

# Flow Cytometry: Principles and Clinical Applications in Hematology

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The use of flow cytometry in the clinical laboratory has grown substantially in the past decade. This is attributable in part to the development of smaller, user-friendly, less-expensive instruments and a continuous increase in the number of clinical applications. Flow cytometry measures multiple characteristics of individual particles flowing in single file in a stream of fluid. Light scattering at different angles can distinguish differences in size and internal complexity, whereas light emitted from fluorescently labeled antibodies can identify a wide array of cell surface and cytoplasmic antigens. This approach makes flow cytometry a powerful tool for detailed analysis of complex populations in a short period of time. This report reviews the general principles in flow cytometry and selected applications of flow cytometry in the clinical hematology laboratory. © 2000 American Association for Clinical Chemistry

Flow cytometry provides rapid analysis of multiple characteristics of single cells. The information obtained is both qualitative and quantitative. Whereas in the past flow cytometers were found only in larger academic centers, advances in technology now make it possible for community hospitals to use this methodology. Contemporary flow cytometers are much smaller, less expensive, more user-friendly, and well suited for high-volume operation. Flow cytometry is used for immunophenotyping of a variety of specimens, including whole blood, bone marrow, serous cavity fluids, cerebrospinal fluid, urine, and solid tissues. Characteristics that can be measured include cell size, cytoplasmic complexity, DNA or RNA content, and a wide range of membrane-bound and intracellular proteins. This review will describe the basic principles of flow cytometry and provide an overview of some applications to hematology.

## General Principles

Flow cytometry measures optical and fluorescence characteristics of single cells (or any other particle, including nuclei, microorganisms, chromosome preparations, and latex beads). Physical properties, such as size (represented by forward angle light scatter) and internal complexity (represented by right-angle scatter) can resolve certain cell populations. Fluorescent dyes may bind or intercalate with different cellular components such as DNA or RNA. Additionally, antibodies conjugated to fluorescent dyes can bind specific proteins on cell membranes or inside cells. When labeled cells are passed by a light source, the fluorescent molecules are excited to a higher energy state. Upon returning to their resting states, the fluorochromes emit light energy at higher wavelengths. The use of multiple fluorochromes, each with similar excitation wavelengths and different emission wavelengths (or "colors"), allows several cell properties to be measured simultaneously. Commonly used dyes include propidium iodide, phycoerythrin, and fluorescein, although many other dyes are available. Tandem dyes with internal fluorescence resonance energy transfer can create even longer wavelengths and more colors. Table 1 lists clinical applications and cellular characteristics that are commonly measured. Several excellent texts and reviews are available (1-6).

Inside a flow cytometer, cells in suspension are drawn into a stream created by a surrounding sheath of isotonic fluid that creates laminar flow, allowing the cells to pass individually through an interrogation point. At the interrogation point, a beam of monochromatic light, usually from a laser, intersects the cells. Emitted light is given off in all directions and is collected via optics that direct the light to a series of filters and dichroic mirrors that isolate particular wavelength bands. The light signals are detected by photomultiplier tubes and digitized for computer analysis. Fig. 1 is a schematic diagram of the fluidic and optical components of a flow cytometer. The resulting information usually is displayed in histogram or two-dimensional dot-plot formats.

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Received February 15, 2000; accepted April 10, 2000.

**Table 1. Common clinical uses of flow cytometry.**

Field	Clinical application	Common characteristic measured
Immunology	Histocompatibility cross-matching	IgG, IgM
	Transplantation rejection	CD3, circulating OKT3
	HLA-B27 detection	HLA-B27
	Immunodeficiency studies	CD4, CD8
Oncology	DNA content and S phase of tumors	DNA
	Measurement of proliferation markers	Ki-67, PCNA <sup>a</sup>
Hematology	Leukemia and lymphoma phenotyping	Leukocyte surface antigens
	Identification of prognostically important subgroups	TdT, MPO
	Hematopoietic progenitor cell enumeration	CD34
	Diagnosis of systemic mastocytosis	CD25, CD69
	Reticulocyte enumeration	RNA
	Autoimmune and alloimmune disorders	
	Anti-platelet antibodies	IgG, IgM
	Anti-neutrophil antibodies	IgG
	Immune complexes	Complement, IgG
	Feto-maternal hemorrhage quantification	Hemoglobin F, rhesus D
Blood banking	Immunohematology	Erythrocyte surface antigens
	Assessment of leukocyte contamination of blood products	Forward and side scatter, leukocyte surface antigens
Genetic disorders	PNH	CD55, CD59
	Leukocyte adhesion deficiency	CD11/CD18 complex

<sup>a</sup> PCNA, proliferating cell nuclear antigen; TdT, terminal deoxynucleotidyltransferase; MPO, myeloperoxidase.

### DNA Content Analysis

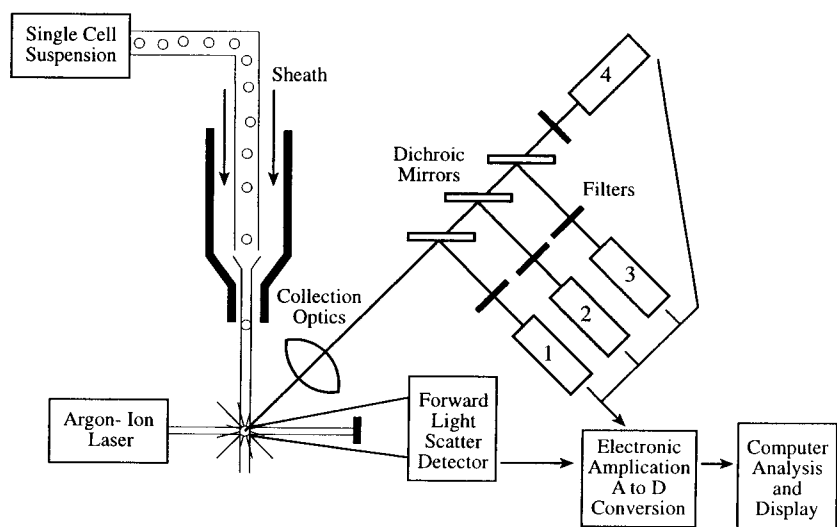
The measurement of cellular DNA content by flow cytometry uses fluorescent dyes, such as propidium iodide, that intercalate into the DNA helical structure. The fluorescent signal is directly proportional to the amount of DNA in the nucleus and can identify gross gains or losses in DNA. Abnormal DNA content, also known as "DNA content aneuploidy", can be determined in a tumor cell population. DNA aneuploidy generally is associated with malignancy; however, certain benign conditions may appear aneuploid (7-12). DNA aneuploidy correlates with a worse prognosis in many types of cancer but is associated with improved

survival in rhabdomyosarcoma, neuroblastoma, multiple myeloma, and childhood acute lymphoblastic leukemia (ALL)<sup>1</sup> (11, 13-16). In multiple myeloma, ALL, and myelodysplastic syndromes, hypodiploid tumors cells portend a poor prognosis. In contrast, hyperdiploid cells in ALL have a better prognosis (11, 13). For many hematologic malignancies, there are conflicting reports regarding the independent prognostic value of DNA content analysis. Although con-

<sup>1</sup> Nonstandard abbreviations: ALL, acute lymphoblastic leukemia; PNH, paroxysmal nocturnal hemoglobinuria; and RBC, red blood cell.

Fig. 1. Schematic of a flow cytometer.

A single cell suspension is hydrodynamically focused with sheath fluid to intersect an argon-ion laser. Signals are collected by a forward angle light scatter detector, a side-scatter detector (1), and multiple fluorescence emission detectors (2-4). The signals are amplified and converted to digital form for analysis and display on a computer screen.



ventional cytogenetics can detect smaller DNA content differences, flow cytometry allows more rapid analysis of a larger number of cells.

### Immunophenotyping Applications in Hematology

The distributed nature of the hematopoietic system makes it amenable to flow cytometric analysis. Many surface proteins and glycoproteins on erythrocytes, leukocytes, and platelets have been studied in great detail. The availability of monoclonal antibodies directed against these surface proteins permits flow cytometric analysis of erythrocytes, leukocytes, and platelets. Antibodies against intracellular proteins such as myeloperoxidase and terminal deoxynucleotidyl transferase are also commercially available and permit analysis of an increasing number of intracellular markers.

#### ERYTHROCYTE ANALYSIS

The use of flow cytometry for the detection and quantification of fetal red cells in maternal blood has increased in recent years. Currently in the United States, rhesus D-negative women receive prophylactic Rh-immune globulin at 28 weeks and also within 72 h of delivery (17). The standard single dose is enough to prevent alloimmunization from ~15 mL of fetal rhesus D+ red cells. If fetomaternal hemorrhage is suspected, the mother's blood is tested for the presence and quantity of fetal red cells, and an appropriate amount of Rh-immune globulin is administered. The quantitative test most frequently used in clinical laboratories is the Kleihauer-Betke acid-elution test. This test is fraught with interobserver and interlaboratory variability, and is tedious and time-consuming (18). The use of flow cytometry for the detection of fetal cells is much more objective, reproducible, and sensitive than the Kleihauer-Betke test (19–21). Fluorescently labeled antibodies to the rhesus (D) antigen can be used, or more recently, antibodies directed against hemoglobin F (19–27). This intracellular approach, which uses permeabilization of the red cell membrane and an antibody to the  $\gamma$  chain of human hemoglobin, is precise and sensitive (21). This method has the ability to distinguish fetal cells from F-cells (adult red cells with small amounts of hemoglobin F). Fig. 2 is a histogram of a positive test for fetomaternal hemorrhage. Although the flow cytometry method is technically superior to the Kleihauer-Betke test, cost, instrument availability, and stat access may limit its practical utility.

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal stem cell disorder that leads to intravascular hemolysis with associated thrombotic and infectious complications. PNH can arise in the setting of aplastic anemia and may be followed by acute leukemia (28). The disease is caused by deficient biosynthesis of a glycosylphosphatidylinositol linker that anchors several complement and immunoregulatory surface proteins on erythrocytes, monocytes, neutrophils, lymphocytes, and platelets (28–31). On erythrocytes, deficiencies of decay-

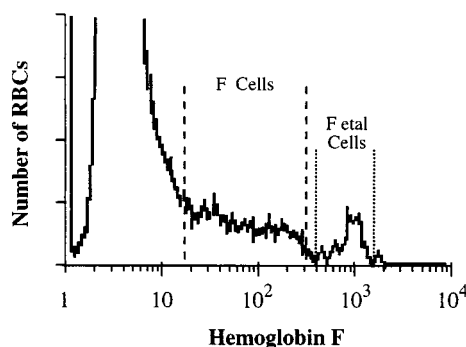


Fig. 2. Hemoglobin F test for fetomaternal hemorrhage.

Most adult RBCs do not have any hemoglobin F and are included in the large peak on the left. A few adult red cells have a small amount of hemoglobin F and are called *F cells*. Higher quantities of hemoglobin F in *fetal cells* yield a higher fluorescence signal and allow discrimination between fetal cells and adult *F cells*.

accelerating factor and membrane-inhibitor of reactive lysis render red cells susceptible to complement-mediated lysis (30, 31). Conventional laboratory tests for the diagnosis of PNH include the sugar water test and the Ham's acid hemolysis test (32). Problems associated with these tests include stringent specimen requirements and limited specificity. Antibodies to CD55 and CD59 are specific for decay-accelerating factor and membrane-inhibitor of reactive lysis, respectively, and can be analyzed by flow cytometry to make a definitive diagnosis of PNH (29, 33–35). In affected patients, two or more populations of erythrocytes can be readily identified, with different degrees of expression of CD55 and CD59 (Fig. 3)

Reticulocyte counts are based on identification of residual ribosomes and RNA in immature nonnucleated red blood cells (RBCs). Traditionally, a blood smear is stained with a dye that precipitates the nucleic acid, and the cells are counted manually (36). This method is subjective, imprecise, labor-intensive, and tedious. The flow cytometric enumeration of reticulocytes uses fluorescent dyes that bind the residual RNA, such as thiazole orange (37, 38). This method provides excellent discrimination between reticulocytes and mature RBCs, with greater precision, sensitivity, and reproducibility than the traditional method (37, 38). However, Howell-Jolly bodies (a remnant of nuclear DNA) are not distinguished from reticulocytes (39). Because the fluorescence intensity is directly proportional to the amount of RNA and related to the immaturity of the RBC, a reticulocyte maturity index has been used clinically to assess bone marrow engraftment and erythropoietic activity and to help classify anemias (34, 38, 40, 41). Some current automated cell counters use similar technology to estimate reticulocyte counts (42).

In the blood bank, flow cytometry can be used as a complementary or replacement test for red cell immunology, including RBC-bound immunoglobulins and red cell antigens (43). In multiply transfused patients, determining the recipient's blood type can be very difficult. Flow cytometry has been used to accurately identify and phe-

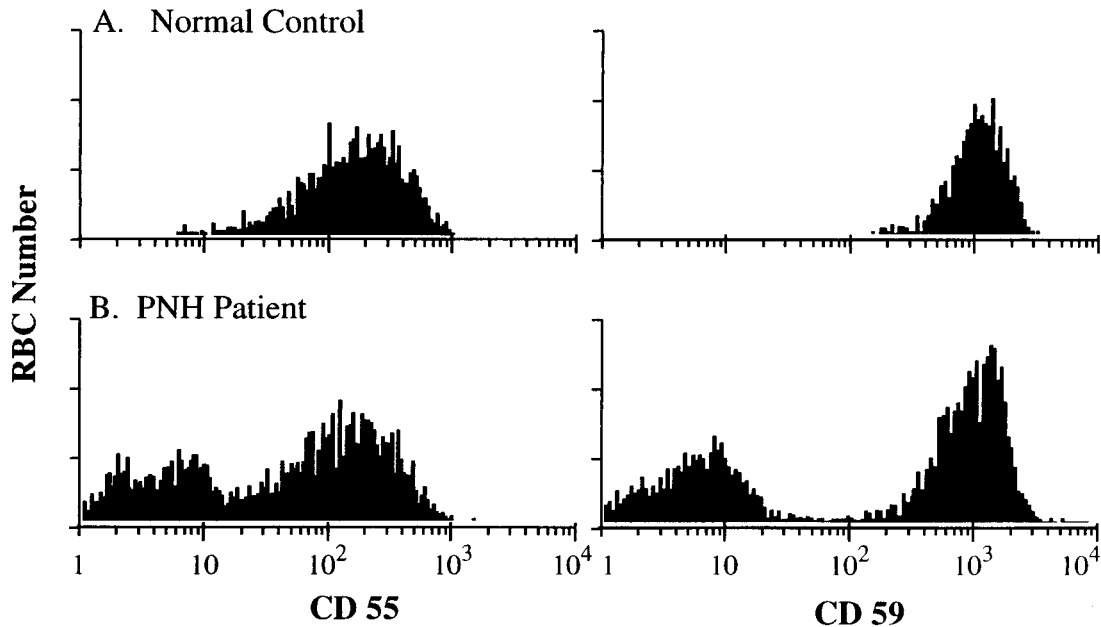


Fig. 3. Diagnosis of PNH.

Control individuals (A) show high expression of CD55 and CD59 on all red cells. In PNH (B), some stem cell clones produce RBCs with decreased expression of CD55 and CD59. In the PNH patient (B), two distinct populations are present: normal red cells with high CD55 and CD59 expression and a second population with low CD55 and CD59 expression.

notype the recipient's red cells (44). Flow cytometry is being used increasingly in the blood bank to assess leukocyte contamination in leukocyte-reduced blood products (45, 46).

#### LEUKOCYTE ANALYSIS

Immunologic monitoring of HIV-infected patients is a mainstay of the clinical flow cytometry laboratory. HIV infects helper/inducer T lymphocytes via the CD4 antigen. Infected lymphocytes may be lysed when new virions are released or may be removed by the cellular immune system. As HIV disease progresses, CD4-positive T lymphocytes decrease in total number. The absolute CD4 count provides a powerful laboratory measurement for predicting, staging, and monitoring disease progression and response to treatment in HIV-infected individuals. Quantitative viral load testing is a complementary test for clinical monitoring of disease and is correlated inversely to CD4 counts (47, 48). However, CD4 counts directly assess the patient's immune status and not just the amount of virus. It is likely that both CD4 T-cell enumeration and HIV viral load will continue to be used for diagnosis, prognosis, and therapeutic management of HIV-infected persons.

Perhaps the best example of simultaneous analysis of multiple characteristics by flow cytometry involves the immunophenotyping of leukemias and lymphomas. Immunophenotyping as part of the diagnostic work-up of hematologic malignancies offers a rapid and effective means of providing a diagnosis. The ability to analyze multiple cellular characteristics, along with new antibod-

ies and gating strategies, has substantially enhanced the utility of flow cytometry in the diagnosis of leukemias and lymphomas. Different leukemias and lymphomas often have subtle differences in their antigen profiles that make them ideal for analysis by flow cytometry. Diagnostic interpretations depend on a combination of antigen patterns and fluorescence intensity. Several recent review articles are available (49–60). Flow cytometry is very effective in distinguishing myeloid and lymphoid lineages in acute leukemias and minimally differentiated leukemias. Additionally, CD45/side scatter gating often can better isolate the blast population for more definitive phenotyping than is possible with forward scatter/side scatter gating. Fig. 4 is an example of CD45/side scatter gating for an acute myeloid leukemia. Although most acute myeloid leukemias are difficult to classify by phenotype alone, flow cytometry can be useful in distinguishing certain acute myeloid leukemias, such as acute promyelocytic leukemia (61, 62). Flow cytometry can also be used to identify leukemias that may be resistant to therapy (63). In ALL, phenotype has been shown to correlate strongly with outcome (64, 65).

The B-cell lymphoproliferative disorders often have specific antigen patterns. The use of a wide range of antibodies allows clinicians to make specific diagnoses based on patterns of antigen expression. Table 2 lists some of the common phenotypes expressed by various B-cell lymphoproliferative disorders. Not only is the presence or absence of antigens useful in making specific diagnoses, the strength of antigen expression can also aid in diagnosis. One example is the weak expression of CD20 and

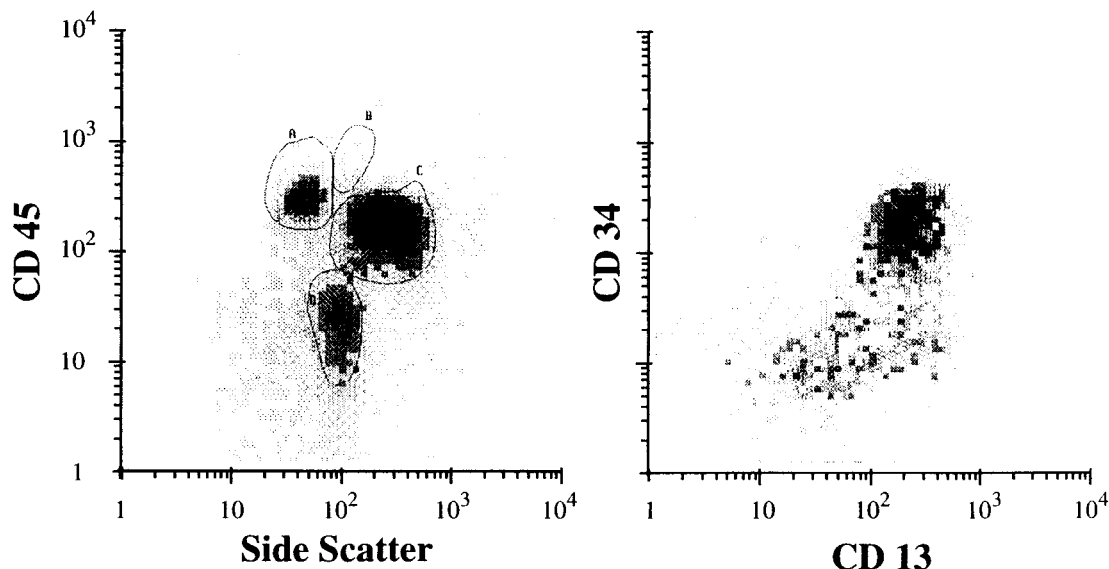


Fig. 4. Immunophenotyping of acute myeloid leukemia.

(Left), CD45/side scatter gating allows discrimination between multiple populations of leukocytes in peripheral blood. Region A, mature lymphocytes; region B, mature monocytes; region C, mature granulocytes; region D, immature myeloid cells. (Right), the population in region D from the left panel expresses CD13, a myeloid marker, and CD34, a stem cell marker. The co-expression of these two markers in the peripheral blood is highly suggestive of an acute myeloid leukemia.

immunoglobulin light chains commonly seen in chronic lymphocytic leukemia. Flow cytometry is particularly good at identifying clonality in B-cell populations. Although T-cell neoplasms may exhibit a predominance of antigens CD4 or CD8, these antigens should not be considered as surrogate markers of clonality. The use of antibodies to the T-cell receptor family may occasionally be helpful in a small percentage of cases; however, many reactive processes can show expansion of particular T-cell receptor clones (66–69). Antigen deletions are common in T-cell lymphomas and may suggest neoplasia, but the only way to definitively diagnose T-cell clonality is by molecular methods. Flow cytometry can be used for lymphoma phenotyping of fine needle aspirates, and is a powerful adjunct to cytologic diagnosis (70). The high sensitivity and capacity for simultaneous analysis of multiple characteristics make flow cytometry useful for the detection of minimal residual disease, especially if abnormal patterns of antigen expression are present (71–75). Flow cytometry is not recommended for the diagnosis of Hodgkin lymphoma,

chronic myelogenous leukemia, or myelodysplastic syndrome, although disease progression in the latter two conditions can often be monitored.

Neutropenia may be immune or nonimmune in nature. The work-up frequently entails a bone marrow examination. Immune neutropenia may result from granulocyte-specific autoantibodies, granulocyte-specific alloantibodies, or transfusion-related anti-HLA antibodies. Flow cytometry can readily identify anti-neutrophil antibodies that are either bound to granulocytes or free in plasma (76). Autoimmune neutropenias may develop in patients with autoimmune disorders such as Felty syndrome, systemic lupus erythematosus, and Hashimoto thyroiditis. When immune-related, flow cytometry can detect anti-neutrophil antibodies and confirm the origin of neutropenia, possibly eliminating the need for a bone marrow procedure. Conversely, the absence of anti-neutrophil antibodies narrows the differential diagnosis to nonimmune causes such as bone marrow failure, myelodysplasia, or marrow-infiltrative processes.

Table 2. Common phenotypes of B-cell lymphoproliferative disorders.

Diagnosis	CD5	CD10	CD19	CD20	CD23	CD79b	FMC-7	CD25	CD11c	CD103
SLL/CLL <sup>a</sup>	+	-	+	+(w)	+	-	-	-/+	+/-	-
Mantle cell lymphoma	+	-	+	+	-	+	+	-	-	-
Follicle center lymphoma	-	+	+	+	-/+	+/-	+/-	-	-	-
Marginal zone lymphoma	-	-	+	+	-	+/-	+/-	-/+	+	-
Hairy cell leukemia	-	-	+	+	-	+/-	+/-	+/-	+	+

<sup>a</sup> SLL/CLL, small lymphocytic lymphoma/chronic lymphocytic leukemia; +, positive; -, negative; +/-, often positive; -/+, occasionally positive; w, weak expression.

Functional deficiencies of leukocytes can be assessed by flow cytometry. Assays for oxidative burst, phagocytosis, opsonization, adhesion, and structure are available (77). One clinical example measures neutrophil adhesion molecules central to a diagnosis of leukocyte adhesion deficiency syndrome type I (78). This syndrome is characterized by an immunodeficiency related to defective neutrophil and monocyte migration to sites of inflammation. The disorder is caused by a congenital deficiency of the leukocyte  $\beta 2$  integrin receptor complex (CD11/CD18 antigen complex) on the myeloid cell surface. This receptor complex binds endothelial cell ligands such as intercellular adhesion molecule-1 (CD54 antigen), which is necessary for neutrophil adherence and transendothelial migration (78, 79). Flow cytometry can be used to identify neutrophils that lack the CD11/CD18 antigen complex to establish a diagnosis that is otherwise difficult to make.

#### PLATELET ANALYSIS

The analysis of platelets by flow cytometry is becoming more common in both research and clinical laboratories. Platelet-associated immunoglobulin assays by flow cytometry can be direct or indirect assays, similar to other platelet-associated immunoglobulin immunoassays. In autoimmune thrombocytopenic purpura, free serum antibodies are not found as frequently as platelet-bound antibodies (80–83). In contrast, in cases of alloantibody formation, serum antibodies may be detected without evidence of platelet-associated antibodies (84). Flow cytometry is an excellent method for direct analysis of platelet-bound antibodies, and it has also been shown to be of benefit in detection of free plasma antibodies (81, 85).

The use of thiazole orange, a fluorescent dye that binds RNA, allows immature platelets (also referred to as reticulated platelets) to be quantified (86–88). The reticulated platelet count can be used to determine the rate of thrombopoiesis. This measurement can separate unexplained thrombocytopenias into those with increased destruction and those with defects in platelet production.

The pathogenesis and molecular defects of many primary thrombocytopathies are well known and relate to defects in structural or functional glycoproteins, such as the abnormal expression of gpIIb/IIIa in Glanzmann thrombasthenia and gpIb in Bernard-Soulier disease (89–94). Flow cytometry is a rapid and useful method of obtaining a diagnosis.

Until recently, functional analysis of platelet activation was used primarily in research. Many immunological markers of platelet activation have been described, and the commercial availability of antibodies permits flow cytometric determination of platelet activation (95–97). Platelet activation may be clinically important in stored blood components, after cardiopulmonary bypass and renal dialysis, and in the treatment of patients with myocardial infarction or thrombotic events.

#### Quantification of Soluble Molecules

Soluble antigens or antibodies can be quantified by flow cytometry if standard cells or beads are used. For example, OKT3 is a mouse anti-human antibody useful in treating transplant rejection. Circulating concentrations of OKT3 can be quantified by incubating with normal CD3-positive lymphocytes, followed by a fluorescently labeled anti-mouse antibody (98). Fluorescence values are compared to a calibration curve generated with known amounts of OKT3. Recently, multiplex assays for several antigens have become possible by the use of beads indexed by incorporating two different dyes (99–102).

#### Summary

Flow cytometry is a powerful technique for correlating multiple characteristics on single cells. This qualitative and quantitative technique has made the transition from a research tool to standard clinical testing. Applications in hematology include DNA content analysis, leukemia and lymphoma phenotyping, immunologic monitoring of HIV-infected individuals, and assessment of structural and functional properties of erythrocytes, leukocytes, and platelets. Smaller, less expensive instruments and an increasing number of clinically useful antibodies are creating more opportunities for routine clinical laboratories to use flow cytometry in the diagnosis and management of disease.

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