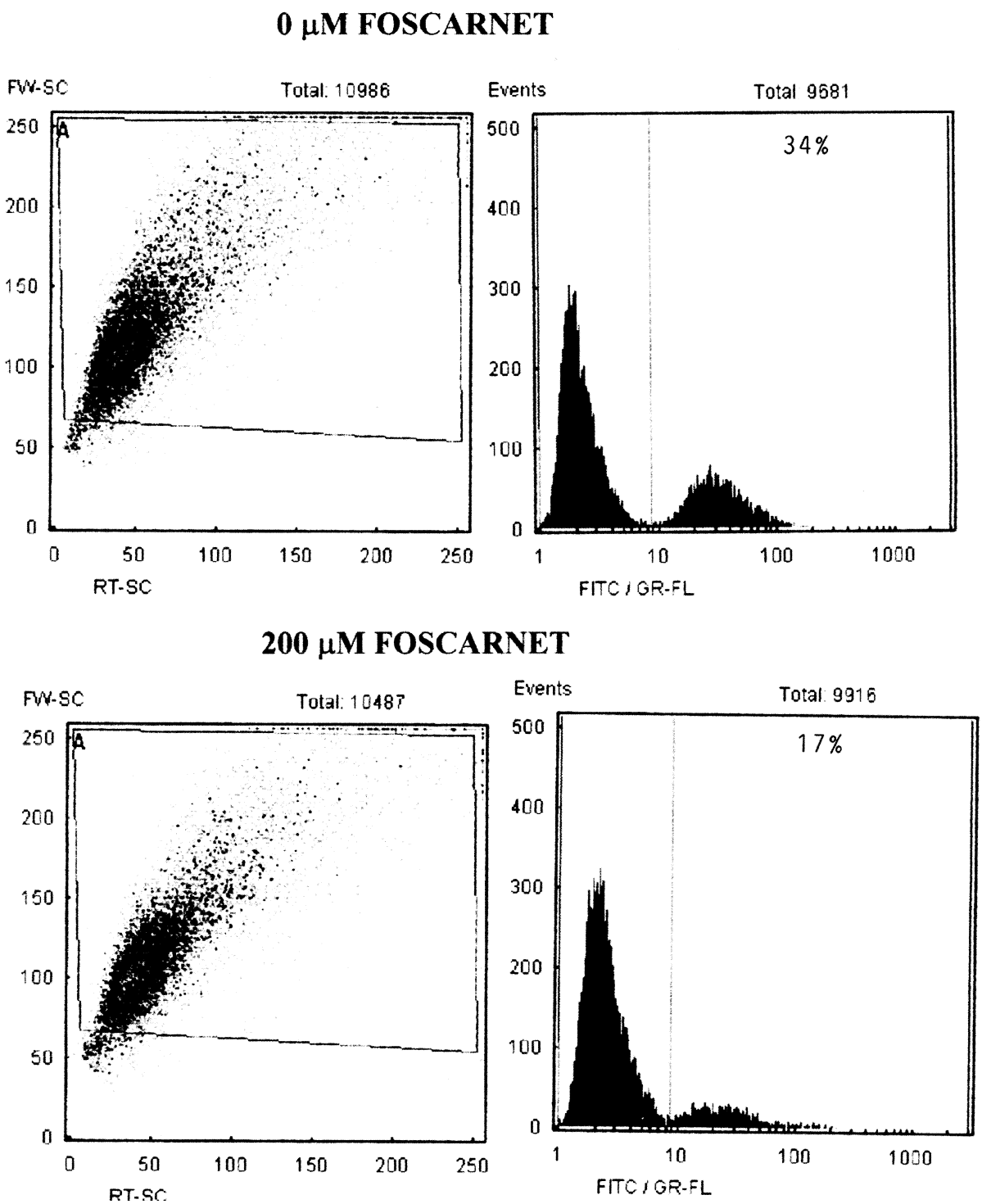
A three color flow cytometric analysis was used to simultaneously measure the effect of ganciclovir on the synthesis of IE and late antigens. Monolayer cultures of HFF, MRC-5 or lung cells were infected with AD169 or its ganciclovir resistant derivative, D6/3/1, at an MOI of 1–10. After 72–96 h of incubation, the cells were harvested, permeabilized, treated with a cocktail consisting of FITC-labeled monoclonal antibody to the HCMV IE antigen and PE-labeled monoclonal antibody to an HCMV late antigen and 7-AAD, and the cells were analyzed for three color fluorescence by flow cytometry. At high MOIs (1–10 PFU/cell) with AD169 or D6/3/1, where only a single round of virus replication can occur, ganciclovir had no effect on the synthesis of the IE antigen but reduced the percentage of cells synthesizing the late antigen from 47% in the absence of drug to 0.7% in the presence of 12 μM ganciclovir. This result is consistent with the mode of action of ganciclovir (Stanat et al., 1991; Sullivan et al., 1992). For cells infected with the ganciclovir resistant derivative, D6/3/1, ganciclovir (12 μM) had no effect on the percentage of cells synthesizing either the IE or the late antigen, confirming that this isolate is resistant to ganciclovir (Lurain et al., 1994). The IC_{50} values for AD169 determined by five different laboratories were between 1.5 and 3 μM ganciclovir, well within the accepted value for this laboratory strain of HCMV (Crumpacker et al., 1996). This mutlicenter study showed that the flow cytometry drug susceptibility assay is transportable to other laboratories and that different laboratories could obtain similar IC_{50} values for the AD169 strain of HCMV.

Cell associated virus. In the same multicenter study the ganciclovir susceptibilities of 19 HCMV clinical isolates were determined using an FITC-labeled monoclonal antibody to an HCMV late antigen and 7-AAD in a two color flow cytometry assay (McSharry et al., 1998a). For analysis of clinical isolates, 10^5 virus infected cells were added directly to medium containing various concentrations of ganciclovir that overlaid confluent monolayers of HFF cells and the flasks were incubated at 37°C for 144 h. At this MOI (0.1 infected cell per uninfected cell) every cell in the culture was not infected at the beginning of the experiment. This assay measured the ability of input virus infected cells to spread infection to adjacent cells in the monolayer in the presence of various concentrations of ganciclovir. PRA were performed in parallel. The results showed that two clinical isolates with known mutations in the UL97 and DNA polymerase genes that lead to ganciclovir resistance had IC_{50} values greater than 96 μM ganciclovir by both the flow cytometry assay and the PRA. Fourteen clinical isolates of unknown phenotypes were sensitive by both assays with average IC_{50} values of 2.79 μM ganciclovir by the flow cytometry assay and 2.80 μM ganciclovir by PRA. Three of the clinical isolates had IC_{50} values by flow cytometry that were discordant with the PRA data; the IC_{50} values determined by the flow cytometry assay were at least 2-fold greater than the values obtained with PRA. Despite the less than perfect correlation between the IC_{50} values derived from each assay, the flow cytometry assay accurately identified the clinical isolates that were susceptible or resistant to ganciclovir

The multicenter group also reported on a more rapid procedure to determine drug susceptibilities on HCMV clinical samples (McSharry et al., 1998b). Medium containing various concentrations of ganciclovir was added to HFF cell monolayers and HCMV infected cells at an MOI of 0.1 were added directly to the medium. At 96 h post infection the cells were harvested, treated with an FITC-labeled monoclonal antibody to the HCMV IE antigen and 7-AAD and the percentage of antigen positive cells was determined by flow cytometry. PRA were done in parallel. Of 25 HCMV clinical isolates four were shown to be resistant by both assays, two were partially resistant by both assays and the remainder were susceptible by both assays. The flow cytometry assay gave slightly higher IC_{50} values than the PRA for the sensitive and partially resistant clinical isolates, but the IC_{50} values for both assays had an acceptable correlation ($r^2 = 0.473$, $P = 0.001$). The discrepancy in the IC_{50} values obtained by the flow cytometry assay and the PRA may be due to the fact that the two assays were performed at different MOI. The flow cytometry assay had an MOI of 0.1 where as the PRA had an MOI of 0.0001. In one recent study of the effect of an experimental antiHCMV drug, 1263W94, where the flow cytometry assay was performed at MOIs of 0.001–0.01, there was no difference in the IC_{50} values obtained from the two assays (McSharry et al., 1999a). Two conclusions can be drawn from these studies. First, it is clear from the published studies that MOI does effect the IC_{50} values of these drugs for HCMV laboratory strains and clinical isolates. Second, both assays clearly distinguish between drug susceptible and drug resistant laboratory strains and clinical isolates of HCMV. Similar results were found for the flow cytometric analysis of drug susceptibilities for herpes simplex virus type 1 (Pavic et al., 1997).

Assay of foscarnet susceptibility of an HCMV clinical isolate. The data presented in this section will illustrate the use of the flow cytometry drug susceptibility assay to determine the effect of foscarnet on the synthesis of IE and late antigens in

Fig. 2. Effect of foscarnet on IE antigen synthesis in cells infected with a foscarnet susceptible clinical sample. Medium containing various concentrations of foscarnet was added to HFF cell monolayers. Then cell associated virus was added to the medium in the flasks at an MOI of 0.01. After incubation at 37°C for 144 h, the cells were harvested, permeabilized, treated with a monoclonal antibody to an IE antigen and analyzed by flow cytometry for the percentage of antigen positive cells. Initially, the cells were analyzed for FW-SC vs. RT-SC to identify intact cells and exclude debris. The intact cells were gated and analyzed for the percentage of antigen positive cells. In the absence of foscarnet, 34% of the cells are expressing the IE antigen. In the presence of 200 μM foscarnet, only 17% of the cells are expressing the IE antigen.


0 μM FOSCARNET
FW-SC
Total: 10986
A
250
200
150
100
50
0
0
50
100
150
200
250
RT-SC
Events
Total 9681
34%
500
400
300
200
100
0
1
10
100
1000
FITC / GR-FL
200 μM FOSCARNET
FW-SC
Total: 10487
A
250
200
150
100
50
0
0
50
100
150
200
250
RT-SC
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17%
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300
200
100
0
1
10
100
1000
FITC / GR-FL

Fig. 2.

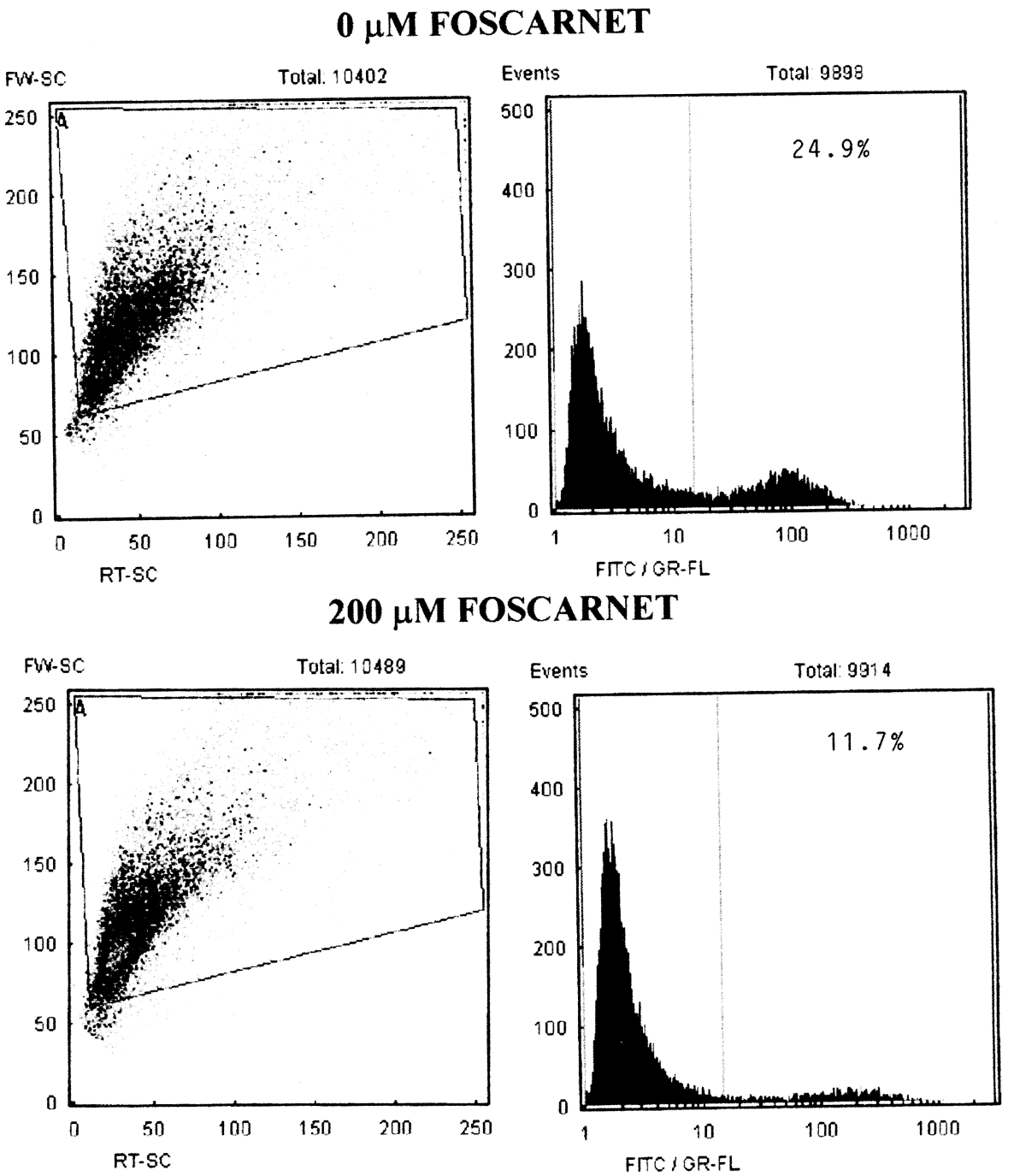

Fig. 3. Effect of foscarnet on late antigen synthesis in cells infected with a foscarnet susceptible clinical isolate. The same as in Fig. 2 except that a monoclonal antibody to the late antigen was used to identify virus infected cells.

cells infected with a foscarnet susceptible clinical isolate. Figs. 2 and 3 illustrate a typical analysis of the effect of foscarnet on the percentage of cells synthesizing the IE (Fig. 2) or late (Fig. 3) antigen. The monoclonal antibody to the HCMV IE antigen (E-13, M-810) used in these experiments recognizes an epitope that is shared by both IE-1 and IE-2 HCMV antigens (Mazeron et al., 1992). It has been widely used for identification of HCMV infected cells and seems to detect clinical isolates obtained throughout the world. A monoclonal antibody to the late antigen, gB, is used to detect HCMV infected cells. Monoclonal antibodies to both IE and late antigens are commercially available from Chemicon International, Temecula, CA, as well as other companies. For the experiment shown in Figs. 2 and 3, HFF cell monolayers were infected with a foscarnet sensitive HCMV clinical isolate. When 90% of the cells in the monolayer exhibited CPE, the cells were harvested and counted to determine the number of cells/ml. Medium containing various concentrations of foscarnet (0, 50, 100, 200, 400, and 800 μM) was added to 25 cm^2 flasks containing confluent monolayers of HFF cells. Then 10^3–10^4 HCMV infected cells were added to each flask. After incubation at 37°C for 144 h, the cells were removed from the flasks and prepared for flow cytometric analysis as described above for cultured cells. The data in Figs. 2 and 3 show the effect of increasing concentrations of foscarnet on the percentage of cells synthesizing the IE or late antigens. In the absence of foscarnet, 34% of the cells are positive for the IE antigen and in the presence of 200 μM foscarnet, only 17% of the cells are positive for the IE antigen (Fig. 2). In the absence of foscarnet 24.7% of the cells are positive for the late antigen and in the presence of 200 μM foscarnet only 11.7% are positive for the late antigen (Fig. 3). Using the percent reduction data obtained from all six foscarnet concentrations tested, the IC_{50} values for this clinical isolate were 99.58 μM foscarnet based on the IE antigen and 102.06 μM foscarnet based on the late antigen. These two IC_{50} values are in excellent agreement and demonstrate that, under conditions of low MOI, IC_{50} values can be determined using either the IE antigen or late antigen. Furthermore, the ability to use the IE antigen to measure IC_{50} values lends itself to analysis after only 72–96 h of incubation with the antiviral drug (McSharry et al., 1998b). The IC_{50} value for this clinical isolate for foscarnet by the PRA was 91.27 μM foscarnet. These results show that under these experimental conditions (MOI of 0.01) the IC_{50} values derived from the flow cytometry drug susceptibility assay using either the IE antigen or the late antigen are in excellent agreement with each other and are only slightly higher than the IC_{50} values obtained with the PRA.

To demonstrate the ability of the flow cytometric drug susceptibility assay to distinguish between drug susceptible and drug resistant HCMV clinical isolates, HFF cell monolayers were infected with cells containing a foscarnet sensitive clinical isolate or a foscarnet resistant clinical isolate in the presence of 0 or 800 μM foscarnet and processed for flow cytometry. Fig. 4 illustrates the analysis of a foscarnet sensitive HCMV clinical isolate (upper panels) and a foscarnet resistant HCMV clinical isolate (lower panels). The analysis demonstrated that in the presence of 0 μM foscarnet, 28.9% of the cells infected with the sensitive isolate expressed the IE antigen and in the presence of 800 μM foscarnet, only 7.2% of the cells expressed the IE antigen. When cells infected with a foscarnet resistant clinical isolate were analyzed (lower panels) 95.0% of the cells were IE antigen positive in the presence of 0 μM foscarnet and 72.3% of the cells were antigen positive in the presence of 800 μM foscarnet. In the presence of 800 μM foscarnet, there was less than a 50% reduction in the percent of antigen positive cells for the foscarnet resistant clinical isolate, confirming that it is resistant to foscarnet. These results show that the assay can clearly distinguish between sensitive and resistant clinical isolates.

The broad applicability of this assay was demonstrated when it was used to determine the susceptibilities for a set of 35 HCMV clinical isolates to six different anti HCMV compounds (McSharry et al., 1998c,d, 1999a,b). A comparison of the average IC_{50} values obtained by the flow cytometry assay and the PRA for these six drugs are presented in Table 1. The data show

that, on a micromolar basis, the antiviral activity of these compounds is CMV423 > 127025 > 1263W94 > cidofovir > ganciclovir > foscarnet. There is excellent correlation between IC_{50} values obtained using the IE and late antigens and between the flow cytometry assay and the PRA for individual drugs. Determination of the drug susceptibility of HCMV clinical isolates was independent of the MOI for most of the drugs tested. However, for some drugs, increasing the MOI above 0.01 had a drastic effect on the ability of the drug to inhibit the synthesis of either IE or late antigens. Therefore, the drug susceptibility assay for cell associated clinical isolates of HCMV should be performed at an MOI of 0.001–0.01. MOIs < 0.001 do not lead to a sufficient number of virus infected cells to perform the susceptibility assay.

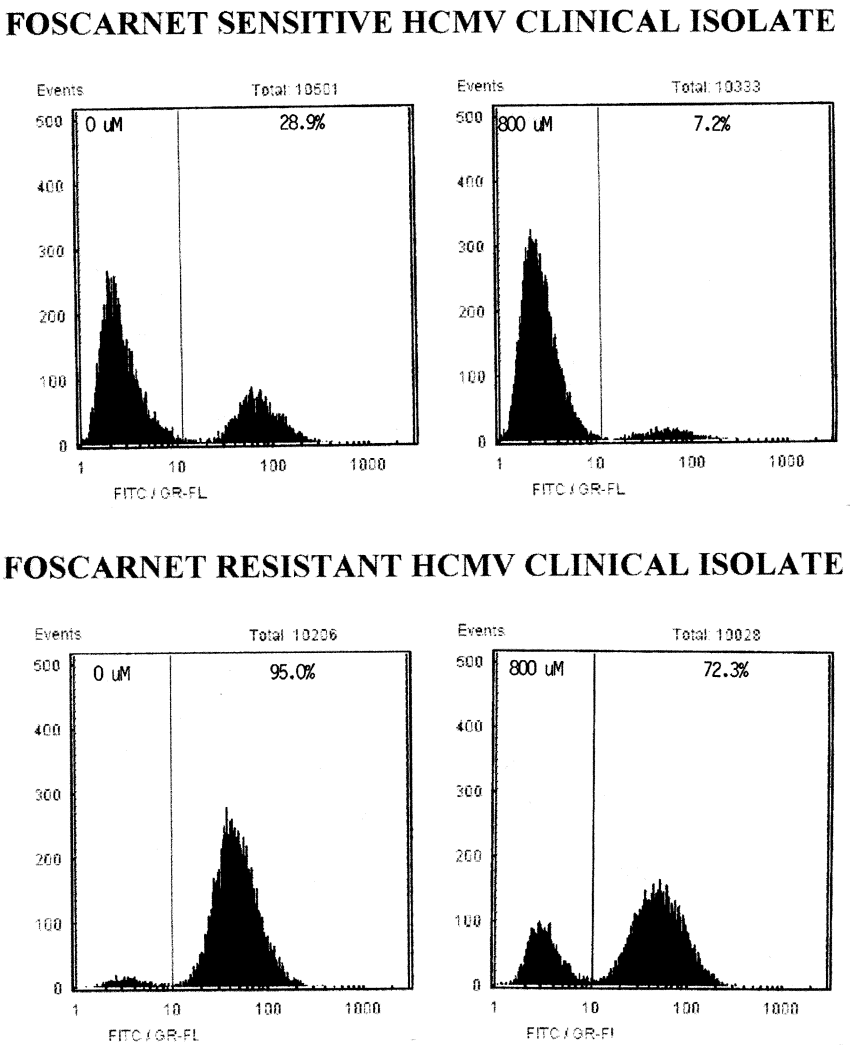

Fig. 4. Effect of foscarnet of the synthesis of IE antigen in cells infected either with a foscarnet susceptible or foscarnet resistant clinical isolate of HCMV. The same as in Fig. 2. Upper panels: foscarnet sensitive clinical isolate; drug treatment reduces the percentage of antigen positive cells from 28.9% in the absence of drug to 7.2% in the presence of 800 μM drug. Lower panel: foscarnet resistant clinical isolate; drug treatment reduces the percentage of antigen positive cells by less than 50% indicating that this is a drug resistant clinical isolate.

Table 1
Comparison of average EC_{50} values for six drugs from HCMV clinical isolates[a]

Drug	Flow cytometry IE antigen (μM)	S.D.	Flow cytometry late AG (μM)	S.D.	PRA (μM)	S.D.
CMV423	0.031	±0.016	0.018	±0.012	0.028	±0.022
127025	0.0497	±0.039	ND	–	ND	–
1263W94	0.379	±0.203	0.384	±0.138	0.312	±0.094
CID	0.33	0.09	0.706	±0.28	0.704	±0.29
GCV	4.93	±1.43	3.76	±1.65	2.77	±1.47
FOS	241.00	±90.92	191.08	±82.50	140.67	±42.24

[a] ND, not determined. CMV423 and 127025 are experimental drugs from Rhone-Poulenc-Rorer and 1263W94 is an experimental drug from GlaxoWellcome. CID, cidofovir; GCV, ganciclovir; and FOS, foscarnet.

In summary, the flow cytometry drug susceptibility assay for HCMV clinical isolates is accurate, rapid, quantitative and potentially automatable. It can be used to measure the susceptibility of HCMV to several antiviral compounds that may have different modes of action. The assay readily distinguished between drug susceptible and drug resistant laboratory strains and clinical isolates. Finally, the assay yields IC_{50} values that are equivalent to those obtained with the PRA. Two caveats that should be kept in mind concerning this assay are: (1) the flow cytometry drug susceptibility assay readily yields IC_{50} values, however, the assay seldom yields IC_{90} or IC_{95} values, values that are achievable with the PRA; and (2) the MOI can effect the IC_{50} value, i.e. higher MOI will lead to higher IC_{50} values. Standardization of the technique will require a constant MOI, a standard time of incubation, and a standard cell type for use in the assay. Comparisons between IC_{50} values obtained with the flow cytometry drug susceptibility assay and other drug susceptibility assays need to be performed in order to validate the assay flow cytometry assay. Furthermore, clinical validation of the assay for HCMV will require prospective studies that will correlate the drug susceptibility of the clinical isolate with clinical outcome. These studies are being carried out in collaborations between the author's laboratory and a number of clinical centers.

4. Herpes simplex viruses (HSVs)

4.1. Detection of HSV infected cell cultures

HSVs are seldom found in the circulation and there are not enough antigen positive cells in vesicular fluid for flow cytometric analysis. Therefore, flow cytometric analysis of cultured cells infected with HSV in vitro is the only practical use of this technology. Flow cytometric analysis of methanol permeabilized HSV infected cells that were treated with PI demonstrated the effect of virus infection on the binding of PI to DNA in virus infected cells (Rosenthal et al., 1987). Uninfected and HSV infected cells were permeabilized with methanol, treated with RNAse and PI to specifically stain the DNA, and the PI fluorescence intensities were measured by flow cytometry. Uninfected cells showed a typical DNA profile of increasing fluorescence intensity indicative of the G_o/G_1, S and G_2/M portions of the cell cycle. In contrast, HSV infected cells showed a broad peak of fluorescence intensity where the three peaks had coalesced into one broad peak. These authors also showed that this assay could be used to determine the effect of antiviral drugs on HSV replication. When HSV replication was blocked by antiviral drugs, the DNA profile resembled uninfected cells instead of HSV infected cells. Therefore, this method could be used as an inexpensive method for screening antiviral compounds.

The light scattering properties and fluorescent intensities of HSV-2 infected Vero cells and HSV-2 transformed mouse embryo cells were compared by flow cytometry (Goolsby et al., 1988). Flow cytometric analysis of the light scatter patterns of HSV-2 transformed mouse embryo cells and HSV-2 infected Vero cells showed little difference. However, when these cells were treated with a polyclonal antibody raised in goats to HSV-2 virions followed by FITC-labeled rabbit antigoat antibody, the HSV-2 infected Vero cells showed increased fluorescence intensity compared to the transformed cells. These results showed that uninfected cells could be distinguished from HSV infected cells by the increased fluorescence intensity associated with the fluorochrome labeled antibody. In addition, they showed that the HSV-2 transformed cells did not produce HSV-2 structural proteins.

Another report showed that flow cytometry could be used to detect and quantitate HSV infected cells in culture (McSharry et al., 1990). HSV-1 and HSV-2 clinical isolates were used to infected MRC-5 cells. After overnight incubation, the cells were harvested, permeabilized, and treated with type specific monoclonal antibodies to IE and early antigens followed by FITC-labeled goat anti-mouse antibodies and PI and analyzed for the percent of antigen positive cells by flow cytometry. The data showed that uninfected cells could be distinguished from HSV infected cells. These authors suggested that by using type specific antibodies to HSV-1 or -2 this procedure could be used to rapidly identify and type HSV infections. The flow cytometry assay for detecting HSV infected cells can be performed after overnight incubation with the clinical isolate making this a very rapid assay. Furthermore, the flow cytometer does all of the quantitative analysis making this assay far less labor intensive than isolation of virus in cell culture and quantitation with the plaque assay.

4.2. Drug susceptibility assays for HSV clinical isolates

4.2.1. PRA

Currently used phenotypic assays for herpes viruses involve isolation of infectious virus from clinical specimens by inoculation of cell cultures and the production of CPE. A bead agglutination assay specific for HSV can be used to confirm the infection. Herpes simplex viruses replicate rapidly in tissue culture and the virus is released from the cells into the medium. After 5 days in culture a sufficient amount of cell free virus is usually available to determine drug susceptibility. After determining the virus titer by plaque assay or some other titration assay such as dye release (Denizot and Lang, 1986) or end point dilution assays, the drug susceptibility of the isolate is determined by the PRA. Approximately 30–60 PFU are added to a monolayer of susceptible cells in a 24 well plate. After a 2 h adsorption period, the inoculum is removed and agarose containing various concentrations of the antiviral drug is added to different wells. After incubation for a week, the monolayers are stained with neutral red, and the plaques formed at each drug concentration are counted with the naked eye. The percent reduction in the number of PFU is calculated and plotted against the drug concentration to determine the IC_{50} and IC_{90} values for each clinical isolate. For HSV-1 and -2, this procedure takes approximately 2 weeks to a month from the time the clinical isolate was obtained from the patient and sent to the laboratory for virus isolation. A number of papers have reported on modifications of this phenotypic assay where they shortened the time for the PRA and limited the number of drug concentrations used to determine drug susceptibility of clinical isolates (Safrin et al., 1994; Tebas et al., 1998; De La Iglesia et al., 1998). Each of these assays still required microscopic observation of PFU to determine the susceptibility of the isolate to antiviral drugs.

4.2.2. Flow cytometry drug susceptibility assay

A flow cytometry based drug susceptibility assay for HSV type 1 was recently developed (Pavic et al., 1997). These investigators used monoclonal antibody to the HSV glycoprotein, gC, and flow cytometry to study the effect of acyclovir, ganciclovir, and foscarnet on the number of cells synthesizing this late antigen. They demonstrated that they could determine IC_{50} values for a number of clinical isolates that were similar to the IC_{50} values determined by cytopathic effect assay. In the flow

cytometry assay, they could determine IC_{50} values with MOI over a 500-fold range. However, higher MOI yielded IC_{50} values that were slightly higher. Of particular interest was the ability to detect 1–2% resistant viruses in a population of susceptible viruses. These results suggest that flow cytometry may replace the more labor intensive and time consuming PRA or dye up take assays for the determination of drug susceptibility of HSVs.

In summary, these reports show that monoclonal antibodies and flow cytometry can be used to detect HSV infected cultured cells and to determine the susceptibility of HSV-1 to various antiviral drugs. The flow cytometry based IC_{50} values for antiviral drugs for HSV-1 compare favorably with those obtained by the PRA. The assays are rapid, quantitative and easy to perform.

5. Human immunodeficiency virus (HIV)

5.1. Detection of HIV in clinical samples

5.1.1. Fluorochrome labeled monoclonal antibodies and flow cytometry

A number of investigators have used monoclonal antibodies to HIV p24, Nef, p17 or gp120/41 antigens and flow cytometry to detect HIV infected cells in peripheral blood obtained from HIV seropositive patients (McSharry, 1994). These studies showed that the percentage of antigen positive PBMCs increased with disease progression and the number of $CD4^+$ T helper cells, a surrogate marker for disease progression, was inversely proportional to the number of antigen positive cells. When patients were put on effective antiviral therapy, the percentage of antigen positive cells decreased. These results suggested that fluorochrome labeled monoclonal antibodies and flow cytometry could be used for rapid diagnosis of HIV infection and to monitor therapy. Although these assays detected HIV infected cells in AIDS patients they did not consistently detect HIV infected cells in asymptomatic HIV infected patients. Thus, this assay has not lived up to its promise because, as performed, it did not have the sensitivity to detect virus infected cells in asymptomatic HIV infected patients.

5.1.2. PCR amplification and analysis of fluorescent beads by flow cytometry

A number of procedures involving PCR and flow cytometry were developed to improve the sensitivity for detection of HIV infected cells in clinical specimens obtained from HIV seropositive patients (Yang et al., 1994, 1995; Dorenbaum et al., 1997). Proviral DNA was extracted from HIV infected cells, amplified by heminested PCR using biotynilated primers, hybridized to digoxigenin-labeled dUTP probes and then bound to beads via a strepavidin linkage. The beads containing HIV proviral DNA were incubated with an FITC-labeled antibody to digoxigenin and the fluorescent beads were detected and quantitated with flow cytometry. This assay proved useful for detecting HIV infected cells in HIV seropositive patients and in babies born to HIV infected mothers.

5.1.3. In situ PCR and RT-PCR and analysis by flow cytometry

HIV infected cells in peripheral blood were detected by in situ PCR amplification of DNA and/or RNA in peripheral blood cells followed by in situ hybridization with fluorescein-labeled oligonucleotide probes and flow cytometry (Patterson et al., 1993; Re et al., 1994; Gibellini et al., 1995; Patterson et al., 1995). These investigators used digoxigenin–dUTP in the PCR reaction and FITC-labeled antibody to digoxigenin to detect cells with amplified proviral DNA or RT-PCR amplified viral RNA. The early studies using this technique showed that between 2 and 15% of the peripheral blood cells obtained from HIV seropositive patients contained proviral DNA with fewer cells containing viral RNA. As the procedures for detecting PCR amplified DNA and RT-PCR amplified RNA improved, and better permeabilizing reagents such as Permeafix (Ortho-Diagnostic Systems) became available, it was possible to label the cell surface to phenotype the cells and to permeabilize the cells for in situ PCR. Using these techniques these authors demonstrated that 17.3–55.5% of the $CD4^+$ cells obtained from HIV infected individuals contained HIV proviral DNA. These results suggest that using PCR to amplify proviral DNA or RT-PCR to amplify HIV RNA and fluorochrome labeled

DNA probes to detect the amplicons is a feasible method for detecting HIV infected PBMCs obtained from HIV seropositive patients. Furthermore, this technique can be used to monitor the effect of antiviral drug treatment on the percent of HIV infected cells (Re et al., 1994).

5.1.4. Fish method to identify HIV RNA in PBMC and analysis by flow cytometry

In a different flow cytometric approach, a direct FISH assay employing a cocktail of 5′-carboxyfluorescein-labeled probes that cover large segments of the expressed HIV RNAs was used to detect HIV RNA in PBMCs from HIV seropositive and HIV-seronegative individuals (Patterson et al., 1998). The phenotype of the cells was determined simultaneously with monoclonal antibody to either CD4 for T helper cells or CD14 for monocytes. CD14 labeled monocytes expressed HIV RNA whereas $CD4^+$ T cells that were positive for proviral DNA on the basis of PCR amplification described above, did not express viral RNA. These finding suggest that the vast majority of HIV infected $CD4^+$ T cells do not express HIV RNA and that $CD14^+$ monocytes are the source of HIV viral RNA found in the plasma. Thus, in the future, using PCR, RT-PCR or FISH assays flow cytometry may play a more meaningful role in the diagnosis and management of HIV infected patients.

5.2. Drug susceptibility assays for HIV

Nucleoside analogues such as AZT and 3TC, nonnucleoside analogues such as nevirapine and delavirdine, and protease inhibitors such as indinavir, saquinavir, ritonavir, and nelfinavir are available for treatment of HIV infections (De Clercq, 1997, 1998; Boden and Markowitz, 1998). Current combination therapy uses two nucleoside analogues, AZT and 3TC, and one or more protease inhibitors (De Jong et al., 1998). Use of these antiviral agents leads to the selection of HIV mutants that are resistant to these drugs.

5.2.1. Current methods for drug susceptibility testing for HIV

Genotypic assays use genetic probes to detect specific mutations associated with resistance to a specific drug (Cockerill, 1999). Cell free virus in plasma is amplified by RT-PCR then genetic probes specific for each mutation are annealed to the DNA. Since the common mutations associated with resistance to these drugs are known, the probes are useful for detecting known mutations. However, unusual mutations will not be detected with these methods. In another method, the genes for RT and protease are amplified from HIV obtained from patients and transfected into a cell line that contains a replication defective HIV. The genes for RT and protease recombine to form infectious virus via homologous recombination. This cell line is screened against various concentrations of antiviral compounds to determine the susceptibility of the isolated virus to these drugs. Finally, silicon chips containing many genetic sequences are available to screen HIV genomes. These genotypic assays are often rapid and automated. However, they will only detect known mutations. Novel mutations will not be detected by any of these methods. Phenotypic assays attempt to grow HIV in the presence of various concentrations of antiviral drug. The virus is isolated from plasma or peripheral blood, incubated with PHA stimulated PBMCs obtained from HIV seronegative individuals and the production of p24 antigen in the supernatant over the following 14 days is measured by an ELIZA test. The assay is sensitive allowing for the detection of HIV infected cells in approximately 95% of HIV seropositive patients (Lathey et al., 1994; Fiscus et al., 1995). However, it is time consuming and labor intensive. Once a sufficient amount of virus has been produced it can be tested for susceptibility to various drugs. For drug susceptibility testing, a set amount of virus is incubated with PHA stimulated PBMC in the presence of various concentrations of drug. The concentration that inhibits the production of p24 antigen by 50% is the IC_{50}.

5.3. Flow cytometric susceptibility assay for HIV

To my knowledge, only one flow cytometric based assay for HIV drug susceptibility has been published (Gervaix et al., 1997). A stable reporter T cell line (CEM-GFP) that express CD4, CXCR4 and green fluorescent proteins (GFP) was established and used to detect the presence of HIV. These investigators used this cell line to determine the effect of antiretroviral drugs on the percentage of cells expressing GFP. They used two color immunofluorescence involving PE-labeled monoclonal antibody to gp120 and GFP expression and flow cytometry to show that CEM-GFP cells infected with HIV express both gp120 and GFP in a time dependent manner. Furthermore, they showed that AZT, nevaripine, and saquinavir reduced the percentage of HIV infected cells that expressed GFP. The analysis of GFP expression by flow cytometry correlated well with the amount of virus added to the CEM-GFP cells. The only drawback to this very exciting finding is that the CEM-GFP cells only express the CXCR4 co-receptor and may not allow infection with macrophage tropic viruses that use the co-receptor CCR5. This problem was recently alleviated when a number of permanent cell lines that express GFP under direction of the HIV LTR, CD4 and the HIV co-receptors, CXCR4 and CCR5 were constructed (Cecilia et al., 1998). These authors used these cell lines to determine the differential neutralization sensitivities of primary HIV isolates in the context of co-receptor usage. To my knowledge no papers have been published that describe the use of these cell lines to screen HIV clinical isolates for sensitivity to antiviral drugs, but their potential is great.

6. Summary

This mini review has demonstrated the use of flow cytometry for the direct detection of virus infected cells in clinical samples (HCMV and HIV) with the aim of using these procedures for rapid diagnosis of virus infections. Although these studies are in their infancy, the data presented suggest that these procedures, either as presented or with some modifications, could be used for the rapid diagnosis of viral infection. Comparisons between the flow cytometry based techniques and genetic probe techniques for the rapid diagnosis of viral infections will eventually determine which technology will carry the day. The remainder of the mini-review illustrated the use of flow cytometry for drug susceptibility testing. The data presented showed that the flow cytometry drug susceptibility assay for HCMV clinical isolates is rapid, easy to perform, and the IC_{50} values obtained with the flow cytometry assay correlate with those obtained with the PRA. Similar results were presented with the flow cytometry drug susceptibility assay for HSV type 1. Because of the dramatic rise in drug resistant HIV clinical isolates, there is great need for drug susceptibility testing for HIV. The one flow cytometry paper that was reviewed suggests a reasonable approach to HIV drug susceptibility testing. Using other modified cell lines with the primary HIV receptor, the two co-receptors, and GFP should begin to open up the field for the use of flow cytometry for HIV drug susceptibility testing. Furthermore, with the availability monoclonal antibodies to human T cell leukemia viruses (HTLV I and II), varicella zoster virus (VZV), Epstein Barr virus (EBV), human herpes viruses 6, 7 and 8 (HHV6, 7 and 8), similar phenotypic flow cytometry based drug susceptibility assays could be developed for antiviral compounds that inhibit the replication of these viruses.

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