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Review

Flow Cytometry Applications in Pharmacodynamics and Drug Delivery

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We highlight recent advances in flow cytometry that have potential applications in the pharmaceutical sciences, particularly in pharmacodynamics and drug delivery. These advances are discussed in the context of the preclinical development of anticancer agents, immunosuppressants and immunomodulators, and oligonucleotides and gene therapy.

KEY WORDS: antibodies; fluorophore; fluorescence activated cell sorting; anticancer agents; immunosuppressants; cytokines; gene therapy; oligonucleotides.

INTRODUCTION

Flow cytometric methods have had considerable impact as a clinical diagnostic tool and are used in assays such as leukemia phenotyping, the CD4 cell count in HIV and the crossmatch in transplantation. Recent advances have extended the versatility of flow cytometric approaches and they can be a sensitive and accurate quantitative method for many applications in pharmacodynamics, pharmacokinetics and drug delivery.

The flow cytometer is remarkable and unique among biomedical analysis instruments because it makes *simultaneous* and *multiple* optical measurements on *individual* cells at *high rates*. This allows the *quantitation* of cellular macromolecules, the *delineation* of cell populations and the subsequent *sorting* of cells that meet specific criteria. The versatility of a flow cytometer is further extended by the high specificity and sensitivity of fluorescent molecular probes such as fluorophore labeled monoclonal antibodies, fluorescent nucleic acid intercalators, and pH and ion sensitive probes. Instrument capability can be further enhanced by installing additional lasers or an ultraviolet light source. With appropriate experimental design, quantitative measurements can be made on each cell type in a mixture containing several cell types and cell function can be studied in the physiological milieu of a complex tissue.

Despite its impressive capabilities, the flow cytometer has not found widespread use in pharmaceutical laboratories and many pharmaceutical scientists tend to view the instrument as a diagnostic tool. This review will therefore focus on flow cytometry applications useful in pharmaceutical research with particular emphasis on methods useful in the development and preclinical assessment of three drug classes: anticancer agents, immunosuppressants and immunomodulators, and oligonucleotides and gene therapy. Diagnostic and immunophenotyping applications will be discussed only if directly relevant to drug development.

CURRENT STATUS

Sensitivity, Limits of Detection, and Linearity of Flow Cytometry

Flow cytometric instrumentation has become increasingly sophisticated and the electronic components such as photomultiplier tubes show predictable behavior over several decades of dynamic range (see Figure 1A). Instrumental linearity is usually not a limiting factor in most applications (1).

Absolute quantitation in the flow cytometer is very reliable particularly when antibodies directly conjugated to fluorophore are used to detect the molecule of interest. A calibration curve for the antibody is easily obtained by using a (commercially available) mixture that contains several microsphere populations each with a known number of antibody binding sites (2). The mixture is stained with saturating amounts of the antibody of interest and, as illustrated in Figure 1B, the flow cytometric fluorescence of each population is plotted against the number of binding sites on the beads to yield the calibration curve. The linearity of the calibration on semi-log axis can extend over three decades or more. The biological factor that determines the limit of detection for many biological specimens is cellular autofluorescence and in some cases, nonspecific binding (1).

Fluorescence quantitation in two or more colors is limited by the overlap between the spectra of the fluorophores used. A correction (referred to as compensation) can be applied electronically to account for the spectral overlap but its effectiveness for absolute quantitation may be compromised when the fluorophores have widely differing intensities.

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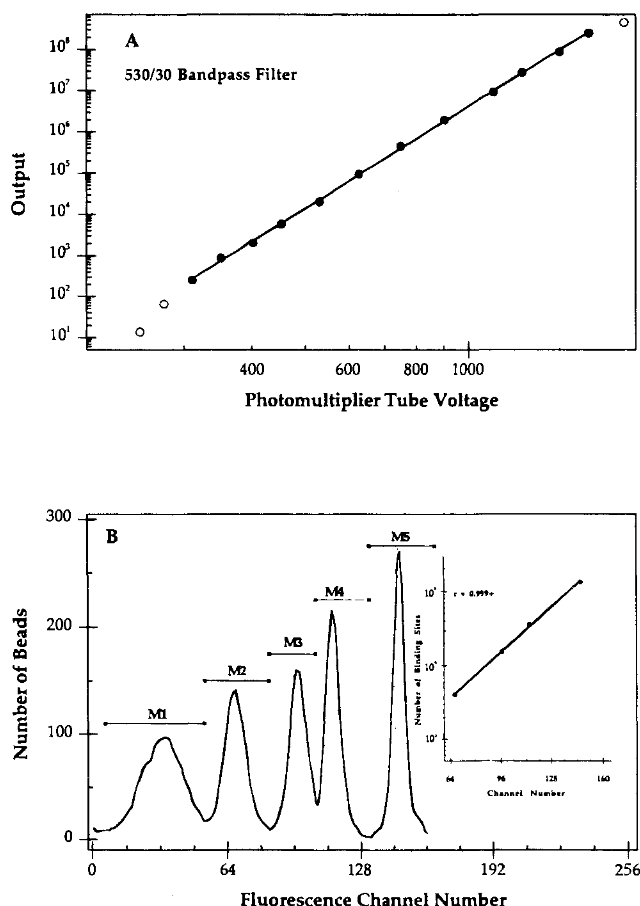

Fig. 1. Figure 1A (redrawn from (61) with permission) demonstrates the dynamic range and linearity of the photomultiplier tubes used in flow cytometers. The results were obtained using fluorescent microspheres and neutral density filters. The response is described by a power function and is linear on a log-log plot over 6 decades (closed circles). Figure 1B shows a typical calibration curve that allows the number of antibody binding sites to be calculated from flow cytometric data. The data are for an anti-human Major histocompatibility complex Class I antibody (Olympus Corp., Lake Success, NY) using Quantum Simply Cellular calibration beads (Flow Cytometry Standards Corp., Research Triangle, NC) on a FacScan flow cytometer (Becton Dickinson, San Jose, CA). The five bead populations indicated contain 0, 4100, 15500, 35300, and 134000 sites, respectively. The regions indicated by M1 through M5 were used to select the populations for calculating the mode. The number of antigen binding sites was plotted against the modes of the populations on a semilog graph to yield the calibration curve shown in the inset.

APPLICATIONS IN THE DEVELOPMENT OF ANTICANCER AGENTS

Potency and Mechanism Determination of Anticancer Agents

Flow cytometry provides the most rapid and accurate methods for assessing the potency and cell cycle specificity of anticancer agents. The viability of thousands of cells can be rapidly assessed in minutes using fluorescent vital stains and cell cycle analysis can be carried out easily on a flow cytometer using agents such as propidium iodide that fluoresce upon intercalating DNA.

The data in Figure 2 (redrawn from (3)), obtained by propidium iodide staining of PC-3 human prostate carcinoma cells treated with 10 nM paclitaxel (Taxol®), provide a direct quantitative demonstration that paclitaxel causes an arrest in the G2/M phase of the cell cycle. Paclitaxel also sensitizes prostate cancer cell lines to radiation in a dose and time-dependent manner (3). Kawamoto (4) used propidium iodide staining and incorporation of the thymidine analog, bromodeoxyuridine, to show that the antineoplastic effects of interferon-α are the result of S phase accumulation, while those of ACNU (nimustine) and cisplatin are caused by a G2/M phase block. Triphenylethylene antiestrogenic agents such as tamoxifen and toromifene block breast cancer cell growth in G0/G1 and cell cycle analysis has also been used to detect the occurrence of clonal selection and drug resistance in response to these drugs (reviewed by (5)).

Because many leukemic cell lines die by apoptosis *in vitro*, the accuracy of drug potency determination can be compromised if the growth conditions are inadequate. Frequently, companion cell types are required to support *in vitro* cell growth

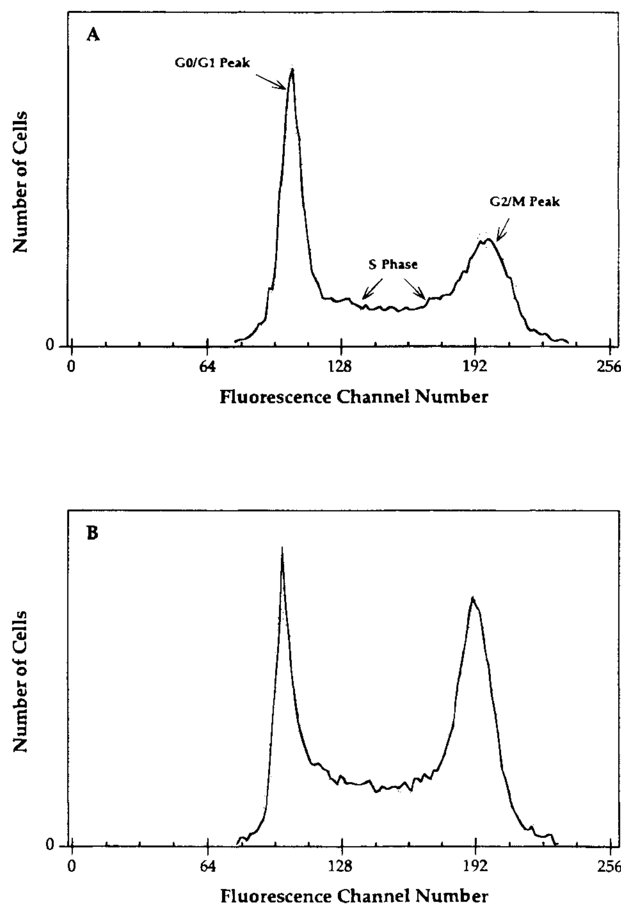

Fig. 2. Figure 2 (redrawn from (3) with permission) shows fluorescence histograms for PC-3 prostate carcinoma cells treated with 10 nM paclitaxel (Taxol®) for 0 hours (Figure 2A) or 6 hours (Figure 2B) and stained with propidium iodide. The fluorescence on the x-axis is on a linear scale. Notice that the fluorescence of the peak corresponding to the cells in G2/M is twice that of cells in G0/G1 and S phase cells have intermediate fluorescence. The y-axis is cell number in arbitrary units. Paclitaxel causes an increase in the percentage of G2/M cells.

and a proliferation assay as such as the stromal-supported immunocytometric assay (SIA) which provides bone marrow stromal cells must be used (6).

In recent years, exciting progress has been made in elucidating molecular mechanisms of apoptosis, a form of programmed cell death. These developments in turn, have stimulated the development of rapid flow cytometric methods for apoptosis quantitation (7) which are likely to prove very useful for studying the pharmacodynamics of anticancer agents and in the screening of apoptosis-modulating drugs. Apoptosis and programmed cell death may also play a role in the mechanism of action of a variety of other drug classes as well. For example, apoptosis/programmed cell death is induced by glucocorticoids such as dexamethasone and inhibited by drugs such as cyclosporin A, RU 486, linomide and trans-retinol (8).

Cell death via apoptosis occurs in response to specific stimuli and has distinct biochemical and morphological signatures such as: nuclear condensation, a reduction in cell volume, blebbing of the plasma membrane, nuclear fragmentation and formation of a 200 base pair DNA ladder derived from internucleosomal degradation of chromatin. Membrane integrity and mitochondrial function are not compromised until apoptosis is advanced (9,10). In both necrosis and apoptosis, the phospholipid asymmetry of the cell membranes is lost, and phosphatidylserine, usually present only in the inner leaflet, is translocated to the outer leaflet (11). Apoptotic cells translocate phosphatidylserine at an early step—after nuclear condensation has begun but before membrane integrity is compromised.

The differences in membrane integrity provide the basis for the flow cytometric method for delineating cell populations in early apoptosis from those in late apoptosis and necrosis. The flow cytometry assay uses (fluorescently labeled) Annexin V, an anticoagulant protein that has a high affinity and selectivity for phosphatidylserine and a dye such as propidium iodide that is excluded from cells with intact membranes (12,13). As shown in Figure 3, viable cells are negative for both Annexin V and propidium iodide, apoptotic cells are positive for Annexin V but exclude propidium iodide, and necrotic cells bind Annexin V and fail to exclude propidium iodide.

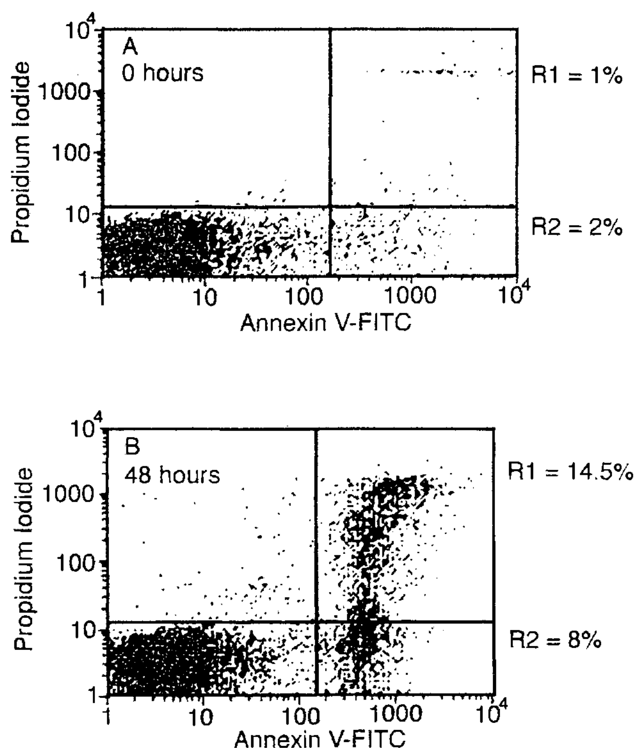

Fig. 3. Figure 3 (adapted from (13)) demonstrates the flow cytometric delineation of the early apoptotic cells (Region R2) from late apoptotic cells and necrotic cells (Region R1) and normal cells (lower left quadrant). Each point represents data from a single cell. Figure 3A shows control, untreated lymphocytes at 0 hours and Figure 3B shows lymphocytes treated with 100 μM dexamethasone for 48 hours. Cells were analyzed after treatment with propidium iodide and fluorescein isothiocyanate-conjugated annexin V. The data in Figure 3B show an increased percentage of early apoptotic (8%) as well as late apoptotic and necrotic cells (14.5%). Reprinted from *Journal of Immunological Methods* with kind permission of Elsevier Science NL.

Uses in Drug Discovery, High Throughput Screening and Combinatorial Chemistry

The flow cytometer is compatible with the products from the solid phase synthesis of peptide, oligonucleotide and other combinatorial chemistry libraries because the instrument has been designed/optimized for the efficient analysis of cells and micron size particles. An instrument with a sorting unit can thus be used to rapidly screen libraries for ligands.

Selection for high affinity ligands is achieved in flow cytometry by using low ligand concentrations during the binding step. Needels *et al.* (14) screened a peptide combinatorial library synthesized on 10 μm beads for high affinity peptide ligands to an anti-dynorphin B antibody using a flow cytometer. A parallel synthesis procedure that placed an encoding oligonucleotide tag and the corresponding peptide on each bead was used. The beads containing high affinity ligands were separated by flow cytometric sorting and peptides were identified by amplifying and then sequencing the oligonucleotide tags. Muller *et al.* synthesized a phosphotyrosine containing peptide library and identified high affinity ligands for the src homology-2 (SH2) domains of Grb2, an adapter protein, and Syk2, a tyrosine kinase (15). In this study, mass spectrometry was used to identify the high affinity ligand sequences sorted by cytometry.

In a direct, functional approach, Midoux *et al.* (16) used flow cytometry to screen membrane permeabilizing α-helical peptide sequences by treating HL60 cells to peptides in the presence of ethidium bromide, a dye that is normally excluded from cells and becomes intensely fluorescent only on binding DNA. Peptides that increased membrane permeability caused increased cellular fluorescence.

Hepatic drug metabolism and cytochrome P-450 activity are of importance in drug development and flow cytometry may be uniquely suited for applications in this area (17,18). Techniques are available for assaying glutathione S-transferase activity (1) but few validated, isozyme specific P-450 assays are currently available. Flow cytometry assays for these drug metabolism applications would certainly be of value to pharmaceutical scientists.

A flow cytometer with sorter is well suited for high throughput screening applications because many flow cytometric staining procedures can be easily automated and because robotic sample handling systems and macro-driven data analysis software are already commercially available.

Tumor Load Determination

Recently, flow cytometry has been combined with *in situ* PCR. The methodology has been used to test for the presence of the disease causing hybrid *bcr/abl* gene in chronic myelogenous leukemia patients (19) and for the presence of proviral DNA and viral mRNA in blood from HIV patients (20,21). Cells are fixed and permeabilized, treated with proteinase K and the cellular mRNA is reverse transcribed into cDNA. The cDNA is amplified with primers that are fluorescently labeled using the polymerase chain reaction. The method is rapid and sensitive and identifies positive populations in a background dominated by negative cells. It can be used to determine viral and tumor loads in patients for pharmacodynamic studies.

The extension of molecular biology techniques to flow cytometry is rapidly increasing. However, it is important to ensure that the probes used are specific for the nucleic acid target. Failure to do so can result in undetected errors because unlike Northern and Southern blotting, flow cytometry does not provide visual endpoints such as autoradiograms that can be evaluated for specificity of binding.

Drug Uptake

When human tumors become multidrug resistant, the responsiveness to anthracyclines and other anticancer drugs is reduced because of increased expression of P-glycoprotein, a transporter which actively effluxes a variety of drugs from the cytoplasm. Because anthracyclines have severe dose limiting toxicities, and because strategies for inhibiting P-glycoprotein-mediated efflux are available, it is very desirable to identify the occurrence of multiple drug resistance in cancer. Flow cytometry can be used to determine the kinetics of uptake and efflux of anthracycline anticancer agents such as doxorubicin and daunomycin because these drugs fluoresce at an emission maximum of 600 nm when excited with the 488 nm argon laser available on most flow cytometers (22–25).

In a conventional flow cytometer, reagent addition requires interruptions in measurement and this can make carrying out transport and kinetics studies inconvenient. To overcome these problems, an on-line flow cytometer which allows reagents to be added directly to cells in the sip-tube of a flow cytometer without interrupting a measurement has been developed (24,25), and sub-second time resolution has been achieved by combining rapid mixing devices with a commercial flow cytometer (26). Doxorubicin uptake measured by flow cytometry using a rapid-injection system yielded results that were in agreement with studies using radiolabeled ^{14}C-doxorubicin (27).

Nooter *et al.* showed (Figure 4) that leukemic cells from patients with refractory acute nonlymphocytic leukemia contained *mdr-1* mRNA and used the on-line flow cytometer to demonstrate that the resistant cells accumulated less daunomycin than control cells obtained from the same patient prior to the appearance of *mdr-1* mRNA (25). The measurements in Figure 4 also show that the addition of cyclosporin-A and verapamil increased daunomycin accumulation, presumably by competing for efflux. Daunomycin uptake increased when sodium azide, a metabolic inhibitor, was added to patient-derived leukemic cells in glucose free medium. This is consistent with an ATP dependent efflux pump. Additionally, the azide-induced increase in uptake could be abolished by the addition of glucose which presumably allowed ATP generation to proceed via the glycolysis, activating the efflux pump.

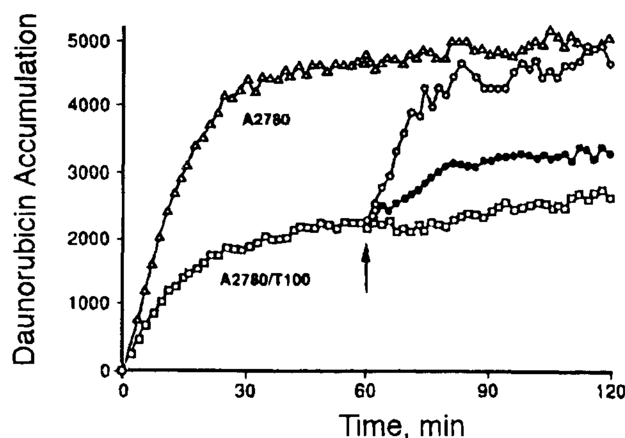

Fig. 4. Data from Nooter *et al.* (25) showing the kinetics of daunorubicin accumulation (expressed as fluorescence intensity in arbitrary units) as measured by flow cytometry in the *mdr*-1 transfected cell line A2780/T100 (open squares) and the control A2780 cell line (open triangles). Cells were treated with 2 μM daunorubicin at time, t = 0 and either 10 μM verapamil (closed circles), 3 μM cyclosporine (open circles) or medium (open squares) was added at time, t = 60 min. Due to the drug effluxing action of the *mdr*-1 gene product, transfected cells accumulated less drug than control cells. However, drug accumulation increased upon treatment with the efflux inhibitors verapamil or cyclosporine. Figure reproduced with permission.

Flow cytometric techniques have been used to demonstrate that the uptake of the anthracyclines, doxorubicin, daunorubicin and daunomycin (22,23), by human solid tumor ascites cells is heterogeneous, and that treatment with the P-glycoprotein efflux inhibitor, chlorpromazine, abolishes the heterogeneity. As shown in Figure 5, a single population with high drug fluorescence emerged in the flow cytometric dot-plots after verapamil treatment suggesting that drug uptake heterogeneity in ascites cells is caused primarily by differences in efflux.

However, flow cytometric techniques have also demonstrated that the tumor response to efflux blockers such as chlorpromazine and verapamil is heterogeneous and this can compromise the usefulness of these drugs as sensitizers for chemotherapy (23).

Flow cytometry can also be used to measure other driving forces for transport, e.g., membrane potential, intracellular pH and calcium ion concentrations that are often of relevance in drug uptake studies. In the interests of space these assays are not discussed.

APPLICATIONS IN THE DEVELOPMENT OF IMMUNOMODULATORY AND IMMUNOSUPPRESSIVE AGENTS

Quantitating Anti-Protein Antibodies

Flow cytometric methods can potentially be used for the rapid quantitation of proteins and peptide drugs in serum for therapeutic drug monitoring. OKT3 is an immunosuppressive mouse monoclonal antibody that is used in rejection and a flow cytometric assay for serum anti-OKT3 antibodies is widely used in transplantation for making dosing decisions because

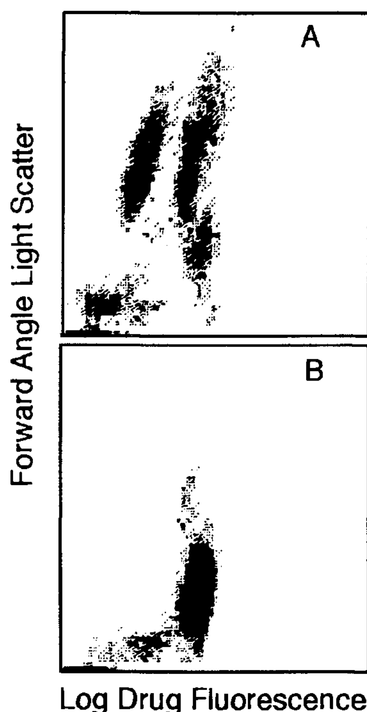

Fig. 5. Dot plot from Krishan (22) showing uptake of doxorubicin by human solid ascites. In (A) there are four subsets demonstrating that drug uptake is heterogeneous. When the cells are incubated with the P-gycoprotein efflux inhibitor, chlorpromazine, a single population emerges and this consistent with a higher efflux rate in the subpopulations with lower accumulation. Figure reproduced with permission.

patients frequently develop antibodies that reduce the effectiveness of OKT3 (28). We discuss this assay in some detail because it is a prototype for pharmaceutical applications and because therapeutic proteins, including those with human sequences, can elicit antibodies in patients. Like the anti-OKT3 antibodies, these antibodies can have a significant impact on pharmaceutical care if they neutralize administered drug or alter its disposition.

The approach used in flow cytometry assays for anti-OKT3 antibodies and other "soluble antigens" is similar to that of an enzyme linked immunosorbent assay (ELISA) and is frequently referred to as the fluorescent microsphere immunoassay (FMIA). As illustrated in Figure 6, the assay for serum anti-OKT3 antibodies uses OKT3 immobilized on polystyrene beads to which serial dilutions of serum are added. The anti-OKT3 antibodies present in serum bind the immobilized OKT3 and after washing, the bound antibodies are labeled with a fluorescently labeled secondary antibody that recognizes the constant region of the human immunoglobulins. The bound fluorescence is quantitated in a flow cytometer. Such bead based flow cytometry assays take 1–2 hours and are more rapid than the corresponding ELISA (28,29). The experience with anti-OKT3 antibody demonstrates that they can prove useful for therapeutic drug monitoring. Similar assays have been proposed for monitoring soluble IL-2 receptors in serum (29) and for a variety of immunoglobulins associated with autoimmune disease (see (30) for a summary).

The FMIA can be used to simultaneously assay for multiple analytes in a single sample by using ligands bound to microspheres of different sizes. Microspheres of different sizes are easily discriminated by using forward light scatter measurements and the fluorescence of each size of beads can be independently quantitated. McHugh (30) used beads of diameter 4, 5, 7 and 9 μm conjugated to antigens from *T gondii*, cytomegalovirus, rubella and herpes simplex virus, respectively, and measured serum antibodies to all four pathogens simultaneously.

Assessing Immune Cell Activation

During immunosuppressant and immunomodulator development, measures of both overall immune status and of the responses of individual lymphocyte subsets are frequently required (31,32). Methods such as lymphocyte proliferation and cytokine secretion have been widely used as measures of immune status. However, these assays take 3–7 days to complete and require relatively large blood volumes. Additionally, lymphocytes must be separated from red blood cells using density gradient centrifugation prior to use.

Although whole blood proliferation assays that use smaller volumes and eliminate lymphocyte isolation have been developed (31–34), these methods still take several days to complete and are vulnerable to variations in initial white blood cell count. A rapid flow cytometric assay for immune activation that takes about 5 hours is a potential replacement for the lymphocyte proliferation assay in many pharmacodynamic applications.

The flow cytometry assay (35) involves monitoring CD69, a cell surface marker that is rapidly and transiently expressed within 4 hours of activation on all T, B and natural killer lymphocytes (36). The assay can be carried out in whole blood making it very suitable for use as a quantitative pharmacodynamic measure. The CD69 assay is useful particularly when potent stimuli are used for T cell activation and the results obtained can parallel those obtained the tritiated thymidine proliferation assay (37). However, weak stimuli can cause increased CD69 expression without causing proliferation, suggesting that the two assays measure complementary aspects of immune activation (38).

Intracellular Cytokines

Cytokines play a central role in mediating normal and pathological immune responses and most immunomodulatory and immunosuppressive drugs have profound effects on cytokine release (39). From immunological studies, it is now clear that cytokine production is qualitatively and quantitatively heterogeneous even in cell populations, for example, helper T cells, that are homogeneous with respect to multiple cell surface markers. Increasingly, the quantitation of the *in vivo* pharmacodynamic effects of immunomodulatory agents and vaccines will require determination of cytokine secretion profiles and measurement of the frequency of cytokine producing cells (40,41). Tedious, time and labor consuming assays such as limiting dilution analysis, immunofluorescence microscopy and the ELISPOT assay that were used for this application have been largely supplanted by flow cytometric methods (42,43). The method is rapid, sensitive and it has been widely used to determine the relative proportions of T helper 1 (Th1) cells and T helper 2 cells (Th2) in peripheral blood (42). The ability to measure the Th1-Th2 balance is important because the interferon-gamma, interleukin-2, tumor necrosis factor and other

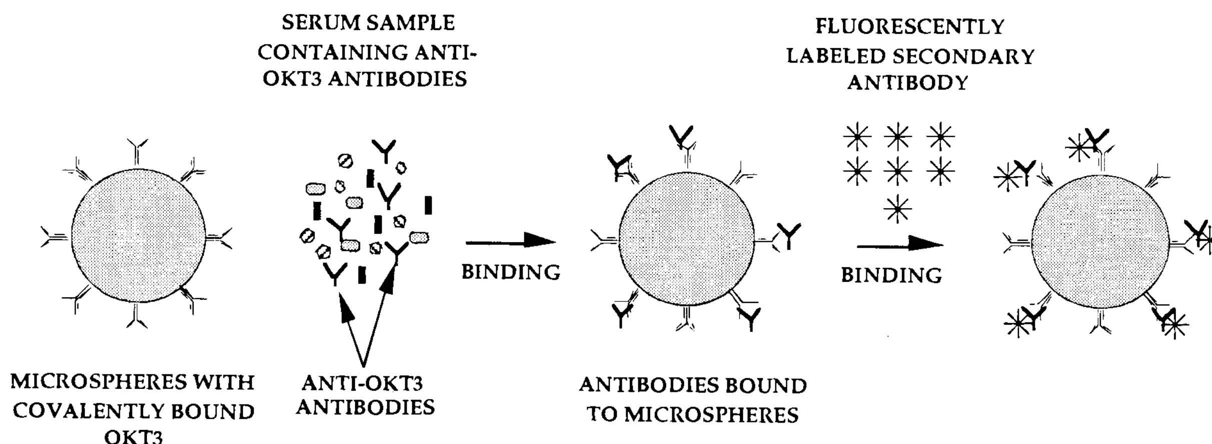

Fig. 6. Schematic of the flow cytometric assay for anti-OKT3 antibodies. The test serum containing anti-OKT3 antibodies is added to microspheres containing covalently coupled OKT3. The anti-OKT3 antibodies bind OKT3. Nonspecific binding is minimized by washing and a fluorescently-labeled anti-human immunoglobulin antibody is added. This allows the bound antibodies to be visualized in a flow cytometer. Figure reproduced from (62) with permission.

cytokines produced by Th1 cells promote cellular immune responses and antagonize the humoral reponse promoted by the Th2 cells which characteristically produce cytokines such as interleukin-4 and interleukin-10.

The flow cytometric intracellular cytokine assay (43,44) can be carried out with heparinized whole blood or isolated lymphocytes. The immune cells are stimulated for 4–6 hours to produce cytokines, usually with nonspecific agents such as phorbol myristyl acetate plus ionomycin. Cytokine release into the medium is blocked with brefeldin A or monensin which prevents egress of newly synthesized cytokine molecules from the cell. The cells are then fixed and permeabilized prior to staining with fluorophore labeled antibodies against the cytokine of interest.

However, care must be taken to use antibodies that are optimized for intracellular staining because fixation can result in epitope alterations. Currently, the technique is used for measuring the percentage of "cytokine-positive cells" and relationship between the actual intracellular cytokine concentration and fluorescence intensity is poorly understood.

APPLICATIONS IN THE DEVELOPMENT OF GENE OLIGONUCLEOTIDE THERAPIES

Gene Therapy Applications

Target Cell Purification

In *ex vivo* gene therapy, the goal is to transform cellular subsets and allow the cells to proliferate *ex vivo* before introduction into patients. Considerable initial gene therapy research effort was directed toward identifying and transfecting hematopoietic stem cells which, in principle, are ideal target cells for gene therapy of chronic blood-related genetic diseases because they yield progenitors for a variety of cell types and have the capacity for self renewal. However, difficulties have been encountered in practice because hematopoietic cells constitute only a small fraction of the marrow, are relatively quiescent and appear to either have limited capacity for self renewal or lose viability upon transplantation.

Goodell *et al.* (45) have shown that mouse cells with phenotypic markers characteristic of hematopoietic stem cells could be distinguished from other contaminating bone marrow cells based on the retention of the fluorescent DNA binding dye, Hoechst 33342. Stem cells retained less Hoechst 33342 and a thousand-fold purification of stem cells capable of reconstituting both lymphoid and myeloid lineages was achieved by monitoring dye fluorescence at two wavelengths, 475 ± 20 nm and greater than 675 nm. In a somewhat surprising twist, the low Hoechst 33342 fluorescence of hematopoietic stem cells is caused by increased expression of P-glycoprotein or a P-glycoprotein-like efflux pump that is inhibited by verapamil (45). Human bone marrow stem cells can be isolated and sorted by using a combination of Hoechst 33342 and rhodamine. (46,47).

This strategy is simple and may prove useful for gene and cell therapy for hematopoietic diseases. Its usefulness for human clinical applications however, would be enhanced considerably if safer alternatives to Hoechst 33342 are identified.

Promoter and Transfection Efficiency

Vector development and delivery are two focus areas for much of the gene therapy research currently in progress. Promoter and transfection efficiencies have frequently been optimized using heterologous proteins such as chloramphenicol acetyl transferase, β-galactosidase and luciferase. These reporter proteins are not native to the cell and these assays have limited usefulness from a pharmaceutical standpoint because native mRNAs and proteins of therapeutic interest can have widely different intracellular half-lives and disposition. Flow cytometry has also been used to identify native and heterologous proteins but extrinsic fluorophore labeled antibodies are necessary.

The cloning by Chalfie *et al.* (48) of the fluorescent green fluorescent protein (GFP) of the luminescent jellyfish, *Aequorea victoria*, has had a significant impact on the design of gene therapy studies because for the first time, a useful fluorophore can be generated in cells using molecular biology techniques. The wild-type fluorophore has two absorption peaks, a major

peak at 395 nm and a lower amplitude peak at 470 nm that is more compatible with the standard argon laser excitation sources in most cytometers (49). The p-hydroxybenzylidene-imidazolidinone chromophore responsible for the fluorescence is short and is generated by cyclization and oxidation of the protein's own Ser-Tyr-Gly sequence at positions 65–67 (50). The active chromophore is spontaneously generated in the presence of oxygen (50) in a variety of cell types and it can be incorporated into native proteins using genetic engineering techniques. No special enzymes or cofactors appear to be needed, and because the chromophore is small, it is likely to have a lesser impact on the protein three-dimensional structure, function and disposition. These properties make GFP a powerful tool for reporting of gene expression, tracing cell lineage, and monitoring protein localization. However, wild-type GFP has a relatively low fluorescence intensity and the need for oxidation introduces a delay (approximately 4 hours at 22°C (50)) between fluorescence development and protein synthesis (51).

Some of these disadvantages can be overcome by using genetically engineered mutant GFPs (52). A serine 65 to threonine mutant, S65T GFP (52), has a single absorption peak at 490 nm and affords increased sensitivity in a flow cytometer (49). Cormack *et al.* (53) have produced mutants with shifted excitation spectra that are more fluorescent under flow cytometric conditions. A plasmid expression vector for GFP with "humanized" codons is also available commercially. The emission wavelength of GFP can be blue shifted by substituting tryptophan or histidine for tyrosine 66 but these variants exhibit lower fluorescence.

Oligonucleotide Therapy Applications

Oligonucleotide delivery is the principal rate limiting factor in the development of antisense and antigene therapies (54) and considerable developmental effort is directed at identifying nontoxic, serum-resistant compounds that enhance oligonucleotide transport *in vivo* (55). The negative charge and molecular weight of oligonucleotides prevents diffusion across cell membranes and oligonucleotide uptake into cells occurs via the energy-dependent adsorptive endocytosis and pinocytosis mechanisms.

Flow cytometry has found increasing use in oligonucleotide uptake studies because it allows dead cells, which tend to accumulate polyanions, to be excluded from the analysis (56). Fluorescein labeled oligos have been commonly used because these are readily synthesized in automated synthesizer but a caveat/drawback with using a fluorescein label in uptake studies is that its fluorescence intensity is pH sensitive and can be reduced in the acidic environment of the endosome.

Physicochemical Characterization of Oligonucleotide Transport

Stein *et al.* (57) used flow cytometry to determine the equilibrium constant and the pseudo-first-order association and dissociation rate constants for the cellular association of a 15-mer fluorescein-labeled, thymidine-containing phosphodiester oligonucleotide in HL-60 at cells at 4°C. The dissociation equilibrium constant was 22 nM and the association process ($t_{1/2}$ = 1 min) was more rapid than dissociation ($t_{1/2}$ = 3.7 h). At physiological temperatures, pinocytosis and not adsorptive endocytosis, was shown to be the primary pathway for oligonucleotide uptake. Inhibitors of protein kinase C inhibited oligonucleotide uptake by inhibiting pinocytosis and oligonucleotides in turn, were found to be sequence independent inhibitors of protein kinase C.

Oligonucleotide Distribution in Blood and Bone Marrow

Oligonucleotide uptake in lymphoid cells has been similarly determined using multi-color flow cytometry. In human blood and bone marrow, oligonucleotide uptake is heterogeneous and myeloid cells and B cells show greater uptake than T cells. CD4 positive helper T cells and CD8 positive cytolytic T cell subsets have similar levels of uptake, and leukemic cells had greater oligonucleotide uptake than their normal counterparts (58). Uptake is also a function of activation and is altered by growth factors.

In murine lymphoid cells, the uptake of an 18–20-mer fluorescein-labeled phosphodiester oligonucleotide was also heterogeneous and only 5% of all lymphoid cells accumulated substantial amounts of oligonucleotide after 3 hours (59). Approximately 5% of the B cells and 10–15% of the T cells were positive for the fluoresceinated oligonucleotide and as in human blood, uptake in the CD4 positive helper T cells and CD8 positive cytotolytic T cells subsets was similar—both subsets showed 6% positives. In contrast, 30–60% of the CD4, CD8 double negative T cells were fluoresceinated oligonucleotide positive. Pre-culturing *in vitro* for 48 hours increased the percentage of B cells positive for oligonucleotide fluorescence almost ten-fold to greater than 50% and this percentage was further increased upon activation with mitogens.

In a study of murine bone marrow B cell precursors using four color cytometry with differentiation specific markers, relatively low uptake was observed among pre-pro- and early pro-B cells and late pro-B- and pre-B cells had increased oligonucleotide uptake (60). The uptake of fluorescein-labeled dextran sulfate in B cell precursors also showed a similar distribution suggesting that the polyanionic nature of oligonucleotide, not sequence or oligonucleotide structure, was an important cause of heterogeneity.

Intracellular Distribution of Oligonucleotides

By carrying out flow cytometry-based oligonucleotide uptake studies in the presence of endosomal acidification inhibitors such as monensin and bafilomycin, Tonkinson and Stein (56) were able to infer that the intracellular processing and trafficking of phosphodiester and phosphorothioate oligonucleotides in HL-60 was different. Phosphorothioate oligonucleotides were processed in an endosome-like compartment that was more acidic than phosphodiesters.

SUMMARY

The current rate of flow cytometry instrument development is likely to continue and significant improvements in sorting rates and in the ability to carry out transport and kinetic studies can be expected in the near term.

In summary, flow cytometry has tremendous promise for quantitative applications in drug discovery, pharmacokinetics, pharmacodynamics and drug delivery. The increasing emphasis on high throughput drug screening and on the development of

targeted therapeutic agents such as proteins, oligonucleotides and gene therapies are likely to make flow cytometry an important if not indispensable analytical tool in the pharmaceutical sciences.

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