Uses of Flow Cytometry in Virology

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INTRODUCTION

This review will focus on the uses of flow cytometry in the study of virus-cell interactions. This topic was last reviewed in 1990 (88, 98, 105). With the exception of simian virus 40 (SV40)-infected cells, there were few virus-cell systems that had been analyzed by quantitative flow cytometry. Since that time, a significant number of papers describing the use of this technology for basic, clinical, and applied research have been published. These include studies on (i) methods for permeabilizing virus-infected cells; (ii) methods for staining viral antigens on the cell surface, in the cytoplasm, and in the nucleus; (iii) effects of virus infection on cellular constituents; (iv) attachment of virus to cells; (v) detection and quantitation of virus-infected cells in vitro and in vivo; and (vi) applications to clinical virology. A few virus-cell systems have been studied in detail by multiparametric quantitative flow cytometry, and

these will be critically reviewed. The application of quantitative flow cytometry in the analysis of many other virus-infected cell systems is less well defined; however, that literature will be reviewed to demonstrate the broad applications of this technology for detecting and quantitating virus-infected cells.

BRIEF DESCRIPTION OF FLOW CYTOMETRY

Cytometry can be defined as the measurement of physical and/or chemical properties of cells. These measurements are often performed with a light or fluorescence microscope. Microscopy is qualitative but, with the exception of computerized image analysis (77), seldom quantitative. Flow cytometry is the measurement of the physical and/or chemical characteristics of cells while they pass single file in a fluid stream through a measuring apparatus (185). The fluorescence-activated cell sorter (FACS) was developed in the early 1970s to study isolated populations of viable cells for immunology. The original instruments were large, contained powerful dual lasers that were water cooled, and were able to analyze and sort cells. Although flow cytometry continues to function in the field of immunology, its use in other fields such as cell biology,

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FIG. 1. Diagram of a flow cell with attached optical systems. As the cells pass through the flow cell (the small black square box in the center left), a laser beam intersects the stream of cells and scatters light. FALS passes through the forward light scatter lens, and the energy is collected by the forward light scatter photodiode. RALS passes through the objective lens, the beam splitter, laser line filter, and diffuser, and the energy is collected by the right-angle scatter photomultiplier tube (PMT). If the cells are labeled with fluorescent molecules, the laser will excite these molecules, which emit light at higher energies. The emitted energies of different wavelengths pass through the objective lens and various filters and are collected and amplified by the various photomultiplier tubes. The amplified signals are converted into digital information and stored in a computer for further analysis. The numbers refer to the following: 1, beam splitter; 2, laser line filter, 396- to 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- to 530-nm band pass; 7, dichroic mirror 2, 610-nm long pass; 8, orange filter, 565- to 592-nm band pass; and 9, red filter, 660-nm long pass.

molecular biology, pathology, and diagnostic microbiology is on the increase. With the expanded use of flow cytometry for studies that do not require the cell sorting capabilities of a FACS instrument, the machinery has been simplified to a more compact, air-cooled, single-laser, analytical instrument. During analysis, the sample is completely contained within the instrument, leading to a degree of operator safety. Currently available multiparameter flow cytometers use a single laser beam to allow the simultaneous quantitative measurement of a number of cellular properties, including cell number, cell size, cellular granularity, and up to three different fluorochromes per cell. In addition to the number of fluorescently labeled cells, the amount of each fluorochrome associated with each cell can be measured by flow cytometry. Since a flow cytometer can analyze cells at rates up to several thousand cells per second, it is possible to analyze a statistically significant number of cells in a short time. Modern analytical flow cytometers with fixed optical benches and internal computers allow the operator to set parameters for various analyses with ease and to store these parameters in internal computers to be recalled when needed. Dual-laser flow cytometers can detect additional fluorescent molecules, but they are not required for the types of analysis presented in this review.

Figure 1 illustrates the business end of a modern, single-laser, analytical flow cytometer. It is a diagram of the argon ion laser and the rectangular flow cell (the small black square on the left center of the diagram) with attached optical assembly, including lenses, mirrors, filters, and photomultiplier tubes. All flow cytometers have four basic components: (i) a fluidic system to carry a suspension of single cells from the sample tube into a quartz flow cell containing sheath fluid; (ii) a focused light source, usually an argon ion laser with an excitation energy of 488 nm; (iii) filters and photodetectors to collect scattered light and light emitted from fluorescently labeled cells and processors to convert these light signals to analog electrical impulses and then to digital signals; and (iv) a computer system to collect, store, and analyze the data. Cells to be measured are introduced into the sheath fluid that keeps the cells in the center of the quartz flow cell and forces the cells to flow single file at uniform speed through a small orifice. The cells pass by a point where the laser intersects the path of the fluid stream. As each cell passes this point, the laser light will be scattered. The scattered light is collected as forward-angle light scatter (FALS), which yields information on the number and size of cells, and right-angle light scatter (RALS), which yields information on the granularity of cells. If the cells are

stained with one or more fluorescent labels, the laser will excite the fluorescent molecules that emit energy at higher wavelengths. For example, fluorescein has a peak emission spectrum at 520 nm; phycoerythrin, at 580 nm; and propidium iodide (PI), at 580 and 620 nm (149). The emitted energies of different wavelengths pass through filters (green filter, 515- to 530-nm band pass; orange filter, 565- to 592-nm band pass; red filter, 660-nm-long pass) that allow light of only a particular wavelength to pass; then they are collected and amplified by photomultiplier tubes. The amplified signals are converted into digital information which is stored in a computer for further analysis and printed in graph form as a hard copy. For more detailed information on the mechanics and practical uses of a flow cytometer, the reader is referred to references 149, 185, 208, and 212.

With the advent of monoclonal antibodies, flow cytometry began to be useful in the clinical laboratory, particularly for quantitating the various subsets of lymphocytes. Today, one of the major uses of flow cytometry in the clinical laboratory is the determination of the number of CD4⁺ cells in normal and human immunodeficiency virus (HIV)-infected patients. Despite some uncertainties concerning the use of CD4 cell counts as surrogate markers in HIV-infected individuals (99), the absolute number and percentage of CD4⁺ cells are being used as surrogate markers for defining which HIV-infected individuals will be classified as having AIDS and for determining those who will be treated with antiretroviral chemotherapy (28). Excellent reviews on the use of flow cytometry for phenotyping cells in health and disease have been published (28, 44, 64, 69, 101, 109, 137, 148, 212).

SAMPLE PREPARATION AND PERMEABILIZATION METHODS

Intact single cells are the major requirement for flow cytometric analysis of virus-infected cells. Blood, bronchoalveolar lavage (BAL), and urine specimens are ideal because they are single cell suspensions. Soft organs, lymph nodes, and spleens can be mechanically manipulated to release single cells. Cells obtained from suspension cultures or cultured cells removed from solid surfaces with EDTA and/or trypsin and resuspended as single cells are suitable for analysis. Many of the cell surface proteins that serve as markers in flow cytometry are removed with enzymatic treatment of cells in tissues or cultured cells grown on solid supports, making it impossible to stain cells for these surface markers. However, if only cytoplasmic and/or nuclear antigens are to be detected and quantitated, treatment with proteolytic enzymes under conditions that yield intact cells can be useful for obtaining single cell suspensions from cultures or tissues.

When cytoplasmic and/or nuclear antigens or the nuclear DNA content of cells is to be studied by flow cytometry, methods to permeabilize the cell must be employed to allow monoclonal antibodies and hydrophilic molecules, such as PI, to diffuse into the cell's interior. Most of the published work on permeabilizing virus-infected cells for analysis by flow cytometry has employed methanol. Jacobberger et al. (89) used 90% methanol to permeabilize SV40-transformed and spontaneously transformed Chinese hamster ovary (CHO) cells so that the cytoplasmic and nuclear compartments of the cell were permeable to both antibodies and the nucleic acid binding dye, PI. Intact cells with 2 N (diploid) or greater DNA content were identified by the amount of PI bound to the DNA after RNase treatment, allowing for a distinction between intact cells and

debris. By using high-affinity monoclonal antibodies to the SV40 T antigen, affinity-purified fluorescently labeled second antibodies, and 50% goat serum in all reactions, these researchers were able to reduce the fluorescent background so that they could attain excellent coefficients of variation. Cell clumping was minimized by keeping the cells cold and adding goat serum containing trace amounts of Triton X-100. Methanol permeabilization yields optimal staining of cellular DNA by PI and retains cytoplasmic and nuclear antigens in a state in which they remain reactive with polyclonal and monoclonal antibodies. A major advantage of this procedure is that cells in 90% methanol can be stored at -70°C for more than a year without detectable loss of internal antigens or DNA content. This permeabilization procedure has been used successfully for many studies on SV40-infected and -transformed cells as well as on many other virus-infected cells including human lymphocytes (13, 38, 54, 78, 80, 89, 96, 97, 103, 104, 106, 124, 125). Major disadvantages of the methanol fixation technique are that it destroys the light scatter properties of cells, reduces the antigenicity of cell surface proteins, and causes destruction of some cells. Figure 2 illustrates the effect of methanol treatment on the light scatter properties of leukocytes obtained from a healthy person. Panel A depicts the FALS (FW-SC) and RALS (RT-SC) patterns of fresh leukocytes treated with Ortho-mune lysing reagent (Ortho Diagnostic Systems, Inc., Raritan, N.J.) to lyse erythrocytes. Note the good separation of lymphocytes (L), monocytes (M), and granulocytes (G). Panel B shows the FALS (FW-SC) and RALS (RT-SC) patterns of leukocytes obtained from the same individual. In this case, the cells were treated with methanol after erythrocyte lysis by Ortho-mune lysing reagent. Note that the cells have lost their ability to be separated by their light scatter properties in the flow cytometer and have fallen down to the bottom left and right portions of the graph.

To overcome some of the disadvantages of methanol treatment, Schimenti and Jacobberger (169) have compared a number of combinations of paraformaldehyde and methanol for permeabilizing CHO, HL60, and NIH 3T3 cells. Pretreatment of cells with paraformaldehyde before methanol treatment stabilized the cells, gave good coefficients of variation for DNA staining with PI, and allowed indirect immunofluorescence staining of SV40 T antigen in the cell nucleus. However, in comparison to methanol treatment alone, the binding of PI to DNA was decreased and the amount of indirect immunofluorescence staining of T antigen in the nucleus was increased. Police et al. (154), who used 0.25% paraformaldehyde before staining with PI, also reported decreased binding of PI to cellular DNA.

A recent report describes a permeabilizing reagent that overcomes many of the disadvantages of the methanol procedure. Connelly et al. (33) used Permea-Fix (Ortho Diagnostic Systems) to permeabilize human leukocytes in whole blood. They report that, after Permea-Fix treatment, (i) leukocytes scatter light at least as well as untreated cells; (ii) CD3, CD4, and CD8 cell surface antigens can be labeled with fluorescently labeled monoclonal antibodies; and (iii) cytoplasmic antigens, such as vimentin or the HIV p24 core antigen, can be labeled with specific monoclonal antibodies. HIV-infected and uninfected H9 cells treated with Permea-Fix can be separated from each other by detection of intracytoplasmic p24 antigen labeled with a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody. Furthermore, CD4⁺ human mononuclear cells infected with HIV in vitro, treated with Permea-Fix, and stained with phycoerythrin (PE)-labeled OKT4A monoclonal antibody and FITC-labeled monoclonal or polyclonal antibody to the HIV p24 antigen can be distinguished from

FIG. 2. Effect of methanol treatment on light scatter properties of lymphocytes. Whole blood was obtained from a healthy individual. (A) Contour map of whole blood cells treated with Ortho-mune lysing reagent followed by flow cytometric analysis of the leukocytes. Lymphocytes (L), monocytes (M), and granulocytes (G) are separated on the basis of their FALS (FW-SC) and RALS (RT-SC) properties. Note the separation of lymphocytes, monocytes, and granulocytes. (B) Contour map of whole blood cells treated with Ortho-mune lysing reagent followed by 90% methanol and analysis of the FALS and RALS properties of the cells by flow cytometry. Note that the cells have lost their ability to be separated by FALS and RALS and are near the bottom and sides of the graph.

other mononuclear cells on the basis of CD4 antigen staining and from uninfected CD4⁺ cells on the basis of FITC-labeled p24 antigen. The reagent can be used on whole blood and seems to be considerably better than the other systems tested for retaining the light scatter properties of human blood cells while still allowing cell surface and cytoplasmic antigens to be stained with monoclonal or polyclonal antibodies directed against these antigens. Figure 3 illustrates the use of Permea-Fix for determining light scatter, cell surface and cytoplasmic labeling with monoclonal antibodies, and nuclear DNA labeling with PI of leukocytes obtained from an AIDS patient. One-tenth milliliter of blood was used for each determination. Figure 3 shows that Permea-Fix allows separation of lymphocytes from other leukocytes in whole blood by light scatter; the T3 cell surface marker can be stained with PE-labeled monoclonal antibody OKT3 (approximately 75% of the cells in the lymphocyte gate stained with this monoclonal antibody); the FITC-labeled isotype control antibody did not stain the PI

staining cells in the lymphocyte gate; and the monoclonal antibody to the HIV p24 antigen stained approximately 13% of the PI staining cells in the lymphocyte gate. Thus, Permea-Fix can be used to fix and permeabilize cells so that all of the parameters of the flow cytometer can be used for analysis of virus-infected cells.

Permea-Fix and some other reagents used to permeabilize cells contain formaldehyde or paraformaldehyde, which reduces the binding of PI to DNA. Since PI binding to DNA is often used to distinguish debris from cells and to gate on cells with 2 N or higher DNA content for analysis of cell-associated antigens, the effect of various fixatives on PI binding to DNA is an important consideration. Figure 4 illustrates a comparison of four permeabilization reagents on the binding of PI to human mononuclear cells: methanol treatment (panel A) provides the highest fluorescence intensity due to PI binding, pretreatment with paraformaldehyde (panel B) reduced the fluorescence somewhat, and the other treatments yielded considerably less binding of PI to DNA.

Other techniques for fixing and permeabilizing cells have been reported, but most have not been used for flow cytometric analysis of virus-infected cells. These include pretreatment of cells with paraformaldehyde followed by treatments with 0.1 to 0.5% Triton X-100 (143), Tween 20 (79, 171), digitonin and Tween 20 (100), or 0.74% *N*-octyl- β -D-glucopyranoside (75). Some have used lysolecithin (47), FACS Lysing Reagent (Becton-Dickinson Immunocytometry Systems, Mountain View, Calif.) (197), or saponin (87) to permeabilize cell membranes. Although these techniques have allowed labeling of cell surfaces, with the exception of FACS Lysing Reagent, they do not result in cell populations that scatter light as well as untreated cells. These disadvantages could be overcome by labeling cell surfaces to identify a specific population of cells that could then be analyzed for the presence of internal cellular or viral antigens without the need for cell separation by light scatter (100). Permeabilized cells could be identified by binding of PI to DNA after RNase treatment and detecting viral antigens with fluorescently labeled antibodies (54). Alternatively, one could use presorted blood cells to study the internal viral antigens.

A number of different procedures can be used to permeabilize cells, and each investigator must choose the procedure that is best suited to his or her needs. Table 1 summarizes three recommended procedures for fixing and permeabilizing cells. The Permea-Fix procedure is excellent for cell surface, cytoplasmic, and nuclear staining with retained light scatter properties (33). FACS Lysing Reagent may also be effective for permeabilizing cells while retaining light scatter properties (197), but no reports of its use on virus-infected cells have been published. For labeling internal antigens and DNA without regard for cell surface antigen staining and light scatter properties, the cells may be stabilized with 0.25% freshly prepared paraformaldehyde followed by permeabilization with 70 to 90% methanol (154, 169). For cell surface and internal antigen staining without concern for good light scatter properties, theoretically, one could employ any of the detergent procedures listed above (47, 75, 79, 87, 100, 143, 171); however, these have not been extensively used for virus-infected cells, and it is not clear how they will work in new situations. Each procedure must be tried to determine how it satisfies individual requirements. Depending on the virus-cell and antigen-antibody combinations used, different procedures may yield different results. For ploidy studies, it is best to use only methanol, because paraformaldehyde impairs binding of PI to the DNA (154, 169).

FIG. 3. Light scatter, cell surface, and internal labeling of lymphocytes permeabilized by Permea-Fix. Whole blood was obtained from a patient with AIDS and permeabilized with Permea-Fix. One portion was treated with a PE-labeled OKT3 monoclonal antibody (Ortho Diagnostic Systems) and analyzed for light scatter and orange fluorescence by flow cytometry. (A) Graph showing separation of lymphocytes from other cells with the lymphocytes gated; (B) graph showing that most of the lymphocytes (75%) in the gate stain with the monoclonal antibody to the T3 cell surface marker. Another portion of the permeabilized cells was treated with an FITC-labeled isotype control (Chemicon International, Inc., Temecula, Calif.) and PI and analyzed for light scatter and two-color fluorescence by flow cytometry. (C) Graph showing that essentially all of the cells stained with PI (RD-FL) but not with the FITC-labeled monoclonal isotype control antibody (GR-FL). A third portion of permeabilized blood cells was treated with an FITC-labeled monoclonal antibody to the HIV p24 antigen (Chemicon International, Inc.) and PI and then analyzed for light scatter and two-color fluorescence by flow cytometry. (D) Graph showing that essentially all of these cells are stained with PI (RD-FL) and a portion (13.2%) of the cells are stained with both PI (RD-FL) and FITC (GR-FL).

STAINING CELL-ASSOCIATED VIRAL ANTIGENS AND NUCLEIC ACID

Surface antigens on unfixed virus-infected cells can be stained with fluorescent-antibody molecules. To prevent non-specific binding of antibodies to cells, the cells are treated with a blocking reagent, such as normal serum, which binds to all antigens and blocks antibodies from binding to all except their specific antigen. This procedure works because specific antibody of high avidity competes successfully for antigen-binding sites with the nonspecific antibodies in normal serum. Nonspecific antigens remain blocked by the antibodies in normal serum. For detecting and quantitating cell surface markers, cells are treated with monoclonal or polyclonal antibodies directed against these molecules. For the antibodies to be detected by the flow cytometer, they must be fluorescently

labeled. In the direct method, fluorescent molecules are covalently attached to the antibody. In the indirect method, unlabeled antibody is first bound to the antigen in question and then detected by reaction with fluorescently labeled antibody directed against the bound unlabeled antibody. For example, FITC- or PE-conjugated goat anti-mouse immunoglobulin G (IgG) is used to detect an unlabeled mouse monoclonal antibody bound to the antigen in question. After any unreacted fluorescently labeled molecules are washed away, the stained cells are detected and quantitated by flow cytometry. Up to three different surface labels can be detected with a single-laser instrument (101). Appropriate controls must be included to rule out nonspecific binding of antibodies to cells. These include unlabeled cells (to rule out background fluorescence) and fluorescently labeled nonspecific antibodies of the same

FIG. 4. Effect of permeabilization procedures on binding of PI to DNA. Fresh blood was obtained from a healthy person, and PBMC were isolated by centrifugation on Histopaque (Sigma Chemical Co., St. Louis, Mo.). The PBMC were divided into four portions, and each portion was permeabilized by a different procedure, treated with RNase and PI, and analyzed for red fluorescence by flow cytometry. The gain of the red photomultiplier tube was kept constant for the determination of PI binding by all four procedures. Shown is the fluorescence intensity of PBMC treated with methanol (A), paraformaldehyde followed by methanol (B), FACS Lysing Reagent (C), and Permea-Fix (D). The data show that methanol treatment permitted the highest binding of PI to DNA (A); pretreatment with paraformaldehyde followed by methanol (B) gave slightly lower fluorescence intensity, and treatment with FACS Lysing Reagent (C) and Permea-Fix (D) gave the same fluorescence intensity, which was approximately half that of methanol treatment alone.

isotype as the primary antibodies for the direct method or unlabeled heterologous antibodies of the same isotype followed by fluorescently labeled second antibodies for the indirect method. Paraformaldehyde is often added to the cell suspension after staining in an attempt to inactivate infectivity and to stabilize the cells before they are analyzed by flow cytometry (37, 141, 168). However, when virus-infected cultured cells or clinical specimens are examined, it is of utmost importance to follow universal precautions (e.g., buttoned lab coat, gloves, eye protection, etc.) because none of the fixatives will completely inactivate all viruses and other infectious agents under all circumstances (136).

For labeling cytoplasmic or nuclear components, the cells must be permeabilized by one of the procedures described above and then treated with monoclonal or polyclonal antibodies to the antigens of interest. These components can also be stained by the direct or indirect antibody staining method (185). For labeling the DNA, permeabilized cells can be treated with hydrophilic dyes, such as PI. The binding of PI to the cellular DNA is directly proportional to the amount of DNA in the cells and is useful for determining the ploidy of cells, i.e., 2N, 4N, or heteroploid. This reaction can be made specific for DNA by treating the cells first with RNase to destroy the RNA, leaving only DNA to interact with the PI.

This procedure has been used extensively to identify cells in G_0/G_1 , S, or G_2/M phase of the cell cycle or aneuploid cells on the basis of the amount of PI bound to DNA (185). A useful combination of fluorescent labels for these studies is FITC-conjugated antibody to viral antigen and PI to stain the DNA, because their emission spectra do not interfere with each other (149). When using PI, one must avoid skin contact because PI is a carcinogen.

STUDIES OF VIRUS-CELL INTERACTIONS, USING FLOW CYTOMETRY

Each week, papers reporting the use of flow cytometry to study virus-cell interactions are published. Thus, it would be both impossible and inappropriate to review all of the papers on this subject. Instead, I will review only a small number of papers to illustrate the utility of flow cytometry for these kinds of studies. Analyses of virus-cell interactions by flow cytometry have included (i) virus-induced programmed cell death, (ii) regulation of the expression of cellular antigens in response to virus infection, (iii) interaction of fluorescently labeled viruses with cells and identification of cell surface virus receptors, (iv) detection and quantitation of both viral and cell surface

TABLE 1. Recommended permeabilization procedures for virus-infected cells

Permeabilization procedure	Advantages	Disadvantages	Reference(s)
Methanol	Excellent permeabilization of plasma membrane and nuclear envelope; excellent PI binding to DNA. Cytoplasmic and nuclear antigens retain their ability to react with antibody over long periods of time when stored at -70°C .	Destroys light scatter properties of cells, removes cell surface antigens, and often destroys cells.	89
Paraformaldehyde-methanol	Excellent permeabilization of plasma membrane and nuclear envelope; increased stability of cells. Cytoplasmic and nuclear antigens retain their ability to react with antibody over long periods of time when stored at -70°C .	Destroys light scatter properties of cells; removes cell surface antigens; and reduces binding of PI to DNA	154, 169
Permea-Fix	Good permeabilization of plasma membrane and nuclear envelope. Cells retain light scatter properties; the ability to label the cell surface and to bind PI to DNA is retained. Both cytoplasmic and nuclear antigens retain their ability to react with antibody.	Reduces binding of PI to DNA.	33

antigens on virus-infected cells, and (v) identification and quantitation of cytoplasmic and/or nuclear viral antigens.

Virus-Induced Programmed Cell Death

Virus infection usually leads to destruction of cells via necrosis or persistent viral infection. However, some virus-cell interactions have been shown to lead to programmed cell death, or apoptosis. Table 2 lists the virus-cell interactions leading to apoptosis that are discussed below.

Historically, apoptosis has been characterized by morphological and molecular criteria (6, 7, 31). Apoptotic cells have shrunken nuclei with condensed chromatin tightly appending the nuclear envelope and a ruffled plasma membrane that remains intact. The DNA is fragmented due to endonucleolytic cleavage of the linker regions between nucleosomal cores (6). The fragmented DNA, isolated from apoptotic cells, can be detected by electrophoresis on agarose gels, on which a ladder effect is seen after the gel is stained with ethidium bromide.

Recently, flow cytometry methods have been developed to measure apoptosis (1, 43, 115). Cells undergoing apoptosis leak some of the small DNA fragments (1). When apoptotic cells are permeabilized and treated with RNase and PI, the apoptotic cells exhibit a PI binding peak with less fluorescence intensity than a normal G_0/G_1 peak (145). This is illustrated in Fig. 5, where the A_0 peak represents the fluorescence intensity of the PI binding of cells undergoing apoptosis. This procedure

is simple, rapid, inexpensive, and quantitative. It has been used to measure the increase in the percentage of apoptotic cells in peripheral blood mononuclear cells (PBMC) obtained from HIV-infected patients when the PBMC were incubated over time (145). A second method has been developed to specifically identify the fragmented DNA, using digoxigenin-dUTP and deoxynucleotidyl transferase to label the 3'-OH of the DNA segments and FITC-conjugated antibody to digoxigenin (83). The fluorescence intensity of the FITC-labeled DNA is quantitated by flow cytometry. A third method involves the detection of increased cell surface expression of the Fas antigen on apoptotic cells. Some apoptotic cells express the Fas antigen on their cell surfaces and have lower levels of bcl-2 expression (31). Flow cytometry has been used to quantitate the levels of the Fas antigen and bcl-2 expression in cells undergoing apoptosis (48, 200). Figure 6 illustrates the enhanced expression of the Fas antigen on gp160-transfected U937-2 cells (112). Both U937-2 cells (panel B) and the UE160 clone that was transfected with the gene for HIV gp160 (panel A) express the Fas antigen; however, a small percentage of the gp160-expressing cells have an enhanced expression of the Fas antigen, as indicated by the increased fluorescence intensity of that cell population (panel A). Thus, flow cytometric detection of two- and three-color fluorescence is becoming the method of choice for detecting apoptosis (172, 173).

In vitro and in vivo studies have implicated HIV in the induction of apoptosis in lymphocytes and bone marrow cells obtained from HIV-infected patients. In some cases, apoptosis is induced by viral antigens without the need for complete virus replication. Oyaizu et al. (145) used flow cytometry to measure the binding of PI to DNA to show that PBMC obtained from HIV type 1 (HIV-1)-infected individuals undergo apoptosis when cultured in vitro. Carbonari et al. (27) used flow cytometry to identify apoptotic lymphocytes obtained from AIDS patients. Apoptotic lymphocytes were identified on the basis of light scatter properties and cell surface markers, which showed that lymphocytes from AIDS patients undergo apoptosis when cultured in vitro, whereas those from healthy individuals do not. Phenotypic analysis of the lymphocytes undergoing apoptosis in vitro showed that the apoptotic cells were $\text{CD}8^+$ T cells and $\text{CD}19^+$ B cells, suggesting that the increased apoptosis seen in cultured lymphocytes from AIDS patients may not explain the slow decrease in $\text{CD}4^+$ T cells seen in HIV-

TABLE 2. Virus-cell interactions leading to apoptosis

Virus	Apoptotic cells	Reference(s)
HIV	EBV transformed, lymphoblastoid	48
HIV	$\text{CD}34^+$ bone marrow, progenitor	161, 213
HIV	$\text{CD}8^+$ T, $\text{CD}19^+$ B	27
HIV	gp160 expressing $\text{CD}4^+$ cells	112
HIV	PBMC	145
HIV	$\text{CD}4^+$	26
Simian immunodeficiency	PBMC	46
EBV, varicella-zoster	$\text{CD}45^+$ RO^+	2
Influenza	HeLa	200
LCMV	Murine T	160

FIG. 5. Detection of apoptotic cells by analysis of PI binding to DNA by flow cytometry. Peripheral blood was obtained from HIV-seropositive and HIV-seronegative individuals, the mononuclear cells were isolated by centrifugation over Ficoll-Hypaque density gradients, and the PBMC were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, L-glutamine, and antibiotics for 4 days. PBMC from each culture were permeabilized with 70% ethanol and then treated with RNase and PI. The red fluorescence intensity was measured by single-color flow cytometry. Shown is the fluorescence intensity resulting from PI binding to DNA of PBMC from an HIV-seronegative individual (A) and that of an HIV-seropositive individual (B). A distinct cell cycle region, A_0 , with less fluorescence intensity than the G_0/G_1 peak was denoted in both histograms but is considerably larger in the PBMC obtained from the HIV-seropositive individual. Reprinted from reference 145 with permission of the publisher.

infected individuals. Re et al. (161) showed that uninfected hematopoietic progenitor cells ($CD34^+$) obtained from AIDS patients underwent apoptosis when cultured in vitro in the presence of interleukin-3. No proviral DNA was present in these cells as determined by PCR with primers that identified as few as five proviral genomes in 8E5/LAV cells, which are known to contain only one HIV genome per cell. These results suggest that exposure of progenitor cells to other virus-infected cells, such as $CD4^+$ cells in the bone marrow, may induce apoptosis in the uninfected $CD34^+$ cells. Zauli et al. (213) showed that 2-h exposure of $CD34^+$ cells to HIV induced apoptosis as measured by PI binding to DNA analyzed by flow cytometry.

Cameron et al. (26) used flow cytometry to show apoptosis in $CD4^+$ T cells activated by HIV-infected dendritic cells. These

FIG. 6. Detection of the Fas antigen on cells undergoing apoptosis. The expression levels of surface Fas antigen on UE160 cells, which were transfected with the HIV envelope gene and express gp160 (A), and U937-2 cells (B) as analyzed by flow cytometry are shown. Cell suspensions from each sample were mixed with a mouse monoclonal antibody to the Fas antigen. Bound monoclonal antibody was revealed by FITC-conjugated mouse immunoglobulin antibodies and analysis by flow cytometry. The dotted lines represent the autofluorescence of cells treated with FITC-conjugated anti-mouse immunoglobulin antibody only. The darker solid line in each panel represents fluorescence intensity of the FITC-labeled Fas antigen. Note that both the U937-2 and the UE160 cells contain a significant portion of cells that express the Fas antigen; however, a small portion of the UE160 cells express the Fas antigen at higher fluorescence intensity than do the U937-2 cells that do not express the gp160 envelope gene of HIV. Reprinted from reference 112 with permission of the publisher.

results suggest a role for apoptosis in the decline of $CD4^+$ T cells early in HIV infection. De Rossi et al. (48) used flow cytometric analysis of PI binding to DNA and expression of bcl-2 and morphological and molecular analyses of DNA ladders to show that HIV-infected Epstein-Barr virus (EBV) lymphoblastoid B cells (Mt) expressing the gp120 cell surface antigen undergo apoptosis in culture whereas EBV lymphoblastoid B cells that do not express the gp120 surface antigen do not. These results implicate the HIV gp120 envelope protein in apoptosis of activated cells, in this case an EBV-infected B-cell line. As described in the legend to Fig. 6, Lu et al. (112) showed that $CD4^+$ cells expressing gp160 undergo apoptosis as measured by flow cytometric analysis of PI binding to DNA and reaction with anti-Fas antibody. These results suggest that lymphocytes that express HIV antigens, such as gp120 and gp160, can induce apoptosis in neighboring cells and could be a significant factor in the decline of $CD4^+$ cells in HIV-infected individuals.

del Llano et al. (46) showed that apoptosis in cultured mononuclear cells, as assessed by flow cytometric analysis of PI binding to DNA with the formation of the apoptotic peak, along with mitochondrial metabolic activity and proliferative responses to pokeweed mitogen, provides a prognostic tool for disease progression in simian immunodeficiency virus-infected rhesus macaques. Razvi and Welsh (160) showed that murine T lymphocytes infected with lymphocytic choriomeningitis virus (LCMV) undergo apoptosis after activation with anti-CD3 monoclonal antibody, suggesting a mechanism for the immune deficiencies associated with these infections in mice. Takizawa et al. (200) showed that influenza virus infection of

TABLE 3. Analysis of the effect of virus infection on cell surface proteins by flow cytometry

Virus	Cells	Antigen	Reference(s)
Pseudorabies	L-929; Neuro 2A	MHC class I expression decreased	130
HSV	Mouse embryo fibroblasts	MHC class I expression decreased	91
Cytomegalovirus	Human foreskin fibroblasts	MHC class I expression decreased	68
Mouse hepatitis	Cerebral endothelial	MHC class II expression decreased	92
West Nile	Lewis Rat Schwann	MHC class I & II expression increased	8
HSV	Endothelial	GMP140 expression increased	56
HIV	T	CD4 expression decreased	181, 198
EBV	B	Bcl-2 expression decreased	120

HeLa cells induced the expression of the apoptosis-related Fas antigen, suggesting that influenza virus causes cell death via apoptosis. Akbar et al. (2) showed that patients with acute viral infections, such as those caused by EBV or varicella-zoster virus, have circulating CD45⁺RO⁺ cells with low levels of bcl-2, which tended to undergo apoptosis in culture. Apoptosis could be prevented by the addition of interleukin-2 to the culture medium. Thus, apoptosis seems to be a factor in the demise of many virus-infected cells. With the aid of flow cytometry, it will be possible to examine the contributions of viral and cellular antigens to this process.

Effects of Virus Infection on Cellular Proteins

Virus infection often modulates the expression of cellular proteins. Table 3 lists a number of virus-cell systems in which the effect of virus infection on the expression of cellular proteins has been studied by flow cytometry.

In a defense against cell-mediated immunity, infection of cells by a number of viruses results in the downregulation of the major histocompatibility complex (MHC) antigens. Figure 7 illustrates the effect of pseudorabies virus infection on the expression of the class I MHC antigens of L929 and Neuro A

FIG. 7. Effect of virus infection on cell surface expression of mouse MHC antigens. L-929 (A) or Neuro A (B) cells were infected with pseudorabies virus at a multiplicity of infection of 10. At various times postinfection, the cells were removed from the flasks and incubated with mouse monoclonal antibodies, anti-*H-2K^k* and anti-*H-2D^k*, followed by FITC-labeled anti-mouse IgG. The fluorescence intensity of mock-infected and infected cells was determined by flow cytometry. In both cell types (panels A and B), the mock-infected cells (—) expressed MHC antigens throughout the time course, whereas pseudorabies virus-infected cells (---) had reduced amounts of MHC on their cell surfaces. Background fluorescence (····) was determined on unstained mock-infected cells. Reprinted from reference 130 with permission of the publisher.

cells. As shown by fluorescence intensity, over a 12-h period the surface expression of class I K^k and D^k MHC antigens on pseudorabies virus-infected L929 cells decreased by 60 to 70% compared with uninfected L929 cells. Similar results are shown for pseudorabies virus infection of Neuro 2A cells. The expression of other surface antigens, such as the transferrin receptor, was not suppressed, suggesting a selective regulatory mechanism (130). Infection of B6/WT-3 mouse embryo fibroblasts with herpes simplex virus type 1 (HSV-1) or type 2 (HSV-2) resulted in the expression of HSV-1 or HSV-2 surface glycoproteins on the plasma membrane but a reduction in the expression of class I H-2K^b and H-2D^b (91). Furthermore, as shown by differences in fluorescence intensity, infection with HSV-2 resulted in a greater reduction of the MHC antigens than infection with HSV-1. This observation was confirmed by demonstrating that the cytolytic activity of HSV-2-specific, cytolytic T lymphocytes against HSV-2-infected cells was reduced compared with the activity of HSV-1-specific cytolytic T lymphocytes against HSV-1-infected target cells. In a similar manner, flow cytometric analysis of human cytomegalovirus (HCMV)-infected human foreskin fibroblasts demonstrated a reduction of the expression of the MHC antigens (68). Treatment of cerebral endothelial cells with gamma interferon induces the MHC class II antigen complex, and infection of these cells with mouse hepatitis virus blocks the expression of MHC class II (92). This effect was shown by using flow cytometry on surface-labeled cells, which indicated the loss of this antigen, and by Northern (RNA) analysis, which demonstrated the loss of mRNA for the MHC class II molecule. The opposite effect was demonstrated when Schwann cells from Lewis rats were infected with West Nile virus: the expression of both MHC class I and class II cell surface proteins was enhanced (8). The quantitative aspects of flow cytometry allowed these types of determinations, which would be impossible with fluorescence microscopy, to be made.

Infection of endothelial cells with HSV induces GMP140, an adhesion protein that is implicated in tissue injury and is not expressed on the cell surfaces of uninfected cells (56). Flow cytometric analysis of HSV-infected endothelial cells showed that expression of GMP140 is dependent on the expression of the gC glycoprotein of HSV.

In addition to the effects of virus infection on the expression of MHC surface antigens, flow cytometry has been used to demonstrate the downregulation of the CD4 molecule on HIV-infected cells (181, 198). Martin et al. (120) showed that infection of B cells with the B95-8 strain of EBV increased the expression of the cellular gene, *bcl-2*; however, the increased expression of *bcl-2* had little effect on cell transformation, which is related to the EBV latent membrane protein gene expression.

The results of these studies demonstrate the utility of flow cytometry in the measurement of the effect of virus infection on the expression of particular cellular antigens. This type of analysis can be enhanced by staining individual cells for both viral and cell surface antigen expression and by using the multiparametric analytical capabilities of the flow cytometer to detect these changes in small populations of infected cells.

Binding of Virus to Cells

A number of investigators have used flow cytometry to detect and quantitate cells that have fluorescently labeled virus attached to them. Both direct conjugation of FITC to virus and conjugation of biotin to virus followed by detection of biotinylated virus bound to cells by treatment with FITC- or PE-labeled avidin are methods that have been used to label virus

TABLE 4. Detection of cell-bound virus by flow cytometry

Virus	Cells	Receptor	Reference(s)
EBV	B lymphocytes	CR2	76, 85
EBV	Human epithelial	CR2	107
SV40	CV-1	MHC class I	24
SV40	Monkey kidney	ND	14
LCMV	MC57, BHK, Vero, HeLa	ND	20
HTLV	T and B	ND	49, 63
Echovirus	BK, Vero, P2002	ND	121
Coxsackievirus A9	BK, Vero, P2002	ND	121
Murine leukemia	NIH 3T3, HeLa	ND	94
Measles	Jurkat	CD46	51, 139
Poliovirus	Human mononuclear	CD14	60
Sindbis	Mouse neuronal	ND	204
HIV	MT-4	CD4	176, 178

for these binding studies. As shown in Table 4, this technique has been used to identify cell surface receptors for a number of viruses. Figure 8 illustrates the use of biotinylated EBV for identifying PBMC that bind EBV. Binding of biotinylated EBV to cells was detected by FITC-labeled avidin, and purified B cells were identified by the B1 monoclonal antibody that reacts with B cells and FITC-conjugated goat anti-mouse antibody. EBV efficiently bound to all subpopulations of B lymphocytes but not to T cells. The receptor was identified as the CR2 molecule, which is the receptor for the Cd3 complement component as well as EBV (85).

In another study, FITC-labeled EBV was used to determine which human B-cell subpopulations were susceptible to virus infection (76). The different subpopulations of B cells were determined by treating them with the following murine monoclonal antibodies: (i) OKB7 to identify the Cd3 receptor; (ii)

FIG. 8. Binding of fluorescently labeled virus to cells. (A) Biotinylated EBV was incubated with purified peripheral blood B lymphocytes followed by incubation with FITC-labeled avidin, mounted under glycerol, and viewed under a fluorescence microscope. Three patterns of fluorescence are shown. (B) The solid line shows the flow cytometric analysis of purified B cells exposed to biotinylated EBV followed by FITC-labeled avidin. (Inset) Flow cytometric analysis of purified B cells treated with a B-cell-specific monoclonal antibody followed by an FITC-labeled F(ab')₂ fragment of goat anti-mouse immunoglobulin. In each case, the dotted line represents purified B cells treated with FITC-labeled avidin or FITC-labeled goat anti-mouse IgG (inset). Note the similarity between the B-cell peak in the inset and the higher fluorescent peak in panel B, suggesting that EBV binds to B cells. Reprinted from reference 85 with permission of the publisher.

OKB9 to identify the transferrin receptor, an indicator of B-cell activation; (iii) OKB10 to identify highly differentiated B cells; and (iv) specific monoclonal antibodies to IgG, IgA, IgD, or IgM to identify B cells expressing surface immunoglobulins. The specifically labeled cells were treated with PE-labeled goat anti-mouse IgG as the second antibody, and two-color immunofluorescence was used to identify the cell type (PE-labeled cell surface receptors) and the cells that had virus attached to them (FITC-labeled cells). Both resting B cells and B cells expressing surface IgM, IgG, IgD, and IgA were positive for FITC-labeled EBV, suggesting that these cells had the EBV receptor on their cell surface. A portion of activated and highly differentiated B cells lacked binding by FITC-labeled EBV, suggesting that the expression of the EBV receptor is developmentally regulated. To prove this point, the authors could have used different fluorescently labeled monoclonal antibodies to the Cd3 receptor, the transferrin receptor, and differentiation antigens to demonstrate that the activated and highly differentiated cells did not express the Cd3 receptor on their cell surfaces.

EBV also replicates in human epithelial cells, leading to a productive infection. The receptor for EBV on human epithelial cells is not known. To study the replication of EBV in human epithelial cells, Li et al. (107) transfected human epithelial cell lines with an expression vector containing the gene for CR2 (the receptor for the Cd3 complement component and EBV). Successful transfection was measured by incubating the cells with a monoclonal antibody to CR2 followed by FITC-labeled second antibody, and the fluorescence intensity was quantitated by flow cytometry. Human epithelial cells that expressed the CR2 receptor also bound EBV, as determined by incubating EBV-treated cells with human serum containing EBV antibodies followed by FITC-conjugated anti-human IgG antibody and quantitating fluorescently stained cells by flow cytometry.

A brief description of other virus-binding studies follows. FITC-labeled SV40 was used in competition studies to confirm that the class I major histocompatibility proteins are an essential component of the virus receptor on lymphoblastoid cell lines (24). The SV40 receptor was detected on polarized monkey kidney cells, using biotinylated SV40 and streptavidin-conjugated rhodamine to detect the bound virus (14). Flow cytometric analysis of polarized cells treated with the biotinylated SV40 showed that different populations of cells had low, medium, or high concentrations of SV40 receptors and that the expression of the receptors was cell cycle dependent. Binding LCMV to HeLa, Vero, and BHK cells was detected by flow cytometry after biotinylated LCMV was incubated with these cells and then treated with FITC-conjugated streptavidin (20). Using this technique, the authors showed that LCMV did not bind to murine lymphocytes. Biotinylated human T-cell leukemia virus type 1 (HTLV-1) was used to identify which cells were infected with HTLV-1 (49). The results suggested that both T and B lymphocytes and monocytes bound the virus. The unusual finding that B cells were infected with HTLV-1, a T-cell tropic virus, was confirmed by nucleic acid hybridization studies with HTLV-1 probes. Further elucidation of the receptor for HTLV-1 was obtained with monoclonal antibody 34-23, which inhibits biotinylated HTLV-1 but not HIV from binding to activated PBMC (63). Biotinylated Echovirus 11 was used in competition studies to determine the receptor for Echoviruses and coxsackievirus A9 (121). Binding of ecotropic or amphotropic murine leukemia viruses to NIH 3T3, HeLa, and CHO-K1 cells was assayed by flow cytometry (94). The different viruses were incubated with different cell types, and then the mixtures were treated with antibody to gp70, the major

viral envelope protein shared by these viruses, followed by incubation with FITC-conjugated second antibody. Bound virus was then quantitated by flow cytometry. The specificity of the binding was confirmed when resistant cells were made susceptible to binding by transfection and expression of the receptor gene from susceptible cells. The human membrane cofactor protein, CD46, was shown to be the receptor for measles virus by transfecting measles virus-resistant murine cells with an expression vector containing a cDNA isoform of CD46 with exons 1 to 6, 9 to 12, and 14 and isolating cells expressing the CD46 cell surface protein. This was done by incubating the cells with a CD46-specific monoclonal antibody followed by a fluorescently labeled second antibody and sorting by FACS (51, 139). Cells expressing the CD46 antigen were incubated with measles virus and then treated with antibody to the measles virus hemagglutinin, followed by FITC-labeled second antibody, and quantitated by flow cytometry. The results showed that cells that express CD46 bind measles virus whereas those that do not express CD46 do not bind virus, suggesting that CD46 is a receptor for measles virus. Two-color immunofluorescence was used in conjunction with flow cytometry to detect the poliovirus receptor on the majority of CD14⁺ mononuclear cells (60). The authors also showed that poliovirus will replicate in these cells, suggesting an extraneural site for poliovirus replication in humans. A possible receptor for Sindbis virus on mouse neural cells was identified by using a fluorescently labeled anti-idiotypic antibody to the neutralizing antibody for the viral E glycoprotein (204). This receptor is developmentally regulated, being present at birth, but is reduced in amounts shortly after birth. The loss of this receptor may explain the increased resistance of mice to Sindbis virus infection with age.

Many compounds inhibit virus replication by preventing binding of virus to susceptible cells, and flow cytometry has been used to study this effect. Schols et al. (176) used a polyclonal antibody to HIV-1 and FITC-labeled second antibody in conjunction with flow cytometry to detect HIV-1 bound to MT-4 cells (a transformed T-cell line). Inhibition of binding of HIV-1 to MT-4 cells by heparin, dextran sulfate, and pentosan polysulfate was quantified by flow cytometry. Neither zidovudine (ZDV) nor dextran inhibited the binding of HIV-1 to MT-4 cells. In further studies, Schols et al. (178) showed that compounds that inhibit binding of HIV-1 to MT-4 cells react directly with gp120 on the virus to block the interaction of gp120 with the CD4 receptor on susceptible cells. Thus, flow cytometric analysis of the interaction of fluorescently labeled virus with cells is a rapid method for quantitating the effect of antiviral compounds on attachment of viruses to cells. In summary, the data presented in these papers demonstrate the use of one- or two-color immunofluorescence in conjunction with flow cytometry for studying the attachment of viruses to cells, identifying virus receptors on cells, and identifying the cells to which these viruses attach.

Detection of Viral Antigens on the Cell Surface

In recent years, a large number of papers that show the use of flow cytometry to detect the presence or absence of viral glycoproteins on the cell surface of virus-infected cells have been published. Table 5 lists some of the viruses and their antigens and represents the kinds of studies that have been performed. Figure 9 illustrates the use of flow cytometry to detect feline immunodeficiency virus glycoprotein on the cell surfaces of feline immunodeficiency virus persistently infected FL-4 cells. In all of these studies, virus-infected cells were treated with antibody to the viral antigen in question, followed

TABLE 5. Detection of viral antigens on cell surfaces by flow cytometry

Virus	Antigen(s) detected	Reference(s)
HSV	gB, gC, gD, gE	50, 90, 187, 194
Bovine HSV	gIII; gIV	108, 205
Duck hepatitis B	Precore protein	170
Japanese encephalitis	E protein	167
Mouse mammary tumor	gp52	164
Varicella-zoster	gpI	192
Vesicular stomatitis	G protein	210
Rabies	G protein	209
HCMV	gB	15
HIV	gp160/120	57, 155-157
Influenza	HA	114
Parainfluenza	F	114
Feline immunodeficiency	gp120	111
Human herpesvirus 6	gp110/60	58
Measles	H and F	175
FLV	env	162

by an FITC-labeled second antibody, and then surface glycoprotein expression on cells was detected and quantitated by flow cytometry. In addition to determining the presence or absence of viral proteins on the cell surface, some of these flow cytometric studies include data on the kinetics of expression of the different viral glycoproteins, an analysis that would be impossible to do by fluorescence microscopy (90). The ability to readily quantitate the number of cells expressing surface viral antigens and the relative amount of antigen per cell demonstrates the advantage of using flow cytometry over fluorescence microscopy, which yields only qualitative data.

Detection of Intracellular Viral Antigens

Detection and quantitation of viral antigens by flow cytometry have been extensively studied in three groups of viruses: the papovavirus, SV40; the lentiviruses, HIV-1, and -2; and the herpesviruses, HCMV, HSV-1, and HSV-2. The use of flow cytometry in studies of these viruses will be presented in detail, and the flow cytometric results will be compared with data from other techniques to give the reader some understanding of the potential use of flow cytometry in virology. Many other viruses have been studied with flow cytometry, but since there are so few papers on each virus, the experiments will be outlined without any attempt at a critical comparison between different studies. Table 6 lists the viruses, permeabilization procedures, antigens, and antibodies that are covered in this section.

SV40. One of the first and most thoroughly studied virus-cell systems has been SV40-infected permissive and nonpermissive cells. Multiparameter flow cytometric analysis of SV40-infected permissive and nonpermissive cells has allowed elucidation of the role of the SV40 T antigen in the induction of cellular DNA synthesis required to initiate cellular transformation. In the initial studies using flow cytometry, the DNA content of SV40-infected Chinese hamster cells, which are nonpermissive for SV40 replication, was analyzed by flow cytometry (82). Infected and uninfected cells were treated with formalin, the DNA was stained with the acriflavine-Feulgen procedure, and the DNA content was measured. The results showed that SV40 induced these cells to undergo DNA synthesis without mitosis, resulting in the production of cells with a heteroploid DNA content. In a follow-up study, flow cytometry was used to detect and quantitate the amount of SV40 T antigen in CV-1 cells productively infected with SV40

(81). The data showed that under these experimental conditions T antigen was not detected until 12 h postinfection and reached a maximum at 96 h postinfection. The quantitative aspect of flow cytometric analysis allowed the measurement of the actual amounts of T antigen over time in a population of virus-infected cells. Jacobberger et al. (89) used multiparametric flow cytometry to study the role of SV40 T antigen in transformation of Chinese hamster embryo cells. In this study, an SV40-transformed, T-antigen-positive cell line (A58-b) and a T-antigen-negative cell line (B1) were permeabilized with 90% methanol, and two-color immunofluorescence was used to detect and quantify the amount of cellular DNA and SV40 T antigen in each SV40-infected cell by flow cytometry. The intact cells were identified as those containing a 2 N DNA content as measured by the binding of PI; the number of SV40-transformed cells was accurately quantitated, and the average content of SV40 T antigen in a population of SV40-transformed cells was estimated by flow cytometry. The results indicated that 97% of the cell-associated green fluorescence was associated with T antigen and only 3% of the green fluorescence was due to the nonspecific binding of the FITC-labeled secondary antibody. The low-fluorescence background was due to the use of high-affinity monoclonal antibodies and affinity-purified secondary antibodies and the inclusion of goat serum in all reaction mixtures. In further studies, a multiparametric analysis was undertaken to determine the cellular DNA content and the expression of SV40 T and V antigens in SV40-infected CV-1 cells, which are permissive for SV40 infection (106). The cellular DNA content was quantitated by determining the binding of PI to the DNA, and the quantity of SV40 T and V antigens was determined by the indirect immunofluorescence technique. T antigen was detected in all phases of the cell cycle by 12 h postinfection, and at later times postinfection there was an increased percentage of T-antigen-positive cells and tetraploid cells. This finding is in agreement with earlier studies (81). V antigen, detected immediately after infection, was due to input virions; de novo synthesis of V antigen began in late S and $G_2 + M$ phases of the cell cycle, and it was expressed at a constant maximum level as the cells became tetraploid. Multiparametric analysis of SV40-infected permissive cells allowed these investigators to monitor the appearance of each viral product and cellular DNA synthesis independently over time.

Lehman et al. (104) used multiparametric flow cytometry to show that the level of SV40 T antigen correlates with the amount of cellular DNA during the cell cycle in both permissive and nonpermissive cells. In each case, the amount of T antigen was highest at the G_2 (or greater than G_2) portion of the cell cycle. Permissive cells were characterized by T-antigen levels in G_2 that were more than twice those found in G_1 . In contrast, in nonpermissive cells levels of T antigen in G_2 were less than twice those found in G_1 .

Multiparametric flow cytometric analysis of permissive cells infected with the temperature-sensitive mutant, tsA30, demonstrated that T-antigen function was required for induction of tetraploid cells (61). Confluent monkey kidney cells infected with tsA30 at the nonpermissive temperature of 40.5°C were stimulated into cellular DNA synthesis but did not become tetraploid. The infected cells accumulated in the G_2 phase of the cell cycle, suggesting that the induction of the second round of DNA synthesis was dependent on a T-antigen function that was not required for the first round of cellular DNA synthesis. This effect of SV40 infection on cellular DNA synthesis, as measured by binding of PI to DNA, was recently confirmed by using two other dyes that bind DNA, chromocycin A₃ and mithromycin (103).

FIG. 9. Detection of viral antigens on the cell surface as assessed by flow cytometry. (A) FL4 cells persistently infected with feline immunodeficiency virus P were incubated with anti-V3.3 serum (—), preimmune serum (·····), or a high-titer serum obtained from a specific-pathogen-free cat infected with feline immunodeficiency virus M2 (—) for 30 min on ice. The cells were then washed, incubated with FITC-conjugated goat-anti-cat IgG for 30 min, washed again, fixed in paraformaldehyde, and analyzed for fluorescence intensity by flow cytometry. (B) Results of a similar experiment using a nonreactive antipeptide antibody. In panel A, note that the antipeptide antibody, V3.3, binds significantly to the surface glycoprotein of feline immunodeficiency virus on the FL-4 cells but not as well as the polyclonal serum from an acutely infected cat. The results from panel B show that other antipeptide antibodies do not bind to the feline immunodeficiency virus persistently infected cells. Reprinted from reference 111 with permission of the publisher.

Flow cytometry has been useful for studying the role of SV40 gene products in cell transformation. In a multiparameter flow cytometric study of SV40-infected human diploid fibroblasts, cellular DNA content was correlated with the expression of cellular p53, SV40 T antigen, and SV40 late V antigen (97). The results show a shift in the DNA content toward tetraploidy with a concomitant increase in the expression of cellular p53 and SV40 T antigen. A multiparameter two-color flow cytometric analysis was used to study the interactions of p53 and the SV40 T antigen in pre- and postcrisis normal human diploid fibroblasts infected with SV40 (96). Flow cytometric data, in combination with immunoprecipitation, suggest that ploidy, T antigen, p53, and T antigen-p53 complexes contribute to the formation of a stable SV40-transformed human cell line. Using a pSB3-neo plasmid that contains the genes coding for the SV40 T antigen, Goolsby et al. (72) showed that large T antigen and p53 gene expression increased during transformation of a human cell line whereas small t antigen decreased. Similarly, Sladek and Jacobberger (188, 189) showed that expression of the large T antigen of SV40 decreased the G_1 phase and increased the G_2 and M phases of the cell cycle in NIH 3T3 cells without affecting the S phase of the cell cycle. Taken together, these results suggest that large T antigen

pushes the cell cycle toward the G_2/M phase, leading to more rapid or less controlled cell division, a characteristic of transformed cells.

HCMV. Several groups have used flow cytometry to detect and quantitate HCMV-infected cells. Elmendorf et al. (54) demonstrated that viral antigen and DNA content could be measured in HCMV-infected cell cultures by using two-color fluorescent flow cytometry. The presence of HCMV early antigen could be detected within 1 h after infection (3 h after addition of virus to cells), and its fluorescence intensity increased with time after infection. The number of cells expressing this antigen increased with increasing multiplicities of infection. HCMV infection of cell cultures caused a broadening of the DNA profile, as measured by fluorescence intensity of PI bound to DNA, that could be attributable to increased cellular DNA synthesis induced by virus infection, binding of PI to newly synthesized viral DNA, or both.

The ability of HCMV to infect PBMC in vitro has been studied with flow cytometry (116, 193). PBMC were obtained from healthy HCMV-seropositive and HCMV-seronegative individuals and infected in vitro with laboratory strains or clinical isolates of HCMV. The identity of the infected cells was determined by using FITC-labeled monoclonal antibodies

TABLE 6. Antigen detection of selected virus-infected cells by flow cytometry

Virus	Permeabilization procedure	First antibody	Second antibody	Nucleic acid stain	Location	Reference
SV40	Formalin	NA ^a	NA	Acriflavin-Feulgen	Nucleus	82
SV40	Acetone-methanol	Polyclonal antibody to T antigen	FITC-labeled antibody	NA	Nucleus	81
SV40	Methanol	Monoclonal antibody to T antigen	FITC-labeled antibody	PI	Nucleus	89
SV40	Methanol	Monoclonal antibody to T antigen; polyclonal antibody to V antigen	FITC-labeled antibody	PI	Nucleus	106
HCMV	Methanol	Monoclonal antibody to an early HCMV antigen	FITC-labeled antibody	PI	Nucleus	54
HCMV	Ethanol	Monoclonal antibodies to immediate early and late HCMV antigens	FITC-labeled antibody	NA	Nucleus	116
HCMV	NA	Monoclonal antibodies to major immediate early and matrix HCMV antigens	PE-labeled antibodies	NA	Membrane	193
HIV	Methanol-acetone	Monoclonal antibody to p24 antigen	FITC-labeled antibody	NA	Cytoplasm	35
HIV	Methanol, para-formaldehyde, Triton X-100	Monoclonal antibodies to p17, p24, and p55 antigens	FITC-labeled antibody	NA	Cytoplasm	36
HIV	Methanol	Monoclonal antibody to p17, p24, nef, gp120, and gp160/41	FITC-labeled antibody	PI	Cytoplasm	125
HIV	Methanol	Monoclonal antibodies to p24, p17, and gp41	FITC-labeled antibody	NA	Cytoplasm	78
HSV-1 and -2	Methanol	Monoclonal antibodies to DNA binding protein; ICP4, and DNase	FITC-labeled antibody	PI	Nucleus	124
BTB	Methanol	PE-labeled antibody to VP7	NA	NA	Cytoplasm	13
ASFV	NA	Polyclonal antibodies to ASFV	FITC-labeled antibodies	NA	Membrane	3
REV-A	NA	FITC-labeled monospecific antibodies to envelope protein	NA	NA	Membrane	203
WHV	NA	FITC-labeled polyclonal antibody to envelope and core antigen	NA	NA	Membrane	29
HCV	Formaldehyde, acetone	Monoclonal antibody to core antigen	FITC-labeled antibody	NA	Cytoplasm	22
BVDV	Saponin	Biotinylated polyclonal antibodies to BVDV	FITC-labeled streptavidin	NA	Cytoplasm	159
FeLV	Triton X-100	Monoclonal antibody to p27 antigen	FITC-labeled antibody	NA	Cytoplasm	45
F-MuLV	NA	Monospecific antibody to gp70	FITC-labeled antibodies	NA	Membrane	135
EBV	NA	Monoclonal antibodies to cell surface antigens	Fluorescently labeled antibodies	NA	Membrane	134
AcMNPV ^b	Acetone	CAT-specific monoclonal antibody ^c	FITC-labeled antibody	NA	Nucleus	138

^a NA, not applicable.^b AcMNPV; *A. californica* nuclear polyhedrosis virus.^c CAT, chloramphenicol acetyltransferase.

to cell surface markers and PE-labeled monoclonal antibodies to immediate early and matrix HCMV antigens. The results showed that up to 25% of the CD14⁺ monocytes and a small percentage of CD8⁺ T cells were infected whereas CD4⁺ T cells and CD19⁺ B cells were not infected by HCMV in vitro. These results agree in part with published work on the infection of human blood cells with HCMV in vivo by Gerna et al. (67), who used immunofluorescence microscopy to show that polymorphonuclear leukocytes and monocytes were infected with HCMV in vivo. However, Soderberg et al. (193) used mononuclear cells obtained from Ficoll-Hypaque gradi-

ents, which eliminate the majority of polymorphonuclear leukocytes for their analysis. This difference in techniques prevents a direct comparison with the in vivo studies reported by others, which suggest that the polymorphonuclear leukocytes are the primary source of HCMV-infected cells in vivo (65-67).

Others have used flow cytometry to assay antiviral compounds against HCMV in vitro (5, 140, 191). Andrei et al. (5) used a monoclonal antibody to a late HCMV antigen to demonstrate that (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)-adenine (HPMPA), (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)-cytosine (HPMPC), and ganciclovir (DHPG) in-

hibit the replication of laboratory strains and clinical isolates of HCMV. The quantitative aspects of flow cytometry allowed the measurement of the effects of different concentrations of these compounds on the replication of HCMV in MRC-5 and HEL cells. Ganciclovir was more effective than acyclovir or phosphonoformate but less effective than HPMP and HPMPA. These results were confirmed by plaque reduction assays that took considerably longer to perform.

Neyts et al. (140) have used fluorescein diacetate, a nonfluorescent compound that becomes fluorescent upon hydrolysis by cytoplasmic esterases found in HCMV-infected cells, to detect and quantitate the number of HCMV-infected cells in culture. Uninfected cells exhibited less fluorescence than HCMV-infected cells in this assay. This assay was used to quantitate the effect of antiviral compounds on HCMV-infected HEL cells, and the results were compared with those obtained with fluorescence microscopy. The two systems gave similar results, but the flow cytometric assay was quantitative and much easier to use. Snoeck et al. (191) compared flow cytometry with the DNA hybridization technique to determine the effects of HPMP and HPMPA on HCMV replication. Both methods showed that the compounds were effective against HCMV infection, but flow cytometry was more quantitative and gave results faster than the nucleic acid hybridization technique.

With the availability of many different monoclonal antibodies to specific HCMV antigens, it is now possible to use flow cytometry to detect and quantitate the synthesis of immediate early, early, and late HCMV antigens throughout the virus replication cycle (128). The combination of these monoclonal antibodies and flow cytometry will be useful for determining the mode of action of antiviral drugs effective against HCMV.

HIV. The initial study describing the use of flow cytometry to detect HIV-infected cell lines was reported by Cory et al. (35). In this study, the authors compared permeabilization with a 1:1 mixture of methanol-acetone with sequential fixation with 100% methanol, 0.5% paraformaldehyde, and 0.5% Triton X-100. Uninfected and HIV-infected H9 cells were permeabilized, stained by the indirect immunofluorescence method, and analyzed by flow cytometry. Cells were selected by FALS and RALS, and FITC-stained cells were quantitated on a log scale. The results showed little staining of uninfected H9 cells and substantial staining of infected H9 cells, with a minor peak of lower intensity and a major peak of higher intensity. One problem with the methanol-acetone procedure was transfer of fluorescence from the infected cells to uninfected cells when the two kinds of cells were stained together. This problem was eliminated by using the methanol-paraformaldehyde-Triton X-100 permeabilization procedure. In this way, the authors were able to determine the percentage of the cell population that was infected with HIV and the relative amount of p24 antigen per cell. The sensitivity of this flow cytometric assay allowed detection of one HIV-infected H9 cell in the presence of 10^4 uninfected H9 cells. The assay was more rapid and sensitive than the reverse transcriptase assay in that virus-infected cells could be detected by flow cytometry 3 days before reverse transcriptase could be detected in the supernatant. In a subsequent report, these investigators compared 10 monoclonal antibodies to HIV antigens to monitor the replication of HIV-1 in H9 cells (36). HIV-infected and uninfected H9 cells were permeabilized with methanol, paraformaldehyde, and Triton X-100, treated with each of the various monoclonal antibodies followed by FITC-conjugated second antibody, and analyzed by flow cytometry. Monoclonal antibodies to the capsid p24 antigen, the matrix p17 antigen, and the precursor p55 antigen detected HIV-infected H9 cells, whereas monoclonal antibodies to envelope antigens were not

as useful. Using monoclonal antibodies to the p24 antigen, the authors were able to monitor the kinetics of HIV replication in H9 cells by flow cytometry. Five to 6 days after infection, a significant percentage of H9 cells appeared to be infected. The time to positivity was proportional to the amount of infectious virus added to the H9 cells. With this information, the infectious titer of a stock of HIV could be determined by the kinetics of the appearance of infected cells as measured by flow cytometry.

The abilities of monoclonal antibodies to the HIV antigens p24, p17, nef, gp120, gp41, and gp160/41 to detect HIV-infected H9 cells were compared by indirect immunofluorescence in conjunction with flow cytometry (125). Monoclonal antibodies to p24 and the nef antigens detected the same percentage of HIV-1-infected H9 cells as a polyclonal antiserum obtained from an HIV-infected individual which, on the basis of immunoblot analysis, was known to contain antibodies to at least five HIV antigens. At the concentrations used in this study, monoclonal antibodies to p24 and nef antigens detected 81% of the HIV-infected cells, those to the p17 antigen detected 21%, those to the gp41 antigen detected 12%, and those to gp120 antigen detected only 2% of the infected cells. Isotype controls demonstrated the specificity of the p24 and nef antibodies for HIV-infected H9 cells. This inability of monoclonal antibodies to gp120 to detect HIV-infected H9 cells was also reported by Cory et al. (36). Since methanol, which is known to destroy both cellular and viral antigens on the surface of cells, was used in both studies to permeabilize the cells before treatment with monoclonal antibodies, the lack of staining of the viral envelope antigens is not surprising.

The replication of HIV-2ben in the U-937 clone 2 monocyte cell line and the CEM and MOLT-4 lymphoid cell lines has been studied by flow cytometry, reverse transcriptase assays, and syncytium formation (18). Unfixed cells were used in this study. These investigators used the indirect immunofluorescence method, employing polyclonal rabbit antiserum against Tween-ether-treated HIV-2ben as the first antibody and FITC-conjugated goat anti-rabbit IgG as the second antibody to label viral antigens on the surface of virus-infected cells. The results indicated that virus replication occurred in all three cell lines, killing MOLT-4 and U-937 cells and causing a persistent infection in CEM cells. The comparison of the three techniques showed that flow cytometry was more sensitive, quantitative, and rapid than syncytium formation for detecting virus replication in these three cell lines. The reverse transcriptase assay failed to show virus replication in MOLT-4 and U-937 cells but did show virus production in the persistently infected CEM cell line. Since this study used polyclonal antibody that was directed against both the *gag* and *env* gene products and others have shown that *gag* gene products are expressed on the surface of HIV-infected cells (142, 183), it is not clear which viral antigens were being detected on the cell surface by flow cytometry. This could be clarified by repeating the studies with monoclonal antibodies to viral envelope antigens, gp120 or gp41, or *gag* gene products, p17 or p24, to determine which antigens are being detected on the cell surface in this flow cytometric assay. Care must also be taken to ensure that the antibodies cannot penetrate the cell.

Heynen and Holzer (78) used indirect immunofluorescence and flow cytometry to study the expression of p24, p17, and gp41 in CEM cells chronically infected with HIV-1. The authors demonstrated that monoclonal antibodies to all three antigens detected HIV-infected cells at a level of at least 1 infected cell among 10^3 uninfected cells. These studies are the first to show equivalent detection of p24, p17, and gp41 for methanol-fixed cells. The authors did not indicate the location

of the immunofluorescence in the cell: the plasma membrane, the cytoplasm, or the nucleus. Possible explanations for a positive result with their monoclonal antibody to gp41 are that it recognizes a carbohydrate-containing epitope that is retained in the cell after methanol treatment or that the fluorescence is in the cytoplasm where the gp41 is synthesized. When either CEM cells or PBMC were infected with HIV-1 *in vitro*, the time course of infection could be monitored by the production of p24 antigen as measured by flow cytometry. Measurement of released p24 antigen by enzyme-linked immunosorbent assay (ELISA) or syncytium formation showed that both assays became positive before a significant percentage of HIV-infected cells was detected by flow cytometry. This result may be due to the lack of quantitation of syncytium formation and the fact that a very small percentage of HIV-1-infected cells can produce sufficient virus for detection by the sensitive ELISA for p24 antigen. After 4 to 5 days of infection and a number of rounds of virus replication, a sufficient number of cells became infected to be detected by the quantitative flow cytometric assay, which is similar to the time course seen by Cory et al. (36) with HIV-infected H9 cells. These results illustrate the ability of flow cytometry to quantitatively measure the number of virus-infected cells compared with other relatively nonquantitative assays such as syncytium formation, which is difficult to quantitate, and ELISAs for released p24 antigen, which are very sensitive tests that can detect small amounts of p24 antigen being produced by a relatively small number of cells.

Others have used flow cytometry to assay the antiviral effects of several compounds against HIV (10, 12, 131, 152, 176, 177, 178, 180). Mercure et al. (131) used two-color flow cytometry to detect and quantitate HIV-infected CD8⁺ T cells that were infected *in vitro*. Then they used PCR and the production of released p24 antigen measured by the ELISA to determine that ZDV and 2',3'-dideoxyinosine inhibit the production of HIV in these cells. The effects of ZDV and 2',3'-dideoxycytidine on the replication of HIV-1 in MT-4 cells were measured by indirect immunofluorescence and flow cytometry (152). The results compared favorably with assays for cytopathic effect, aggregate formation, and fluorescence as measured by fluorescence microscopy. Schols et al. (180) used fluorescein diacetate, an ester that becomes fluorescent when acted upon by esterases in viable MT-4 cells and fails to fluoresce in dead cells, to determine the effect of ZDV on HIV-1-infected MT-4 cells. The presence or absence of fluorescence was detected and quantitated by flow cytometry. They determined concentrations of ZDV that prevented cell death, thereby leaving fluorescent MT-4 cells to be quantitated by flow cytometry. In other studies, these authors (10, 177, 179) showed that flow cytometry can be used to detect syncytium formation in HIV-infected MOLT-4 cells and that syncytium formation can be blocked by sulfated polysaccharides.

Recent information suggests that ZDV-resistant mutants of HIV induce syncytium formation in MT-2 indicator cell lines whereas ZDV-sensitive viruses do not (21, 95). This observation suggests a rapid *in vitro* assay for determining which virus isolates are resistant to ZDV. Syncytia are large, multinucleated cells that are usually identified by light microscopy. However, for a ZDV resistance assay, MT-2 cells infected with HIV could be stained with PI in the presence of RNase, which would label the DNA in the multinucleated syncytium with a fluorochrome. By using the FALS and fluorescence capabilities of the flow cytometer, the large multinucleated cells could then be detected and quantitated rapidly. When an HIV isolate is cultured in MT-2 cells in inhibiting concentrations of ZDV, cells infected with ZDV-resistant HIV would produce syncytia,

whereas MT-2 cells infected with ZDV-sensitive HIV would not. The concentration of ZDV used and the percentage of syncytium-forming cells should be an indication of the resistance of the virus isolate. The ability to rapidly quantitate the syncytia by flow cytometry may yield a procedure for monitoring disease progression and may be useful for determining a change in therapy before substantial amounts of ZDV-resistant HIV are produced in the host.

HSV-1 and -2. Flow cytometry has been used to detect HSV antigens in HSV-infected cultured cells. HSV-2-infected Vero cells and HSV-2-transformed mouse embryo cells were analyzed by scattered light and, for background immunofluorescence, by flow cytometry (71). Little difference was noted between HSV-2-infected and -transformed cells when they were studied by FALS and RALS; however, when the cells were treated with polyclonal antibodies to HSV-2 raised in goats as the primary antibody and FITC-labeled rabbit anti-goat antibody as the second antibody, there was a significant difference between the fluorescently stained HSV-2-infected cells, which exhibited high fluorescence intensity, and the transformed cells, which did not. HSV-2-infected cells treated with normal goat serum and then with FITC-labeled rabbit anti-goat second antibody did not exhibit significant fluorescence.

HSV-1- and HSV-2-infected MRC-5 cells were treated with a number of murine monoclonal antibodies that reacted with nuclear antigens of HSV-1, HSV-2, or both (201), and the fluorescence intensity was detected and quantitated by two-color immunofluorescence (124). Figure 10, which is a contour map of log FITC-fluorescence intensity along the y axis and linear red fluorescence intensity representing PI binding to DNA along the x axis, illustrates the results of treating uninfected (panel A), or HSV-1 (panel B)- or HSV-2 (panel C)-infected MRC-5 cells with a monoclonal antibody specific for the HSV-2 nuclear antigen and with PI to stain the cellular DNA. The antibody does not stain the uninfected (panel A) or the HSV-1-infected (panel B) cells, whereas significant staining of HSV-2-infected cells is evident (panel C). The PI binding pattern is illustrated on the right half of each panel, where the relative cell number is along the y axis and fluorescence intensity is along the x axis. Uninfected MRC-5 cells (panel A) exhibit a large narrow peak representing cells in G₀/G₁ and a small peak representing cells in G₂/M portions of the cell cycle. Virus infection caused a change in the DNA profile to a broader profile lacking G₀, S, and G₂ peaks (panels B and C), which could be due to increased cellular DNA synthesis, viral DNA synthesis, or both. In further studies with antibodies specific for HSV-1 or HSV-2 nuclear antigens, MRC-5 cells infected with HSV-1 could be distinguished from MRC-5 cells infected with HSV-2. The sensitivity and specificity of the assays were excellent. This report (124) shows that flow cytometry can be used to rapidly and accurately detect and quantitate HSV-infected cells in culture and to monitor the effect of viral replication on cellular DNA synthesis.

Flow cytometry was used to study the effects of antiviral drugs on HSV infection (165). Cellular DNA in HSV-infected and uninfected cells was stained by PI binding, and the DNA profiles were detected by flow cytometry. Uninfected cells gave a normal DNA profile consisting of G₀/G₁, S, and G₂/M peaks. In contrast, HSV-infected cells exhibited a dramatic broadening of the DNA profile where the G₀/G₁, S, and G₂/M peaks merged. Effective antiviral compounds, such as acyclovir, phosphonoacetic acid, and a xanthate derivative, prevented this broadening of the DNA profile in HSV-infected cells. This very simple, rapid, and relatively inexpensive procedure may be useful for screening antiviral drugs against any of the

FIG. 10. Two-color fluorescence of HSV-2-infected MRC-5 cells. Uninfected, HSV-1-infected, and HSV-2 (38111)-infected MRC-5 cells were treated with a monoclonal antibody (SD-1) to an HSV-2 nuclear antigen, followed by FITC-labeled second antibody. After treatment with RNase and PI, the cells were analyzed for two-color fluorescence by flow cytometry. Uninfected MRC-5 cells (A) showed little FITC-labeled antigen and a normal DNA profile. HSV-1-infected MRC-5 cells also showed little FITC-labeled antigen but had a broader profile of PI staining material (B). HSV-2-infected MRC-5 cells showed significant FITC-labeled antigen, with essentially all of the cells staining with the HSV-2-specific antibody (C). The PI staining material was also broader than that found in panel A.

herpesvirus group since this broadening of the DNA profile in herpesvirus-infected cells seems to be a general phenomenon (54, 124, 165, 191).

In another study, polyclonal human or rabbit antibodies were used in conjunction with flow cytometry to detect viral antigens in HSV-1-infected human foreskin fibroblasts and to monitor the effect of antiviral agents, such as acyclovir and interferon, on the HSV-1 infection of these cells (196). Both acyclovir and alpha or gamma interferon inhibited the production of HSV-specific antigens. Acyclovir was considerably more effective than either type of interferon in these inhibition studies. The quantitative aspects of flow cytometry make it a

very useful tool for rapidly screening antiviral compounds for their effect against herpesvirus-infected cells. As described above, determining the effect of herpesvirus infection of cell cultures on the binding of PI to cellular DNA is a very easy and rapid assay for measuring the effect of antiviral compounds on HSV replication.

Miscellaneous Viruses

BTB. Bluetongue virus (BTB) replication has been studied in bovine blood mononuclear cells infected in vitro (13). Peripheral blood was collected from an uninfected, healthy

calf, and the mononuclear cells were obtained and cultured. After 4 h of incubation, nonadherent cells were removed and cultured separately. The nonadherent cells were stimulated with concanavalin A and interleukin-2 24 h before infection with BTV. Both adherent and nonadherent cells were infected with BTV, and infection was monitored by flow cytometry and infectivity assays. Bovine T cells, B cells, and monocytes or granulocytes were identified by staining cell surface markers with FITC-conjugated monoclonal antibodies to their respective antigens. With two-color fluorescence, BTV antigen was detected in adherent and mitogen-stimulated, nonadherent PBMC 2 to 4 h after infection; at 16 h postinfection, 10 to 12% of the cells were infected. Unstimulated, nonadherent cells failed to express the BTV VP7 antigen. Furthermore, virus replication was evident by flow cytometry almost 24 h before it was detected by the infectivity assay. The results suggest that BTV replicates in bovine monocytes and mitogen-stimulated CD4⁺ lymphocytes but not in unstimulated CD4⁺ cells. The ability to do multiparametric quantitative analysis of FITC-labeled surface antigens and PE-labeled internal antigens on the same population of cells enabled these researchers to perform this experiment rapidly and quantitatively. Because of the small percentage of infected cells, this type of analysis would be difficult to perform with the immunofluorescence microscope.

ASFV. In vitro experiments, hyperimmune polyclonal antibody and monospecific antibodies raised in swine to African swine fever virus (ASFV) were used to monitor the expression of ASFV glycoproteins on the surface of ASFV-infected monkey cells (3). Monkey cells were infected with ASFV and analyzed for viral antigen expression at various times ranging from 0 to 20 h postinfection. The results showed that ASFV-specific proteins were expressed on the cell surfaces within 6 h after infection, and maximal expression occurred at 12 h post-infection. The sensitivity of flow cytometry allowed these investigators to determine the number of cells infected, the time course of the appearance of antigen on the cell surfaces, and the amount of antigen on the surface of each cell. In addition to the ease and rapidity of the assay, the quantitative aspect of flow cytometry allowed measurements that would be difficult to make with fluorescence microscopy.

REV-A. The gp90 envelope protein of reticuloendotheliosis-associated virus (REV-A) was detected by flow cytometry on the surface of virus-infected, transformed chicken bone marrow cells (203). A fluorescein-labeled monospecific rabbit antibody to a peptide derived from the carboxy-terminal portion of the REV-A envelope protein was used to stain unfixed, REV-A-infected cells. Greater than 99% of REV-A-infected bone marrow cells expressed gp90 antigen on their cell surfaces, whereas uninfected cells treated with the same FITC-labeled antibody showed little staining. In the presence of antibody to gp90 and complement, bone marrow cells expressing this protein on their cell surface were lysed, suggesting that gp90 is exposed on the cell surface of REV-A-infected cells and that its carboxyl terminus is exposed to site-directed antibodies that cause cytotoxicity by activating the complement system.

WHV. Woodchuck hepatitis virus (WHV) has been detected by flow cytometry in PBMC obtained from experimentally infected animals (29). Polyclonal antibodies to WHV surface or core antigens were obtained from either infected woodchucks or a human hepatitis B virus chronic carrier with a high titer of anti-hepatitis B virus core antibody. These antibodies were conjugated with FITC for direct staining and used to label WHV antigens on the surface of the PBMC obtained from WHV-infected animals. Both WHV surface and core antigens

were detected on the surface of infected PBMC obtained from these animals. The interaction of fluorescently labeled anti-WHV antibodies with WHV antigens expressed on the surface of infected cells could be inhibited by pretreating the infected cells with unlabeled antibody to surface and core antigens. The results of these blocking studies suggest that the reaction is specific and that, under these labeling conditions, both WHV surface and core antigens are exposed on the surface of the cell.

HCV. A monoclonal antibody directed against the first 75 amino acids of the hepatitis C virus (HCV) core antigen was used in conjunction with flow cytometry to detect and quantify HCV-infected cells in PBMC obtained from a patient with chronic hepatitis (22). Neither the surface of the monocytes nor the lymphocytes stained with monoclonal antibody to this epitope on the core antigen, suggesting that the reaction was specific for the internal core antigen. Others have demonstrated the presence of HCV core antigen and virus in the circulation by ELISA and by PCR (199), suggesting that HCV does circulate in the peripheral blood of patients with non-A, non-B chronic hepatitis.

BVDV. In in vivo experiments, bovine viral diarrhea virus (BVDV) was detected by flow cytometry in blood samples from cattle persistently infected with the virus (159). Heparinized peripheral blood was obtained from persistently infected and uninfected cattle, and the erythrocytes were lysed, fixed with 1% paraformaldehyde, and permeabilized with 1% saponin. Biotinylated polyclonal antibody against BVDV or biotinylated normal serum was used, and specifically bound antibodies were detected with FITC-conjugated streptavidin by flow cytometry. Of 143 samples tested, 37 obtained from persistently infected cattle were positive for BVDV by flow cytometry, whereas only 34 of these 37 samples were positive by the standard infectivity assay. The results suggest that the flow cytometric assay is more sensitive and considerably more rapid than the standard infectivity assay for BVDV.

FeLV. Feline leukemia virus (FeLV)-infected lymphoid, erythroid, and myeloid lineage marrow cells in cats were studied by flow cytometry (45). Bone marrow cells were obtained from FeLV-infected cats and fixed with 3% paraformaldehyde followed by 0.5% Triton X-100 to permeabilize the plasma membrane. Indirect immunofluorescence with a monoclonal antibody to the p27 viral core antigen was used to detect FeLV-infected cells. The cells were separated into myeloid, erythroid, and lymphoid populations by light scatter, and the percentage of p27 antigen-positive cells in each population was determined by flow cytometry. The results suggested that all three cell populations are infected with FeLV. This interesting finding was made possible by using the multiparametric properties of the flow cytometer, including light scatter to separate the lymphoid, erythroid, and myeloid cell populations and immunofluorescence detection to detect FeLV-infected cells. This type of analysis would be difficult with a light or fluorescence microscope.

F-MuLV. In one study, the gp70 surface antigen of Friend murine leukemia virus (F-MuLV) was detected on the plasma membrane of infected cells and the number of infected cells expressing this antigen was quantitated by flow cytometry (135). Mouse erythroleukemia cells or splenocytes explanted from F-MuLV-infected mice were stained for the presence of the F-MuLV gp70 surface antigen by indirect immunofluorescence and analyzed by flow cytometry. The results showed that approximately 10% of the splenocytes were expressing F-MuLV gp70 on their cell surfaces. However, the F-MuLV-infected mice were only 1% as susceptible as uninfected mice to superinfection with other retroviruses, such as spleen focus-

forming virus, that use the same receptor. Similar results were found with mice incapable of making antibody or lacking mature T cells, suggesting that the immune response does not contribute to this interference phenomenon. This flow cytometric assay established that F-MuLV-infected mice are resistant to challenge by spleen focus-forming virus because the gp70 antigens of F-MuLV on infected cells are occupying the cell receptor for the spleen focus-forming virus. This is the basis for the interference phenomenon. The ability of flow cytometry to accurately detect small percentages of virus-infected cells has made this type of analysis possible.

EBV. Miller et al. (134) used flow cytometry to determine calcium mobilization and cell surface expression of MHC class II, CD19, CD22, and IgM on lymphoblastoid cells transfected with the gene for LMP2, the latent membrane protein expressed in EBV-transformed cells. The results suggested that these surface proteins were expressed to the same extent in the transfected cells as in control cells. Antibody to IgM, MHC class II, or CD19 cross-link these proteins on the cell surface and induce calcium mobilization in normal lymphoid cells. Expression of LMP2 in the transfected cells inhibited the calcium mobilization. In this case, calcium mobilization was measured by flow cytometry of indol-1-loaded cells.

***Autographa californica* nuclear polyhedrosis virus.** Morris and Miller (138) used flow cytometry to study the expression of *A. californica* nuclear polyhedrosis virus gene products in a variety of cultured insect cells. The expression of early, late, and very late promoters in various cell lines was studied by measuring the amount of chloramphenicol acetyltransferase activity. Cell cultures were infected with recombinant viruses containing chloramphenicol acetyltransferase reporter genes under the transcriptional control of viral early, late, and very late promoters. The expression of the chloramphenicol acetyltransferase gene attached to these promoters was measured by treating the transfected cells with a mouse monoclonal antibody to the chloramphenicol acetyltransferase gene product followed by FITC-conjugated goat anti-mouse IgG as the second antibody and analysis by flow cytometry. The data suggest that productive infection occurred in *Spodoptera frugiperda* cells, with almost 100% of the cells infected, whereas a lower percentage of cells was infected in the other cell lines tested. Nonpermissive cells were blocked in viral DNA replication.

In summary, this brief survey of several virus-cell systems that have been analyzed by flow cytometry should give the reader some idea of the potential of multiparameter flow cytometric analysis for the study of virus-cell interactions. With the ability to differentiate individual antigens stained by as many as three fluorescent labels, it will be possible to expand these kinds of studies in the future.

CLINICAL VIROLOGY

Flow cytometry has been used as a rapid test for the direct detection of virus-infected cells obtained from patients infected with HCMV or HIV-1.

HCMV

In the studies of Elmendorf et al. (54), HCMV-infected BAL samples obtained from patients with HCMV pneumonia were permeabilized, stained by the indirect immunofluorescence method, and analyzed by two-color quantitative flow cytometry. The data indicated that HCMV-infected BAL cells could be distinguished from uninfected BAL cells and that infected BAL cells could be detected and quantitated by

indirect immunofluorescence in conjunction with flow cytometry. The flow cytometric technique for detecting HCMV-infected BAL cells was as accurate as the standard infectivity assays and gave a quantitative answer within 24 h after the specimen was received. This rapid technique for diagnosing HCMV infection is considerably faster and more quantitative than standard cell culture assays, which take weeks to complete, or the shell vial technique for amplification of HCMV in cell culture followed by detection of immediate early antigen by immunofluorescence (40, 70).

Indirect immunofluorescence in conjunction with flow cytometry can be used to detect and quantitate HCMV-infected PBMC obtained from immunosuppressed patients with acute HCMV infection (126). PBMC, obtained from healthy controls or immunocompromised patients with acute HCMV infection, were permeabilized, stained by the indirect immunofluorescence method, and analyzed for two-color fluorescence by flow cytometry. Figure 11 illustrates the flow cytometric analysis of PBMC obtained from a healthy control subject and that obtained from an immunocompromised patient with acute HCMV disease. Panel A shows the gating of PBMC with a 2 N content of DNA on the basis of FALS and PI binding. Panel B shows the lack of staining of PBMC for the FITC-labeled immediate early antigen in cells obtained from a normal individual. Note that very few PBMC are labeled in this control sample. Panel C illustrates the staining of PBMC expressing the HCMV immediate early antigen in cells obtained from a patient with acute HCMV disease. Note that, compared with healthy individuals, a significant percentage of the PBMC obtained from this patient is expressing the immediate early antigen, indicating that these cells are actively infected with HCMV. Table 7 shows the range of values obtained from the flow cytometric analysis of PBMC obtained from uninfected healthy adults (controls) and 10 immunocompromised patients with acute HCMV disease. The data show that a very small percentage of PBMC obtained from healthy adults (controls, 0.1 to 0.3% positive cells) expresses the HCMV immediate early antigen, whereas patients with acute HCMV disease, as confirmed by an infectivity assay, have much higher percentages (0.6 to 8%) of PBMC expressing the HCMV immediate early antigen. Furthermore, the results obtained from flow cytometry were available within 24 h, while the infectivity assays took considerably longer. These results compare favorably with the recently developed HCMV antigenemia assay that is being used to detect HCMV in peripheral blood of immunosuppressed transplant patients with suspected HCMV disease (65–67) and with PCR assay data from patients with HCMV disease (186). The flow cytometric technique is more rapid and much easier to perform and gives more accurate quantitative data than other techniques that attempt to assay HCMV directly from specimens obtained in vivo. This analysis can be performed on freshly isolated PBMC and yields a diagnostic result within 8 h from the time the clinical sample is obtained. Future studies will attempt to use multiparameter flow cytometric analysis of triple-stained leukocytes to determine which blood cells are infected with HCMV in vivo.

Flow cytometry has been used for monitoring the effect of the antiviral drug ganciclovir on virus gene expression in HCMV-infected individuals (126). Table 8 shows the effect of ganciclovir treatment on the expression of HCMV immediate early antigen in PBMC obtained from a kidney transplant patient with acute HCMV disease. Peripheral blood was obtained during the acute stage of the patient's disease and approximately each week thereafter. Whole blood was sedimented over Ficoll-Hypaque, and the PBMC were isolated, permeabilized, stained for the HCMV immediate early antigen

FIG. 11. Detection of HCMV-infected lymphocytes in patients with acute HCMV disease. Peripheral blood was obtained from healthy individuals and patients acutely infected with HCMV. PBMC were obtained by centrifugation on Histopaque. PBMC were permeabilized with 90% methanol, treated with a monoclonal antibody to the HCMV immediate early antigen (Chemicon International), RNase, and PI, and analyzed for two-color fluorescence by flow cytometry. (A) Cells gated on the basis of binding of PI to DNA and forward light scatter. Approximately 70% of the events are in gate A. The cells in gate A were analyzed for green and red fluorescence. (B) Two-color fluorescence of PBMC obtained from a healthy individual; only 0.2% of the PBMC are staining for the FITC-labeled antigen, whereas all of the gated cells stain with PI. (C) Two-color fluorescence of PBMC obtained from a patient with acute HCMV disease. All of the gated cells stain with PI, and approximately 3.1% of the PBMC stain for the FITC-labeled antigen.

TABLE 7. Detection of HCMV in PBMC of immunosuppressed patients: comparison of techniques

Patient no.	Flow cytometry (% HCMV-positive PBMC) ^a	Infectivity (HCMV-positive CPE) ^b
Control	0.1–0.3	Not determined
1	2.9	Blood Urine
2	2.5	Urine
3	1.7	Blood Urine
4	5.4	Urine
5	0.6	Blood Urine
6	1.2	Blood
7	3.9	Urine BAL
8	0.9	BAL
9	8.1	Blood
10	0.6	Urine

^a Permeabilized PBMC were treated with a monoclonal antibody to an immediate early HCMV antigen followed by FITC-labeled goat anti-mouse IgG F(ab')₂, RNase, and PI, and the percentage of antigen-expressing cells was determined by flow cytometry.

^b Blood, urine, or BAL specimens were inoculated into MRC-5 cells and examined for cytopathic effect (CPE).

by the indirect immunofluorescence method, and analyzed for two-color immunofluorescence by flow cytometry. During the acute phase of the infection, 5 to 22% of the PBMC were positive for HCMV immediate early antigen; after 5 weeks of treatment with two different concentrations of ganciclovir, fewer than 1% of the PBMC were positive for the HCMV immediate early antigen. These data show that the flow cytometric technique is useful for monitoring patients on ganciclovir treatment and may be a useful procedure to determine the effectiveness of antiviral therapy in this disease.

HIV

A number of groups have independently reported that HIV antigens can be detected and quantitated in PBMC obtained

TABLE 8. Effect of ganciclovir on expression of HCMV in PBMC

Date	Ganciclovir treatment (no. of doses)	Flow cytometry (% positive cells) ^a	Infectivity ^b	Serology (titer) ^c
01/03/92		ND ^d	ND	IgG, 238; IgM, 4
01/07/92		ND	ND	ND
01/14/92		0.7	ND	ND
01/28/92		4.7	ND	ND
01/12/92	1	8.9	ND	ND
02/19/92	1	6.7	ND	IgG, 818; IgM, 34
02/24/92	1	19.5	ND	ND
03/05/92	2	22.7	Blood and urine positive	IgG, 429; IgM, 35
03/11/92	2	13.2	ND	ND
03/26/92		1.9	ND	ND
04/28/92		1.0	ND	ND

^a Permeabilized PBMC were treated with monoclonal antibody to an immediate early HCMV antigen followed by FITC-labeled goat anti-mouse IgG F(ab')₂, RNase, and PI, and the percentage of antigen-expressing cells was determined by flow cytometry.

^b Blood and urine specimens were inoculated into MRC-5 cells and examined for cytopathic effect.

^c Serum samples were examined for HCMV-specific IgG and IgM antibodies.

^d ND, not determined.

from HIV-seropositive patients. In the initial report, PBMC obtained from uninfected and HIV-infected individuals were isolated on Ficoll-Hypaque, permeabilized, and analyzed for two-color fluorescence by flow cytometry (125). The results showed that HIV p24 or nef antigens could be detected in PBMC obtained from patients with clinical AIDS. The majority of HIV-seropositive individuals who were asymptomatic (Centers for Disease Control [CDC] group II) did not express the HIV p24 antigen in significant numbers of their PBMC, whereas the majority of those with AIDS (CDC group IV) did. These results suggested that this technique can be used to measure disease progression. Furthermore, the percentage of p24 antigen-positive PBMC was inversely proportional to the total CD4⁺ cell count, indicating that the percentage of p24 antigen-positive cells correlates with a frequently used surrogate marker for HIV infection (99). PBMC obtained from HIV-seronegative individuals showed no significant staining with either the p24 or the nef monoclonal antibodies. In a small sample, patients on ZDV therapy showed decreased percentages of p24 antigen in their PBMC, suggesting that flow cytometry may be used to monitor patients on antiretroviral chemotherapy. This technique has been used to monitor virus load in patients on various antiretroviral therapies (129, 163).

Ohlsson-Wilhelm et al. (143) permeabilized PBMC and then used a double-labeling technique employing PE-labeled monoclonal antibody to CD14, which labeled the monocytes, and mouse monoclonal antibody to p24 antigen followed by FITC-labeled second antibody, which labeled the HIV-infected cells. With this procedure, the PE-labeled CD14⁺ monocytes were excluded from the lymphocyte population so that only the lymphocyte population was analyzed for the presence of internal p24 antigen. The results demonstrated that PBMC obtained from both asymptomatic (mean, 2%) and symptomatic (mean, 4.2%) HIV-seropositive patients had significant numbers of p24 antigen-positive blood cells, whereas most of those on ZDV therapy and all normal controls did not express p24 antigen in this assay. The percentage of p24 antigen-positive PBMC was inversely related to the total number of CD4⁺ cells. When cells from patients with higher percentages of p24 antigen-positive PBMC were tested for virus infectivity by coculture with mitogen-stimulated control lymphocytes, the cultures became positive for p24 antigen sooner than those cocultured with PBMC obtained from patients with lower percentages of p24 antigen-positive PBMCs.

In a similar study, Costigliola et al. (38) showed that p24 antigen could be detected in CD4⁺ PBMC obtained from HIV-seropositive patients at all stages of the disease. Those in CDC stage II had lower percentages of p24 antigen-positive CD4⁺ cells, and those in CDC stage IV had higher percentages of p24 antigen-positive CD4⁺ cells. Antiretroviral therapy with ZDV reduced the percentage of p24 antigen-positive cells. Holzer et al. (80) used flow cytometric analysis of CD4⁺ cells obtained from HIV-infected individuals to show that the CD4⁺ cell number declined and the percentage of HIV-p24 antigen-positive CD4⁺ cells increased as the disease progressed. Finally, Gadol et al. (62) reported on the use of frozen PBMC obtained from HIV-seropositive patients for the detection of p24 and p17 antigens by flow cytometry. They compared a number of different fixation techniques, each of which yielded slightly different results. However, they did show that asymptomatic, HIV-seropositive individuals seldom had significant numbers of p24 antigen-positive PBMC, whereas symptomatic individuals were frequently positive for p24 antigen in up to 10% of their PBMC. From the results of these five independent studies, it is clear that by using the rapid and sensitive quantitative flow cytometric technique, cell-associ-

ated HIV p24 antigen expression can be detected most of the time in patients with AIDS but less frequently in asymptomatic HIV-seropositive individuals. Furthermore, when HIV antigens are detected in PBMC from asymptomatic patients, a lower percentage of antigen-positive cells is detected. The quantitative aspects of this technique allow this differentiation to be made rapidly and with ease. This procedure has been used to monitor disease progression in HIV-seropositive patients and to ascertain the effect of antiviral therapy on virus replication in these patients (38, 62, 80, 125, 143). With the ability to detect both cell surface and internal antigens by using fixatives, such as Permea-Fix and possibly FACS Lysing Reagent, it will be possible to identify and quantitate the peripheral blood cells that are infected with HIV by two- or three-color multiparameter flow cytometric analysis of PBMC obtained from HIV-infected patients without the need to sort cells.

This flow cytometric technique has been used successfully to detect p24 antigen-positive cells obtained from HIV-seropositive children 6 months to 12 years of age (127). There is a critical need for an accurate method to identify HIV-infected babies shortly after birth. Recently, a number of investigators have demonstrated the ability to detect free p24 antigen in plasma of newborn babies by the immune complex-dissociated p24 antigenemia assay (9, 25, 84, 133). Since there is sufficient free p24 antigen in the plasma of newborn babies for detection by ELISA, it should be possible to detect cell-associated p24 antigen in PBMC from these babies by direct or indirect immunofluorescence in conjunction with flow cytometry. With these two rapid, quantitative, complementary techniques, it should be possible to detect cell-free and cell-associated HIV antigens in HIV-infected babies born to HIV-seropositive mothers within days to weeks after birth. This time frame is useful for therapeutic intervention for those babies who are infected and for identification of those HIV-seropositive babies who are not HIV infected. In addition, these two antigen detection systems are more rapid and less expensive than the very sensitive, but time-consuming and costly, quantitative infectivity assays (4, 113).

One of the major criticisms of the flow cytometric technique is that the number of p24 antigen-positive cells detected seems to be very high (13 to 25% in earlier studies and 1 to 10% in more recent studies), the reasons for which vary. They include the point at which the cutoff value for a negative sample is set, the lack of isotype controls in many experiments, and high nonspecific binding of antibodies to cells in some of the clinical samples. Percentages of p24 antigen-positive cells as high as 10% are possible in individuals with advanced HIV infection or AIDS. In one report, essentially all patients with 1 to 10% of their PBMC positive for the p24 antigen were also positive for infectivity in coculture assays (146). Quantitative coculture assays will be required to better compare these two assays. That this technique identifies p24 antigen-positive cells in asymptomatic individuals is probably correct for the following reasons: (i) PCR has detected both HIV proviral DNA and mRNA expression in PBMC at all stages of disease (11, 23, 34, 55, 86, 93, 132, 153, 174, 195, 211); (ii) immune complex-dissociated p24 antigen assays have detected free p24 in plasma at all stages of disease (9, 25, 133, 158); and (iii) infectious virus has been detected in PBMC and plasma from HIV-seropositive patients at all stages of HIV infection (4, 42, 84, 146, 147). An additional criticism of flow cytometry for this use has been the possibility that p24 antibodies are detecting p24 antigen in cells that have phagocytized free viral antigen. The only way to address this possibility is to use antibodies

against HIV antigens that accumulate in the nucleus, such as Rev and Tat (41).

In sum, the data indicate that flow cytometry should be a useful, rapid, and relatively inexpensive technique to measure virus load in HIV-infected individuals and to monitor the effect of antiviral therapy on viral load in vivo. However, it must be remembered that these flow cytometric techniques will only detect cells that are expressing the antigen to which the fluorescently labeled antibody is directed. Cells latently infected with HIV will not be detected with this assay.

ANTIBODIES MEASURED BY FLOW CYTOMETRY

In addition to detecting and quantitating virus-infected cells, antibodies to viruses, cells, and bacteria have been detected and quantitated by flow cytometry. McHugh et al. (122, 123), Scillian et al. (182), and Sligh et al. (190) demonstrated that antibodies to HCMV, HSV, and HIV can be detected by binding to microspheres coated with viral antigens. Antibodies to different viruses could be distinguished by binding each virus to microspheres of different sizes that could be separated by light scatter. Once the antibody attached to its specific microsphere, a fluorochrome-labeled antibody directed against the bound antibody was added to the microspheres, and the labeled spheres were analyzed for fluorescence and light scatter by flow cytometry. Gorse et al. (73) used uninfected H9 cells, HIV-infected H9 cells, and H9 cells expressing the recombinant gp160 gene to measure antibody to recombinant gp160 vaccine. After the human sera reacted with the cells, the antibody-bearing cells were treated with a fluorochrome-labeled antibody to human IgG, and then the fluorescence was analyzed by flow cytometry. This technology has been used to detect antilymphocyte antibodies bound to peripheral blood lymphocytes in patients with AIDS, using FITC-conjugated goat antibody to human IgG (19). Antibodies to bacteria have also been detected by flow cytometry. A multicomponent antigen of *Helicobacter pylori* was bound to microspheres and used to test sera for the presence of antibodies to this organism (17). Bound antibodies were detected with FITC-conjugated goat anti-human IgG, and the fluorescence was quantitated by flow cytometry. Antibodies to *Toxoplasma gondii* in human serum have been measured by using fluorescently labeled antibodies against human IgG, IgM, or IgA after these antibodies were attached to fixed tachyzoites (39). The amount of each antibody bound was quantitated by measuring fluorescence intensity by flow cytometry. Thus, it is possible to use this technology for rapid, quantitative assays for both antibodies and cells expressing antigens derived from patients.

BACTERIA, FUNGI, AND PARASITES

Although this review deals with the use of flow cytometry for the detection of viruses in cells and clinical samples, brief mention will be given to the use of flow cytometry for the detection and quantitation of bacteria, yeasts, and parasites. This subject was recently reviewed (52, 184). Phagocytosis and killing of *Candida albicans* and *Staphylococcus aureus* have been quantitated by flow cytometry (117–119). The organisms were treated with a nonfluorescent derivative of carboxyfluorescein that becomes fluorescent when activated by esterases in viable cells. The phagocytic cells in dextran-enriched plasma were labeled with a PE-conjugated monoclonal antibody. After the organisms were mixed with the phagocytic cells and incubated, two-color fluorescence was detected and quantitated by flow cytometry. Phagocytic activity was monitored by the increase of green fluorescence in a constant amount of

orange cells, and cell killing was monitored by a decrease in the amount of green fluorescence in these orange cells. Green et al. (74) used the permeability to PI of *C. albicans* and other organisms and analysis of cell-associated red fluorescence intensity by flow cytometry to determine the minimal fungicidal concentration of various antifungal compounds.

The viability of *Pneumocystis carinii* has been detected in athymic nude F344 rat lung homogenates and human BAL specimens by flow cytometric analysis of PI-stained cells (102). Only nonviable organisms take up the PI, making it possible to detect and quantitate nonviable organisms. This procedure may be useful for rapid diagnosis and for testing anti-infective agents against *P. carinii*.

The fluorescent nucleic acid-binding dye, ethidium bromide, was used to label bacteria, and the light scatter properties of the bacteria and the fluorescent dye were used to determine the antibiotic effect of amikacin on a variety of gram-negative organisms, using flow cytometry (30). Ordonez and Wehman (144) used the fluorescent dye 3,3'-diphenyloxycarbocyanine to develop a rapid test to measure the effect of antibiotics on the induction of membrane potential changes in *S. aureus*. Changes in fluorescence due to antibiotic action were monitored by flow cytometry, and tests could be performed in 90 min compared with overnight culture for standard assays. The effect of immune sera and complement on the killing of the Lyme disease parasite, *Borrelia burgdorferi*, was quantitated by flow cytometry (166). The organisms were treated with a murine monoclonal antibody, anti-OspA, followed by FITC-conjugated second antibody, or PI was used to stain the organism. The organisms were detected by a combination of light scatter and fluorescence. Specific antibody and complement killed the organisms, reducing the number detected by flow cytometry. This assay was rapid and sensitive. Finally, *Plasmodium falciparum* has been detected in intact and lysed erythrocytes of infected individuals by using flow cytometry (150, 206). This brief summary of some applications of flow cytometry for the detection of other microorganisms should give the reader a sense of the wide applicability of this technology in microbiology.

FUTURE PROSPECTS

I trust that this review has given the reader the sense that flow cytometry has a place in the analysis of virus-cell interactions. Four major areas on which flow cytometry will likely have an impact are summarized below.

(i) Flow cytometry will be used for rapid detection of virus-infected cells and other organisms in body fluids if monoclonal or polyclonal antibodies that specifically react with cell surface or intracellular viral antigens are available. Examples are cells infected with viruses, such as HTLV-1 and -2 (16), hepatitis B virus (29), HCV (199), human herpesvirus 6 (32, 53), varicella-zoster virus (192), and measles virus (59), in blood and microbes, such as *P. carinii* (102) and *Mycobacterium tuberculosis*, in pulmonary lavage fluids. Using flow cytometry for direct detection and possible quantitation of these microbes will save time and expense in the clinical laboratory.

(ii) Flow cytometry will be used for rapid detection of viruses after amplification in cell culture. For many viruses that are not cell associated in body fluids, or when there are too few cells for a statistically accurate determination by flow cytometry, it is possible to amplify the number of cells expressing the viral antigens by overnight incubation in cell culture and then to permeabilize, stain, and analyze the virus-infected cells by flow cytometry. Again, this will save considerable time in the clinical laboratory, particularly when those viruses that take several

days to produce cytopathic effects in tissue culture are being studied.

(iii) As has been demonstrated above, flow cytometric analysis of virus-infected cells is an excellent way to monitor the effects of antiviral or anti-infective agents in vitro and in vivo. Further application of this technology will allow screening of numerous compounds for their antiviral and anti-infective activities. Flow cytometry will continue to be useful in monitoring patients on antiviral chemotherapy to determine the in vivo effects of therapeutic agents on their infection.

(iv) With the development of fluorescent in situ hybridization in suspension, it will be possible to use nucleic acid hybridization methods to detect viruses, bacteria, and other microorganisms in cells and quantitate them by flow cytometry (110, 151, 202, 207). Since flow cytometric analysis of cells stained for viral antigens will detect only cells that are actively expressing viral proteins, the combination of in situ hybridization and flow cytometry will allow detection of latently infected cells. In addition, the combination of flow cytometry and PCR will provide the sensitivity required to detect and quantitate viruses in individual cells.

Of course, flow cytometry will continue to be used to monitor lymphocytes in healthy and diseased people, detect DNA ploidy in tumor cells, and serve as an excellent tool for basic research. Increased use and familiarization with this powerful technology will give the clinician, the laboratorian, and the researcher the ability to move from qualitative science to a more quantitative aspect of their work. In addition, the ability of flow cytometry to measure multiple parameters simultaneously will allow those who are interested in studying the interactions of different cellular and viral components within the cell to perform these experiments. The multiparameter flow cytometric analysis of double- and triple-stained cells will give investigators the opportunity to identify which cells are infected as well as to quantitate the number of virus-infected cells. For example, assuming that the CD4⁺ T lymphocyte is the primary target for HIV, peripheral blood cells could be stained with one or more fluorescently labeled cell surface reagents, and the cells could be permeabilized and treated with one or more fluorescently labeled reagents directed against internal viral antigens. These types of experiments should enable one to identify the peripheral blood cells that are infected with HIV or any virus of interest.

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