Principles and Applications of Flow Cytometry

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Flow cytometry is a technique of quantitative single cell analysis. The flow cytometer was developed in the 1970's and rapidly became an essential instrument for the biologic sciences. Spurred by the HIV pandemic and a plethora of discoveries in hematology, specialized flow cytometers for use in the clinical laboratory were developed by several manufacturers. The major clinical application of flow cytometry is diagnosis of hematologic malignancy, but a wide variety of other applications exist, such as reticulocyte enumeration and cell function analysis. Presently, more than 40,000 journal articles referencing flow cytometry have been published. This brief review of the principles and major clinical applications of flow cytometry may be supplemented by several recent review articles and books.[1-5]

The technique of analyzing individual cells in a fluidic channel was first described by Wallace Coulter in the 1950s, and applied to automated blood cell counting. Subsequent developments in the fields of computer science, laser technology, monoclonal antibody production, cytochemistry, and fluorochrome chemistry led to the development of the flow cytometer two decades later. Because the first commercial flow cytometers were large, complex, expensive, and difficult to operate and maintain, they were primarily used in the research laboratory. However, the enormous value of the flow cytometer in the medical and biologic sciences was quickly appreciated, and its cost and complexity gradually decreased as its analytic capability increased.[6] The present "state-of-the art" flow cytometers are capable of analyzing up to 13 parameters (forward scatter, side scatter, 11 colors of immunofluorescence) per cell at rates up to 100,000 cells per second. Automation and robotics is increasingly being applied to flow cytometry to reduce analytic cost and improve efficiency.

Basic Principles of Flow Cytometry

Prepared single cell or particle suspensions are necessary for flow cytometric analysis. Various immunoflurescent dyes or antibodies can be attached to the antigen or protein of interest. The suspension of cells or particles is aspirated into a flow cell where, surrounded by a narrow fluid stream, they pass one at a time through a focused laser beam. The light is either scattered or absorbed when it strikes a cell. Absorbed light of the appropriate wavelength may be re-emitted as fluorescence if the cell contains a naturally fluorescent substance or one or more fluorochrome-labeled antibodies are attached to surface or internal cell structures. Light scatter is dependent on the internal structure of the cell and its size and shape. Fluorescent substances absorb light of an appropriate wavelength and reemit light of a different wavelength. Fluorescein isothiocyanate (FITC), Texas red, and phycoerythrin (PE) are the most common fluorescent dyes used in the biomedical sciences. Light and/or fluorescence scatter signals are detected by a series of photodiodes and amplified. Optical filters are essential to block unwanted light and permit light of the desired wavelength to reach the photodetector. The resulting electrical pulses are digitized, and the data is stored, analyzed, and displayed through a computer system.[7, 8] The end result is quantitative information about every cell analyzed (Fig. 1). Since large numbers of cells are analyzed in a short period of time (>1,000/sec), statistically valid information about cell populations is quickly obtained.

Clinical Applications of Flow Cytometry

Diagnosis of Hematological Malignancies

The identification and quantitation of cellular antigens with fluorochrome-labeled monoclonal antibodies ("immunophenotyping") is one of the most important applications of the flow cytometer.[9-11] Immunophenotypic analysis is critical to the initial diagnosis and classification of the acute leukemias, chronic lymphoproliferative diseases, and malignant lymphomas since treatment strategy often depends upon antigenic parameters. In addition, immunophenotypic analysis provides prognostic information not available by other techniques, provides a sensitive means to monitor the progress of patients after chemotherapy or bone marrow transplantation, and often permits the detection of minimal residual disease. Flow cytometric analysis of apoptosis, multidrug resistance, leukemia-specific chimeric proteins, cytokine receptors and other parameters may provide additional diagnostic or prognostic information in the near future.

Leukemias represent abnormal proliferations of hematopoietic cells that are arrested at a discrete stage of differentiation. Leukemias are classified into acute and chronic forms based on a constellation of clinical and laboratory findings. The acute leukemias are classified into two subclasses; the lymphoblastic (ALL) type and myeloid (AML) type based on morphologic, cytochemical and immunophenotypic features. Nearly three

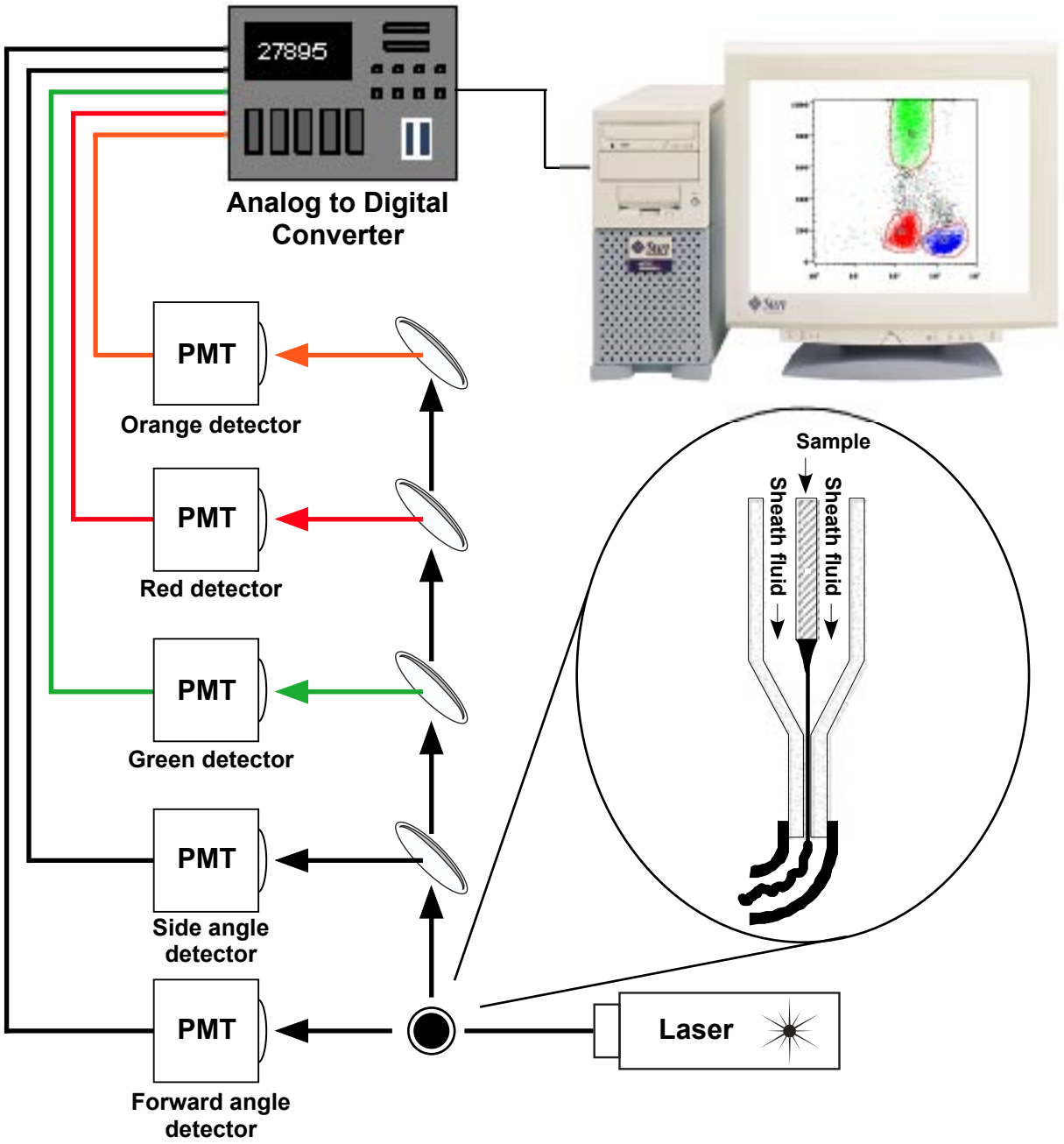

Fig. 1. How the flow cytometer works. Fluorochrome-labeled monoclonal antibody solutions are added to a cell suspension from a peripheral blood, bone marrow aspirate, or lymph node. The tubes are incubated at room temperature for a short period of time. The labeled cell suspensions are passed through the flow cell of a flow cytometer (Insert). Many flow cytometers are automated, but some models require the operator to process the tubes individually. More than 10,000 cells from each tube are typically analyzed to produce statistically valid information. Each cell passes individually through the highly focused laser beam of the flow cytometer, a process termed single cell analysis. The fluorochrome of each labeled monoclonal antibody attached to the cell is excited by the laser light and emits light of a certain wavelength. The cells also scatter light at multiple angles. Photodetectors placed a forward angle and at right angles to the axis of the laser beam collect the emitted or scattered light. Forward and right angle scatter signals, and as many as five fluorochrome signals can be detected from each cell (multiparametric analysis). The signals from each photodiode are digitized and passed to a computer for storage, display, and analysis. Typically, all data recorded from each cell is stored, for possible later recall for further analysis ("list mode data storage"). A variety of histograms for visual display can be generated automatically or at the discretion of the operator. List mode data can also be transferred to a separate computer for analysis. Presently, most commercial flow cytometers utilize a standardized file format for list mode storage, and a variety of computer programs are commercially available for data analysis and display.

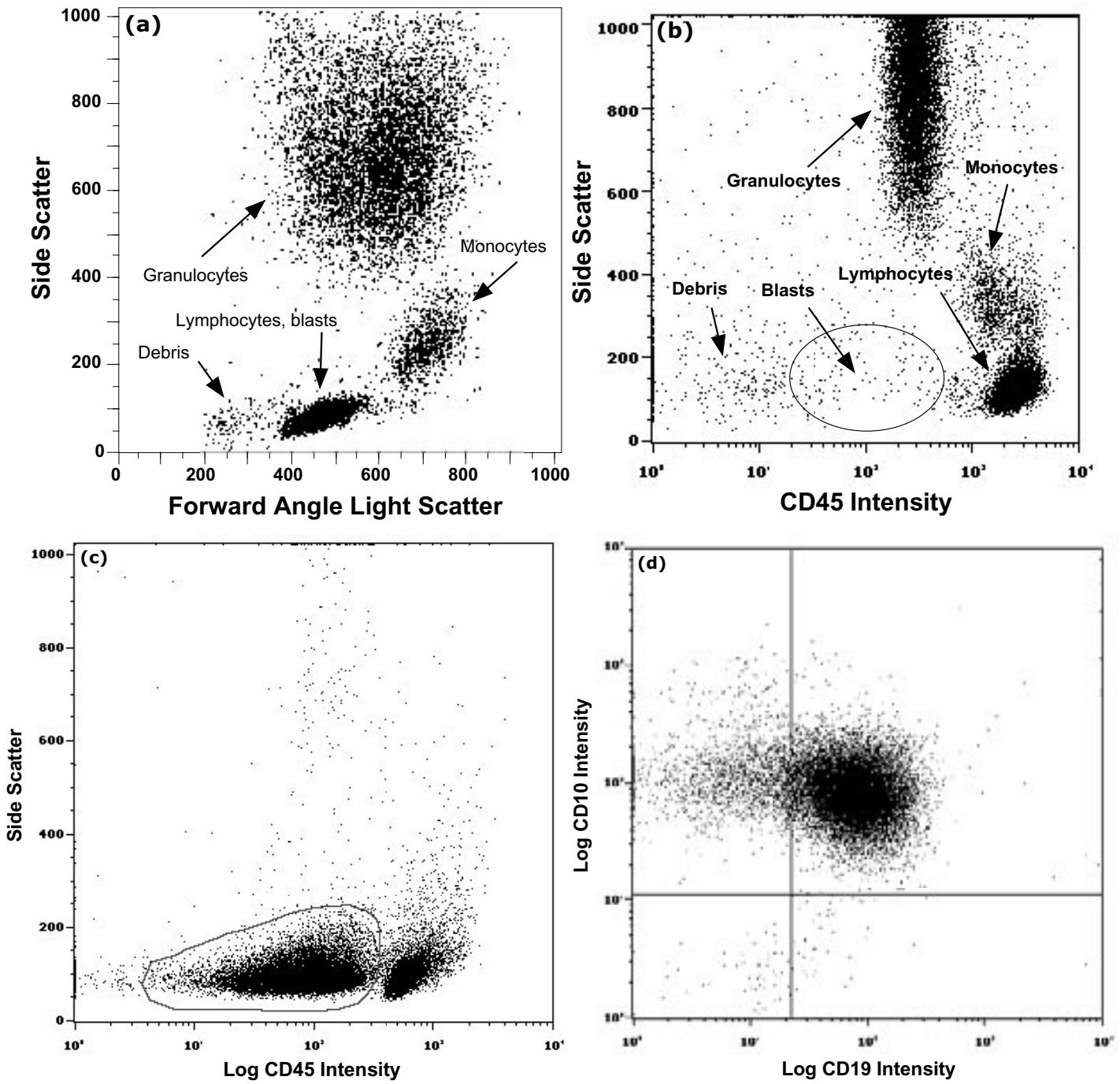

Fig. 2. Flow cytometric data analysis. Data analysis requires selection of the cell population(s) or interest, followed by determination of the proportion of positive cells for each antigen studied in each population. Typically, forward vs. side scatter or CD45 vs. side scatter are used to identify the cell populations(s) of interest. (a) "Dot plot" of forward light scatter vs. side scatter of a bone marrow aspirate demonstrating the characteristic position of different cell populations. Each "dot" represents a single cell analyzed by the flow cytometer. (b) "Dot plot" of a bone marrow aspirate showing CD45 expression and side scatter. This histogram is usually used for leukemia analysis, since leukemic blast cell usually show decreased CD45 expression, and appear in a region of the histogram where few other cells are present. The bottom histograms are bone marrow from a child with acute lymphoblastic leukemia (ALL). (c) The bone marrow predominately consists of blast cells and lymphocytes. A "gate" has been drawn around the blast cells, to restrict analysis to this cell population. (d) The gated blast cells have been analyzed for CD19 (x-axis) and CD10 (y-axis). The blasts show bright expression of CD10 and CD19, typical of childhood ALL.

Table I
Human Leukocyte Antigens for Hematologic Diagnosis

Antigen	Normal Cellular Expression	Major Diagnostic Application	Biological Function
CD1	Cortical thymocytes, dendritic reticular cells, Langerhans cells	Precursor T cell ALL and some lymphoblastic lymphomas.	MHC class I-like molecule, associated with beta-2-microglobulin. Antigen presentation, thymic T-cell development.
CD2	T-cells, thymocytes, NK cells	Hematopoietic neoplasms of T cell lineage.	Sheep erythrocyte rosette receptor. LFA-3 (CD58) ligand. Adhesion molecule, can activate T cells.
CD3	T-cells, thymocytes	Hematopoietic neoplasms of T cell lineage.	T cell antigen receptor. Signal transduction by TCR.
CD4	Thymocyte subsets, helper and inflammatory T cells, monocytes, macrophages	Sezary cell leukemia. SomeT-cell hematopoietic neoplasms	Coreceptor for MHC class II molecules. Receptor for HIV-I and HIV-2 gp120.
CD5	T-cells, thymocytes, B-cell subset	B-CLL and.most hematopoietic neoplasms of T cell lineage.	CD72 ligand Signal transduction. T-cell activation
CD7	Pluripotential hematopoietic cells, thymocytes, major T-cell subset, NK cells, some early myeloid cells	Hematopoietic neoplasms of T cell lineage.	T and NK cell activation.
CD8	Cytotoxic T cells, thymocyte subsets, NK cells	Hematopoietic neoplasms of T cell lineage.	Coreceptor for MHC class I molecules. Regulates function of CD3/TCR complex
CD10	Early B lymphocytes, PMNs, B and T cell precursors, bone marrow stromal cells	Precursor B cell ALL and non-Hodgkin lymphomas of follicular cell center origin.	CALLA. Zinc metalloproteinase. Neutral endopeptidase,
CD11b	Monocytes, granulocytes, NK cells	Myelomonocytic leukemias, particularly of FAB M4 and M5 subclasses.	Mac-1. Cell adhesion molecule. Binds CD54, complement component iC3b and extracellular matrix proteins.
CD11c	Myeloid cells, monocytes	Hairy cell leukemia and related hematopoietic neoplasms.	Cell adhesion molecule. Subunit of integrin CR3 (associated with CD18). Binds fibrinogen
CD13	Myelomonocytic cells	Leukemias of myeloid lineage.	Zinc metalloproteinase. Aminopeptidase N,
CD14	Myelomonocytic cells	Myelomonocytic leukemias, particularly of FAB M4 and M5 subclasses.	Receptor for complex of LPS and LPS binding protein (LBP)
CD15	Granulocytes, monocytes, endothelial cells	Hodgkin's lymphoma, other hematopoietic neoplasms.	Lewis-x (Lex) antigen. Branched pentasaccharide, expressed on glycolipids and many cell surface glycoproteins. Sialylated form is a ligand for CD62E (ELAM).

Table I (Cont'd)

Antigen	Normal Cellular Expression	Major Diagnostic Application	Biological Function
CD16	NK cells, granulocytes, macrophages	Hematopoietic neoplasms of NK-cell lineage.	FcgRIII. component of low affinity Fc receptor (FcgRIII). Mediates phagocytosis and ADCC.
CD19	Pan B-cell antigen	Precursor B cell ALL and non-Hodgkin lymphoma of B-cell lineage.	Forms complex with CD21 (CR2)and CD81 (TAPA-1). Coreceptor for B cells. Regulation of B-cell activation
CD20	B-cell antigen	Precursor B cell ALL and non-Hodgkin lymphoma of B-cell lineage.	Ca++ channel, B cell activation?
CD23	Activated B cells, activated macrophages, eosinophils, follicular dendritic cells, platelets	Leukemia and lymphoma diagnosis.	Low affinity receptor for IgE (Fc_RII). Ligand for CD19: CD21:CD81 coreceptor.
CD25	Activated T cells, activated B cells, monocytes	Hairy cell leukemia, ATL/L, other hematopoietic neoplasms.	Tac. Interleukin 2 receptor alpha chain.
CD30	Activated B and T cells	Hodgkin's lymphoma, anaplastic large cell lymphoma.	Ki-1. Growth factor receptor.
CD34	Hematopoietic precursors, capillary endothelium	Leukemias of early myeloid lineage, lymphoblastic lymphoma.	Ligand for CD62 (L-selectin)."Stem cell antigen"
CD41	Megakaryocytes, platelets	Acute leukemia of megakaryocytic lineage (AML, FAB-M7).	aIIb integrin. Associates with CD61 to form GPIIb. Binds fibrinogen, fibronectin, von Willebrand factor and thrombospondin
CD42b	Megakaryocytes, platelets	Acute leukemia of megakaryocytic origin (AML, FAB-M7).	gpIb, vWF receptor. Binds von Willebrand factor and thrombin. Essential for platelet adhesion.
CD43	T cells, myeloid cells, some B cell lymphomas	Some T-cell lymphoproliferative diseases.	Leukosialin, sialophorin. binds CD54 (ICAM-1)
CD45	Panhematopoietic.	All hematopoietic neoplasms.	Leukocyte common antigen. Tyrosine phosphatase, augments signalling
CD56	NK cells	Hematopoietic neoplasms of NK-cell lineage.	NKH-1. Cell adhesion molecule

Table I (Cont'd)

Antigen	Normal Cellular Expression	Major Diagnostic Application	Biological Function
CD57	NK cells NK cells subsets of T cells, B cells and monocytes	Hematopoietic neoplasms of NK-cell and T-cell lineage.	HNK-1. Oligosaccharide. Many cell surface glycoproteins.
CD61	Megakaryocyte platelets, megakaryocytes, macrophages	Acute leukemia of megakaryocytic origin (AML, FAB-M7).	Integrin b3 subunit, associates with CD41 (GPIIb/IIIa)(fibrinogen receptor) or CD51 (vitronectin receptor).
CD79a	B cells (lineage specific)	Hematopoietic neoplasms of B-cell lineage.	Components of B cell antigen receptor. Cell surface expression and signal transduction.
CD103	Intestinal epithelial lymphocytes Intraepithelial lymphocytes, 2-6% of peripheral blood lymphocytes	T-cell neoplasms	aE integrin.
CD117	Blast cells of myeloid lineage, mast cells	Acute myeloid leukemias.	c-kit. Stem Cell Factor (SCF) receptor. Stem cell survival and progenitor cell replication/differentiation
HLA-DR	B cells, monocytes, activated T cells, myeloid precursors	Hematopoietic neoplasms	HLA Class II recptor.
Glycophorin A	Erythrocytes, erythroid precursors	Erythroleukemia (AML, FAB-M6)	
TdT	Lymphoblasts, thymocytes, myeloblast subset	Acute leukemia and lymphoblastic lymphoma	
Myeloperoxidase			

Data from Protein Reviews on the Web. CD MOLECULES. Human cell surface molecules recognized by the International Workshops on Human Leukocyte Differentiation Antigens. http://www.ncbi.nlm.nih.gov/PROW/guide/45277084.htm

decades ago, the French-American-British (FAB) group recognized the presence of increased immature hematopoietic precursors in the acute leukemias. This resulted in a morphologic and cytochemical criteria for subdividing ALL into three subtypes and AML into seven subtypes. The recent World Health Organization (WHO) classification of the acute leukemias, published in the monograph *Pathology and Genetics of Tumors of the Haematopoietic and Lymphoid Tissues*, incorporates non-morphologic data, including flow cytometry, karyotypic, and molecular data.[12]

A wide variety of monoclonal antibodies against cellular antigens are available for the immunophenotypic analysis of hematological malignancies (Table I). In order to establish a B- or T-cell clonality, a panel of antibodies is used. A pan-B-cell panel would include CD19, CD20, and CD22 and a pan-T-cell panel would include CD2, CD3, CD4 and/or CD7, while additional antibody panels might be necessary to establish the presence of a specific lymphoproliferative disorder. The classic immunophenotypes of common B and T cell lymphoproliferative disorders are highlighted in Tables 2 and 3. Monoclonal antibodies against leukocyte common antigen (CD45) are often included in the panel to differentiate hematological malignancies from other neoplasms and to help detect populations of blast cells, since almost all leukemic cell populations exhibit decreased (dim) CD45 expression compared to normal leukocytes. The CD34 and HLA-DR antigens are markers for hematopoietic stem cells used for the diagnosis of acute leukemia and quality assurance in bone marrow transplantation. Most of the remaining leukocyte surface antigens are lineage associated, but not specific to a single lineage or stage of cellular maturation.

Precursor B-ALL is the most common subtype of ALL, comprising 75% to 85% of ALL cases. These cases usually originate from B-lymphocytes at relatively early stages of development. The diagnosis of B-ALL primarily relies on the reactivity of two monoclonal antibodies, CD10 and CD19 (Fig. 2).[13] Leukemias of T-lineage (T-ALL) comprise 15% to 25% of ALL cases. Clinically, most patients are older males who present with high peripheral blast counts and mediastinal masses. The flow cytometric diagnosis of T-ALL is more difficult than that of B-ALL since "monoclonality" is not as easy to demonstrate and markers that are detected only in the early phases of T-cell maturation and are absent in mature T cells (e.g. CD1b) are few and occur uncommonly in T-ALL. The most sensitive marker for T-ALL appears to be the pan-T 40 kd antigen defined by anti-Leu-9 (CD7). Leukemias of myeloid lineage typically express CD13, CD33, or CD117, while those of monocytic leukemia are positive for CD4, CD11b, CD11c, CD14, CD36, CD64, or CD68.[14] CD41 and CD61 are helpful in establishing megakaryocytic lineage for an acute leukemia, while erythroleukemias express CD235 (glycophorin-A)(Table I).

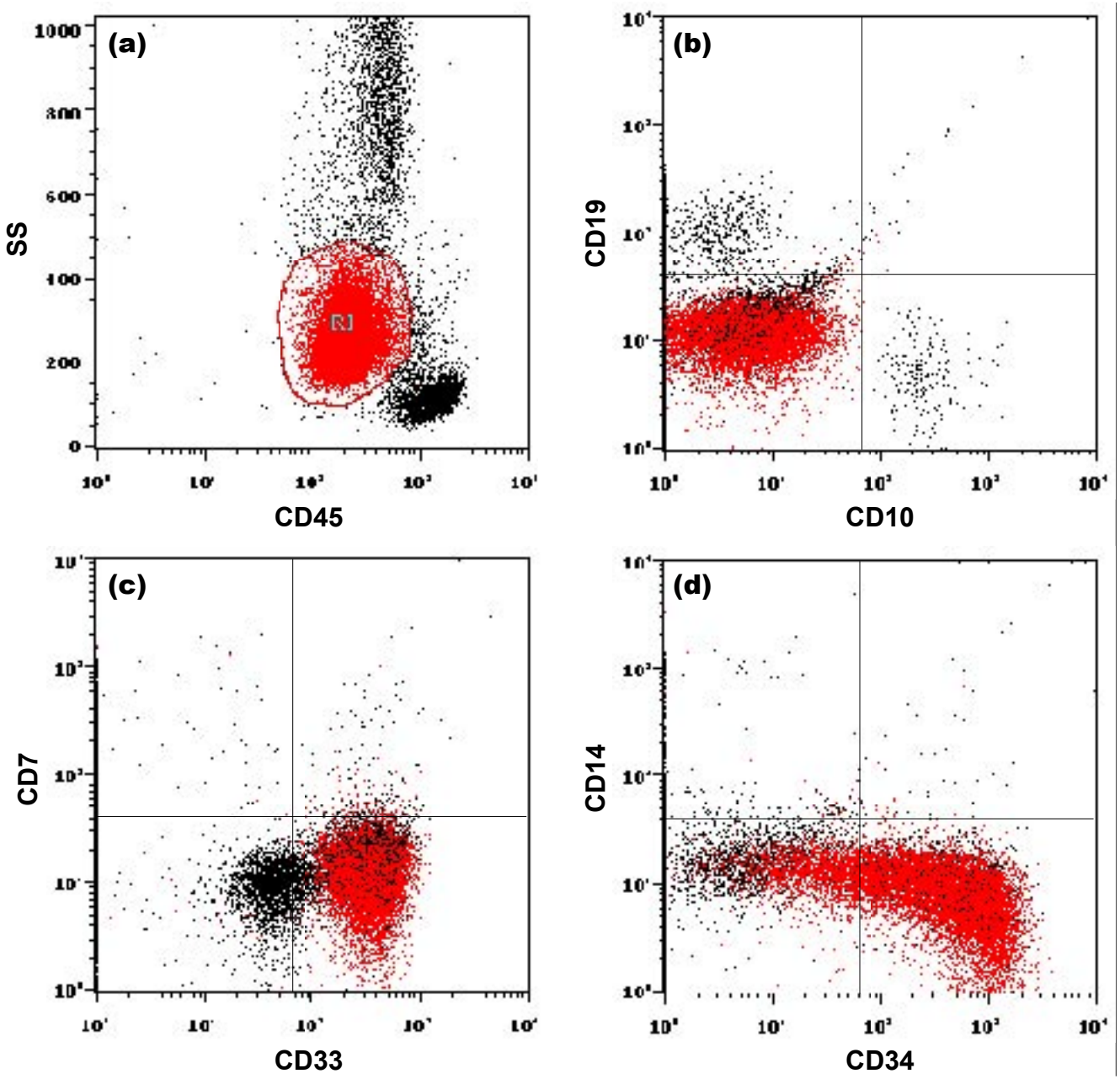

Fig. 3. Immunophenotypic analysis of an adult patient with acute myelogenous leukemia. (a) A histogram of CD45 expression vs side scatter showing a polygonal gate. Approximately 70% of the total cell population showed the dim CD45 expression characteristic of blasts and were included in the gate. (b) A scattergram of CD10 (x axis) and CD19 expression (y axis) showing negative expression of these antigens by the gated cells. (c) A scattergram of CD7 (x axis) and CD33 expression (y axis) showing bright expression of CD33 antigen by the gated cells. (c) A scattergram of CD14 (x axis) and CD34 expression (y axis). The majority of the cells (83%) showed moderately bright CD34 expression, but CD14 is negative.

Flow cytometry has become an essential tool in the diagnosis of hematologic and lymphoid neoplasia by aiding in determining whether a clonal proliferation is B- or T-cell in origin; and in most cases help with a specific diagnosis, when a classic pattern is present.[15]. The unique capability of flow cytometry to rapidly analyze, even in small samples, multiple cell properties simultaneously such as size, granularity, surface and intracellular antigens and DNA content allow for increased sensitivity in the detection of neoplastic cells and should contribute to improving accuracy and precision in the diagnosis and classification of lymphomas and lymphoprolferative disorders.[16, 17] It must be emphasized, however, that flow cytometry is an adjunct to the clinical history and the microscopic examination of cells.

Table II
Immunophenotypic Features of B-Cell Lymphoproliferative Disorders

	sIg	CD5	CD10	CD11c	CD23	CD43	CD103	Cyclin-D
CLL/SLL	Weak	+	-	-	+	+	-	-
MCL	Strong	+	-	-	-/+	+	-	+
FL	Strong	-	+	-	-	+/-*	-	-
MZL	Strong	-	-	+/-	-	+/-	-	-
HCL	Strong	-	-	+	-		+	-
PL	Strong	+/-	-	-	-		-	-
LPL	Strong	-	-	-	-	+/-	-	-

Key

sIg – surface immunoglobulin
CLL – chronic lymphocytic leukemia
MCL – mantle cell lymphoma
FL – follicular lymphoma
MZL – marginal zone lymphoma
HCL – hairy cell leukemia
PL – prolymphocytic leukemia
LPL – lymphoplasmacytic leukemia

- negative
+ positive
+/- may be positive
-/+ usually negative but may be positive

• Occasional cases of grade 3 follicular lymphoma are CD43 positive

Detection of Minimal Residual Disease

Flow cytometry is used as a simple, rapid method for detection of minimal residual disease (MRD), the persistence of malignant cells in the bone marrow or other tissues of patients with hematologic malignancies after remission at levels below the limit of detection by conventional morphologic assessment.[18, 19] It is believed that these residual malignant cells are the source of disease relapse in many patients, although additional therapy to eradicate very small numbers of residual cells does not improve survival in all patients. Researchers are actively evaluating the significance of MDR.

Table III
Immunophenotypic Characteristics of T-Cell Lymphoproliferative Disorders

	CD2	CD3	CD4	CD5	CD7	CD8	CD25	CD30
MF	+	+	+	+	-	-	na	na
SS	+	+	+	+	+/-	-	na	na
ATLL[1]	+	+	+	+	-	-	+	+/-
ALCL[2]	+	-/+	+	-	-	-	+	+
ETL[3]	+/-	+	-	-	+	-/+	na	+/-

[1] Most cases are CD4+, CD8-. Rare cases are CD4-, CD8+ or double positives for CD4 and CD8.
[2] Anaplastic large cell lymphoma kinase (ALK) protein detectable in 60-85% of cases and has prognostic significance; majority of ALCL are positive for EMA; the CD30 stain is usually cell membrane stain.
[3] Usually CD103+; Tumor cells may express CD56

Key

MF Mycosis fungoides
SS Sezary syndrome
ATLL Adult T cell Leukemia/lymphoma
ALCL Anaplastic large cell lymphoma
ETL Enteropathy-type T-cell lymphoma

Laboratory techniques for the detection of MDR must meet four criteria, which include sensitivity (detection limit of at least 10^{-3} cells), specificity (ability to differentiate normal and malignant cells), reproducibility, and applicability (easy standardization and rapid collection of results).[20] Morphologic evaluation, with an overall detection limit of approximately 5%, is clearly not suitable for the detection of MDR.[21] However, immunophenotypic analysis, cytogenetics, fluorescence in-situ hybridization (FISH), Southern blotting, polymerase chain reaction (PCR), and other techniques with detection limits of 10^{-2} to 10^{-4} cells have been applied, as well as the clonogenic assay, which has a detection limit of $\leq 10^{-4}$.[20]

Flow cytometric analysis is less sensitive than the polymerase chain technique for MRD, but it is simple and rapid to perform, provides quantitative data, and has adequate sensitivity in many leukemia cases. Flow cytometric analysis detects the presence of aberrant immunophenotypic features that are not characteristic of normal cell populations in the specimen under study. For example, the discovery of $CD10^+$, TdT^+, or $CD34^+$

cells in the cerebrospinal fluid is diagnostic of MRD, since immature leukocytes with these markers are not normally present in the CSF. The expression of TdT, cytoplasmic CD3, CD1a, or the dual phenotype $CD4^+/CD8^+$ by bone marrow cells is diagnostic of residual MRD in T-ALL, since cells with these phenotypes are normally confined to the thymus. The detection of B-ALL MRD is more difficult, since small numbers of immature B-cells are normally present in the bone marrow. But, the majority of B-ALL cases have aberrant antigenic features, including cross-lineage antigen expression (i.e., TdT, T-cell, or myeloid antigens), asynchronous antigen expression, or changes in the level of antigen expression (i.e. "dropped" or overexpressed antigens). The search for new markers and techniques of immunophenotypic analysis for MRD is also underway by several investigators.

Lymphocyte Subset Enumeration for Immunodeficiency Disease

In the early 1980's, the principal discovery of the pathogenesis of human immunodeficiency virus (HIV) infection was the recognition of an alteration in peripheral blood CD4 T-cell levels. Since that time, the enumeration of the absolute number of CD4+ T-cells by flow cytometry, and the measurement of HIV RNA levels by molecular techniques has proven critical for the diagnosis and prognostication of HIV infection and the management of patients receiving anti-viral therapy. Presently, most laboratories utilize three- or four-color immunophenotypic analysis for lymphocyte subset enumeration, with a CD45-side scatter gate to identify the lymphocyte population and to eliminate dead cells, debris and degranulated granulocytes from analysis. Three-color analysis is usually performed with two labeled specimens (i.e., CD45-CD3-CD4 and CD45-CD3-CD8) while four-color analysis is performed with a single labeled specimen (i.e., CD45-CD3-CD4-CD8). In the past, the absolute lymphocyte count was separately determined on the hematology analyzer and then used in conjunction with flow cytometric data to calculate absolute CD4+ and CD8+ counts. The accuracy of this analysis has been recently improved by introduction of methods that allow for direct measurement of absolute cell numbers with the flow cytometer only.

Studies of HIV-infected patients in the research laboratory have led to a number of discoveries awaiting widespread clinical utilization. At present, the most important is quantitative measurement of immune activation by measurement of CD38 expression on CD8+ T-cells. Some studies indicate CD38 expression superior to measurement of the viral load by HIV RNA for predicting disease progression and survival in HIV-infected patients. Other flow cytometric assays under study for the management of HIV-infected patients include measurement of the frequency of antigen-specific immune responses, functional assays for antigen-specific cytokine responses, measurement of cell turnover, programmed cell death, and HIV viral burden.[22]

Flow cytometric analysis was instrumental in the discovery of some primary (congenital) immunodeficiency diseases, a heterogeneous group of diseases of the host defense systems which commonly present in childhood as chronic or recurrent infection, failure to thrive, unusual infections, allergic disorders[23, 24] and leukocyte adhesion disorders, and is commonly used for the diagnosis and management of these diseases.[25]

Analysis of DNA Ploidy, the Cell Cycle, and Cell Death

The significant medical discovery of understanding the human cell cycle in combination with flow cytometric technology allowed for the development of DNA analysis of neoplasia by flow cytometry. A defined number of cells are stained with a known saturating amount of DNA-specific fluorescent dye under controlled conditions of temperature, pH, and ionic strength. The cells are then analyzed using a flow cytometer where, upon excitation by a light beam of the appropriate wavelength, the amount and intensity of fluorescent emission of the dye bound to DNA of each cell, is measured based on a statistically significant number of cells (i.e. ≥10,000) in a period of a few minutes. The relative total DNA content in an unknown cell population is determined when compared to cells analyzed with known and constant DNA content. In addition, small populations of cells can be detected in a heterogenous mixture, and cell populations with small variations in DNA content (~ 4% with a CV of 2%) can be detected.[26-29]

The advent of univariate flow cytometric DNA analysis in the late 1970's was soon followed by reports of the independent prognostic significance of tumor cell DNA content and/or proliferative activity in a number of human malignancies. Numerous additional publications soon appeared, many of which did not substantiate the independent prognostic significance of DNA analysis. In addition, interlaboratory variation in specimen preparation, analysis, data interpretation, and quality control lead to questions about the validity of some results. In 1996 a committee of the American Society for Clinical Oncology concluded that the existing data did not warrant the routine applica-

tion of measurements of DNA ploidy or proliferation analysis.[30] Since 1996, the utilization of DNA analysis has significantly decreased, and it is most often performed in patients with node-negative breast carcinoma and other tumors where the clinical correlation is strongest. However, recent technological innovations may lead to a revival of interest in clinical DNA analysis. In this regard, Bagwell modified DNA analysis to optimize the accuracy of DNA ploidy and S phase in node-negative breast cancer, eliminate spurious technical inconsistencies, and apply standardized modeling rules to data analysis and interpretation. Furthermore, the authors developed a prognostic model that combines DNA ploidy and the S phase into a Relative Risk Index (RRI).[31, 32] Multiparametric DNA analysis using cytokeratin expression to exclude normal background cells has also been shown to improve the predictive value of the measurements.

Measurement of the Efficacy of Cancer Chemotherapy

Selection of the optimal chemotherapeutic agent is one of the major problems in oncology. Even with the advent of large multicenter therapeutic trials for the determination of chemotherapeutic efficacy, individual variability in tumor characteristics often leads to a poor therapeutic outcome. A major cause of failure to many of the natural products used as chemotherapeutic agents is multiple drug resistance (MDR). The over expression of P-glycoprotein and other proteins involved in cellular transport is a frequent cause of MDR, although detoxification by biochemical means, DNA replication and repair, or other mechanisms may be involved.[33] In conjunction with immunocytochemistry and molecular techniques, flow cytometry has been essential for measuring the expression of cell surface and intracellular markers of MDR, assessing the intracellular accumulation and efflux of chemotherapeutic drugs, and studying the other mechanisms leading to MDR.[33] The identification of intrinsic or acquired MDR is potentially of significant clinical value in planning chemotherapy, and several clinical trials of drug efflux blockers are underway.

The reliable *in vitro* prediction of tumor cell sensitivity to radiation and antineoplastic agents prior to therapy in individual cancer patients is a long-sought goal of oncologists. The clonogenic assay system developed by Hamburger and Salmon in the 1970's is frequently used for the evaluation of cytostatic drugs but this assay is complex, time consuming, manually laborious, and requires cells that form discernable colonies.[34, 35] A variety of flow cytometric techniques have also been explored as alternatives to the clonogenic assay, including microdrop encapsulation and assays of proliferative survival using bromodeoxyuridine (BrdU) incorporation.[36, 37] Flow cytometric measurements of cell viability or apoptosis have been used to design drug treatment protocols and improve the accuracy and reliability of the conventional clonogenic assay.[38]

Ligand, antigen, or molecule-targeted biological therapy utilizing monoclonal antibodies is the most rapidly growing area of pharmacology for a wide variety of human diseases, including cancer and autoimmune disease.[39, 40] These agents work through a number of mechanisms. Some directly disrupt cell proliferation and anti-apoptosis by blocking the cell membrane receptors and circulating ligands associated with signal transduction, while others serve as the targeting system for other cytotoxic products.[41] The first of this new class of pharmaceutical agents was anti-CD3 (OKT3), developed for the immunosuppressive therapy of solid organ transplant rejection. More recently developed monoclonal antibodies are directed against CD20, CD33, CD25, CD45, and CD52.[42] Prior to treatment, flow cytometric analysis is critical for confirming that the antigen is expressed by the aberrant cells. During and after treatment, flow cytometry is utilized to verify binding of the antibody and to monitor the efficacy of tumor cell eradication.

Reticulocyte Enumeration

The enumeration of peripheral blood reticulocytes is often performed to obtain information about the functional integrity of the bone marrow. Reticulocytosis occurs in anemic patients with a functional bone marrow and is marked by an increased number of peripheral blood reticulocytes, while reticulocytopenia occurs in anemic patients with a dysfunctional bone marrow and is marked by decreased numbers of peripheral blood reticulocytes. In addition to the evaluation of anemic patients, reticulocyte enumeration is also of value in monitoring bone marrow regenerative activity after chemotherapy or bone marrow transplantation. In the laboratory, the differentiation of the reticulocyte from the mature red blood cell is based on the presence of RNA and other substances in the reticulocyte, which are lost during differentiation into the mature red blood cell. Manual counting of reticulocytes by light microscopy with supravital dyes for RNA was developed in the 1940's and remains the standard method of reticulocyte enumeration. However, reticulocyte enumeration by flow cytometry is much more accurate, precise, and cost-effective than manual counting and is increasingly being performed in the clinical laboratory. In addition, the flow cytometer provides a variety of additional reticulocyte-related parameters, such as the reticulocyte maturation index

(RMI) and immature reticulocyte fraction (IRF), which are not available with light microscopy and appear valuable in the clinical diagnosis and monitoring of anemia and other diseases.[43]

Dedicated, fully automated flow cytometers specifically designed for reticulocyte enumeration are produced two companies, TOA (Kobe, Japan) and ABX (Montpellier, France).[44] The development of technology to perform reticulocyte enumeration by optical light scatter was a major breakthrough for the clinical laboratory, since it could be incorporated into existing hematology analyzers with little additional cost. Beckman Coulter Inc. (Fullerton, CA) first provided reticulocyte enumeration technology on their STKS™, MAXM™, and MAXM A/L hematology analyzers.[45, 46] The Coulter technique utilizes a new methylene blue stain and differentiates reticulocytes from mature red blood cells, white blood cells, and platelets through the measurement of impedance, radio frequency, and laser light scatter (VCS technology). The CELL-DYN 3500 (Abbott Diagnostika GmbH, Wiesbaden-Delkenheim, Germany) Hematology Analyzer also utilizes optical light scatter and a supravital dye for reticulocyte enumeration, while the Bayer/Miles Technicon H*3 blood analyzer (Bayer/Miles, Diagnostics Division, Tarrytown, N.Y.) utilizes the nucleic acid-binding dye oxazine 750 for reticulocyte enumeration. One hematology analyzer, the Cell-Dyn 4000 (Abbott Diagnostika GmbH, Wiesbaden-Delkenheim, Germany), provides a fluorescent measurement of the reticulocyte count and reticulocyte quantitative maturational data in whole blood using a proprietary fluorescent dye (CD4K530).[47-49]

Platelet Function Analysis

The flow cytometer has been essential for the analysis of platelet structure and function in the research laboratory. Although the small physical size and biovariability of the platelet creates inherent difficulties for flow cytometric analysis, several clinical assays are performed by specialized flow cytometry laboratories. These assays will achieve more widespread practice in the near future as standardized techniques and controls become available. These assays have been classified by Bode and Hickerson to include platelet surface receptor quantitation and distribution for the diagnosis of congenital platelet function disorders, platelet-associated IgG quantitation for the diagnosis of immune thrombocytopenias and for platelet cross-matching in transfusion, reticulated platelet assay to detect "stress" platelets, fibrinogen receptor occupancy studies for monitoring the clinical efficacy of platelet-directed anticoagulation in thrombosis, and the detection of activated platelet surface markers, cytoplasmic calcium ion measurements, and platelet microparticles for the assessment of hypercoagulable states.[50]

Cell Function Analysis

The analysis of cell function can provide relevant clinical information that cannot be acquired from static cellular parameters, such as the expression of surface antigens. Since these considerations are particularly relevant in transplantation medicine and diseases of the immune system, many investigations have focused on functional analysis of the lymphocyte. Virtually every event that occurs during the process of lymphocyte activation can be measured by flow cytometry, but determinations of tyrosine phosphorylation, calcium flux, oxidative metabolism, neoantigen expression, and cellular proliferation have the greatest clinical potential at this time.[51, 52] Tyrosine phosphorylation can be measured within the cell by multiparametric flow cytometry and labeled antiphosphotyrosine monoclonal antibodies, or within activated cell lysates with colored microbeads labeled with monoclonal antibodies specific for different tyrosine kinase substrates (multiplex bead technology).[52] Intracellular calcium flux is measured with ratiometric Ca^{2+} indicators whose spectral characteristics change with Ca^{2+} binding. Calcium flux has been used to study platelet activation in response to different agonists and lymphocyte activation in viral infection and other diseases. Flow cytometric measurement of the oxidative burst in neutrophils has been used as a screening test for chronic granulomatous disease. The most common technique for this purpose uses a nonfluorescing dye (dihydrorhodamine-123) that is selectively concentrated in the mitochondria and is oxidized to a brightly fluorescent compound (rhodamine-123) during the normal oxidative burst.[53, 54] Lymphocyte neoantigens are surface or intracellular proteins, including cytokines, that are up-regulated during lymphocyte activation. The detection of these substances may become one of the most important flow cytometric assays. CD11b/CD18 and CD154 are present examples of diagnostically significant neoantigens.[54]

Applications in Transfusion Medicine

Immune sensitization is a dreaded consequence of fetal maternal hemorrhage in a Rhesus (Rh) negative woman pregnant with Rh positive fetus. Appropriate intrapartum and postpartum administration of Rh immune globulin to prevent such immune sensitization, relies on sensitive detection and accurate quantitation of fetomaternal hemorrhage.

Limitations in the sensitivity, precision and the difficulty in standardization of the manual Kleihauer-Betke test have prompted an increase utilization of flow cytometry for fetal cell detection in maternal blood samples.[55-57]. Flow cytometry offers a simpler, more reliable and precise alternative to the Kleihauer-Betke technique, especially in massive feto-maternal hemorrhage. [56, 58-60] Flow cytometry aids in accurate quantitation and worthwhile reductions in the clinical use of anti-D immunoglobulin.[61] It should be noted that a well-performed Kleihauer-Betke test still appears useful as a screening technique for detection of feto-maternal hemorrhage.[62] However, accurate quantitation of size of feto-maternal hemorrhage is more reliably determined by flow cytometry.[62] Flow cytometry has additional potential application for the study of HbF levels or frequency of adult red cells with low levels of HbF in individuals with hemoglobinopathies, and the medical evaluation of anemic patients, including sickle cell and thalassemic patients.[56, 60, 63]

Organ Transplantation and Hematopoietic Cell Therapy

The detection of cellular antigens and biologic substances critical for investigations of the immunobiology of graft acceptance and rejection and the mechanism of action of immunosuppressive drugs. Without the information acquired through immunophenotypic analysis during the past decade, many of the basic mysteries of transplantation would not have been answered, and the present clinical success of organ transplantation would not have been achieved. Clinical applications of flow cytometry in solid organ transplantation include pre-transplant cross- matching, HLA antibody screening, and post-transplantation antibody monitoring.[64-67] In bone marrow transplantation, the enumeration of $CD34^+$ hematopoietic stem cells in the peripheral blood or bone marrow graft correlates with engraftment success and the length of hematopoietic recovery following stem cell transplantation.[68] Other applications of flow cytometry in bone marrow transplantation include pre-transplantation determinations of the efficacy of *ex vivo* T-cell graft depletion, and post-transplantation evaluation of immune recovery, graft rejection, graft-versus host disease, and the graft-versus-leukemia effect. [69, 70]

Applications in Microbiology

Flow cytometry has been shown to have practical application in microbiology. Classical microbiology techniques are relatively slow in comparison to other analytical techniques, in many cases, due to the need to culture the microorganisms. In addition, it becomes especially difficult when dealing with unculturable microorganisms. The ancestor of the modern flow cytometer was an aerosol particle counter designed to analyze mine dust, and which was, in fact, used during World War II by the US Army for the detection of bacteria and spores, in attempts to detect biowarfare agents.[71] Modern flow cytometry allows single- or multiple- microbe detection (bacteria, fungi, parasites and viruses) in an easy, reliable, and fast way on the basis of their peculiar cytometric parameters or by means of certain flourochromes that can be used either independently or bound to specific antibodies or oligonucleotides.[72-75] Further improvements in flow cytometric discrimination of microorganisms typically rely on fluorescent staining: nucleic acid stains; fluorogenic enzyme substrates and membrane potential-sensitive dyes have all been used for this purpose.[76, 77] In addition, flow cytometry has permitted the development of quantitative procedures to assess antimicrobial susceptibility and drug cytotoxicity in a rapid, accurate, and highly reproducible way.[72] The most outstanding contribution of flow cytometry is the possibility of detecting the presence of heterogeneous populations with different responses to antimicrobial treatments.[72] In the last few years of the 1990's , the applications of flow cytometry in microbiology significantly increased.[78] A cytometric apparatus for laboratory bacteriology should, ideally, be inexpensive and simple to operate and use inexpensive reagents.[76, 77] Despite the advantages, the application of flow cytometry in clinical microbiology is not yet widespread, probably due to lack of access to flow cytometers or the lack of knowledge about the potential of this technique.

Future Expectations

The flow cytometer is a versatile tool with enormous potential for the study of cells and particles. Because of its unique analytic capabilities, the flow cytometer has become an integral part of the medical research laboratory during the past two decades. The ingress of the flow cytometer into the clinical laboratory has been slower and more controversial. Immunophenotypic analysis and lymphocyte subset analysis is widely performed in the clinical laboratory, but most of the other applications are limited to larger and/or specialized laboratories due to economic considerations. However, no other laboratory instrument provides multiparametric analysis at the single cell level, and the flow cytometer or application-variants of the flow cytometer will become more valuable as medical diagnosis and therapy changes. New fluorochromes, including UV-excited, complex of dyes ("tandem dyes"), and nanocrystals are under development, as well as a new

generation of modular flow cytometers using small, solid state lasers, robotics, and advanced, innovative bioinformatics software.[79] An example of the new, specialized flow cytometers of the future is the High Throughput Pharmacological System (HTPS).[80] This system, designed for automated high throughput analysis of novel bioresponse-modifying drugs, permits analysis of 9-10 cell samples/minute from 96-well microplates. Another interesting offshoot of the flow cytometer is the laser scanning cytometer (LSC), a microscope slide-based technology capable of acquiring multiparametric data from selected cells from a heterogeneous population which is proving particularly useful for the analysis of fine needle aspirate and body fluid specimens.[81-83]

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