Flow Cytometric Analysis of Granulocytes

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INTRODUCTION

Neutrophils are derived from pluripotential stem cells in bone marrow. Both monocytes and granulocytes share a common parental stem cell (GM-CFU–granulocyte-macrophage colony forming unit). Several distinct stages of development are recognized: myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell, and segmented neutrophil. The maturation process is regulated by a number of substances including growth factors produced by circulating monocytes and lymphocytes such as GM-CSF (granulocyte-macrophage colony stimulating factor), M-CSF, G-CSF (1), C3e (2), erythropoietin, and IL-3. Neutrophils are normally stored in the bone marrow for five to seven days, after which mature neutrophils are released into the blood. Factors such as G-CSF and IL-1 play a key role in the release of neutrophils from marrow to the circulating pools (3–5). GM-CSF from T-lymphocytes is implicated in the stimulation of neutrophil progenitors at doses as low as 50-100 U/ml (6). Corticosteroids commonly cause a significant increase in circulating leukocytes, primarily as a result of increased release of neutrophils from bone marrow stores. A secondary effect includes a decreased neutrophil adherence to vascular endothelium, a decreased migration of cells out of the vasculature and a slight prolongation of the neutrophil circulating half-life (7–9).

In blood vessels, two pools of neutrophils are recognized: circulating and marginating. The former circulate throughout the body in the blood stream, while the marginating pool consists of neutrophils attached to endothelial cell surfaces of small capillaries and venules. An approximately equal number of neutrophils occupy each pool in the human. Neutrophils in the circulating pool have a half-life of about 7 hr (10) after which they marginate and emigrate through tissue where they remain functional for one to two days. They are subsequently phagocytosed by macrophages or are disposed of through the mucosal surfaces. In an adult, approximately 1.5×10^9 neutrophils/kg (body weight) are manufactured daily (11). The neutrophil is a cell that is affected by, and can be responsible for, a large number of clinical syndromes. It operates as a primary source of toxic oxygen metabolites as well as a major contributor to the early inflammatory response and is, therefore, a cell of significant importance.

The purpose of this chapter is to examine the role of neutrophils and their functions in the immune system in terms of abnormalities of function and methods for analysis. Several aspects of neutrophil physiology and function will be explored and placed in the context of current experimental techniques. Of primary interest will be the use of flow cytometric techniques now available for single-cell analysis in clinical evaluations. Table 24.1 provides a simple overview of some of the clinical disorders of neutrophils, many of which are discussed in this chapter. In so doing, it is intended to provide some explanation as to the value and utility of each technique. Table 24.2 lists a number of drugs known to affect neutrophil function. These will not be discussed individually, except for the specific neutrophil function defects.

GENERAL OVERVIEW OF FUNCTION

Inflammation has been generally recognized by the classic symptoms of calor (heat), rubor (redness), tumor (swelling), and dolor (pain). It has taken several decades to advance our knowledge beyond a peripheral involvement of the neutrophil in illness and disease. The past decade has seen tremendous advances in the understanding of the physiology and biology of neutrophil function. It was Metchnikoff's description of the phagocytic process as part of a host defense system (12) and the subsequent demonstration of leukocyte chemotaxis that began our present understanding of neutrophil function. The measurement of in vitro chemotaxis by Comandon in 1917 (13) and more recently by Boyden in 1962 (14) provided a means for practical evaluation of neutrophils and other phagocytic cells. Boyden's in vitro techniques quickly spurred several major developments that linked an endogenous chemotactic factor with a major chemotactic factor (15–17). More recently, the linkage between leukocyte recruitment and inflammation has become more apparent with the discovery of a number of glycoproteins (CD11/CD18 complex) whose primary purpose is the adhesion to vessel walls (18). CD11b/CD18 is undoubtedly the same protein that facilitated the earlier observations of C3-coated particle adherence to cell membranes (18, 19). There are many techniques now available for the evaluation of neutrophil function. While this chapter cannot cover in detail all of these methods

Table 24.1
Clinical Disorders of Neutrophil Function

Function	Inherited Disorder	Acquired Disorder
Chemotaxis	Job's syndrome (75, 300–302) SCID (304, 305) Chediak-Higashi (159–161, 310, 311) α-mannosidase deficiency Leukocyte adhesion deficiency (149–151) Kartagener's syndrome (73, 75) Actin dysfunction (90)	Malnutrition (303) Periodontal Disease (302, 306–309) Thermal injury (164, 312–316) Diabetes mellitus (317, 318) Hodgkin's disease (162) SLE (319, 320) Rheumatoid arthritis (166, 167, 321, 322) Hepatic cirrhosis (163)
Phagocytosis	Actin dysfunction (323) Tuftsin deficiency (183)	Thermal injury (324) Splenectomy (325) Juvenile periodontitis (182) Neonate (181)
Microbicidal Killing	Chediak-Higashi (160) MPO deficiency (328) Specific granule deficiency (26, 330–332) CGD (334) Actin dysfunction	Malnutrition (326, 327) Thermal injury (329) Diabetes mellitus (333) Sepsis Hypogammaglobulinemia (335) Severe bacterial infections (336) Malnutrition (303) Hepatic cirrhosis (5539) Periodontal disease (30, 337, 338) Paraproteinemia (34, 339) AIDS (340) Splenoctomy (325) Rheumatoid arthritis (341) Diabetes mellitus (301, 342)
Adherence	Adherence glycoprotein (149–151) Deficiencies (265) Cystic fibrosis (344)	Diabetes mellitus (343) Neonates (288, 289)
Locomotion	Lazy leukocyte syndrome (155, 345)	Cytochalasin B (346) Malnutrition (303)
Oxidative Killing	CGD (76, 202, 203, 347) G-6-P Dehydrogenase deficiency (351) Chediak-Higashi (160, 331)	Neonates (216, 348–350) Thermal injury (352) Lassa fever (353)
Granule Functions	MPO deficiency (61–63, 354) Kartagener's syndrome (73–75, 356) Chediak-Higashi (357, 358)	Thermal injury (355)
Membrane Deformability	Chediak-Higashi	Neonates (350) Immature neutrophils (359)
Opsonic Defects	Chediak-Higashi (360)	
LTB₄ Receptors		Thermal injury (361) Rheumatoid arthritis (341, 362)
Fc,III Receptors	Cystic fibrosis (344)	Neonates (363) PNHª (278, 364–366)

ªPNH—Paroxysmal nocturnal hemoglobinuria

(listed in Table 24.3), several are discussed in more detail below.

FLOW CYTOMETRIC PROPERTIES OF NEUTROPHILS

Flow cytometry as a tool for evaluation of neutrophils is particularly useful because of the properties neutrophils display on the cytometer. Figure 24.1 shows a typical two-parameter histogram of leukocytes run on a conventional cytometer. The single-parameter projections of the scatter are also shown in this figure. Because of their "granulocytic" properties, neutrophils are easily distinguished from other leukocytes. This property has been one of the easiest methods by which to separate neutrophils for lymphocytes or monocytes. Separation of eosinophils can be further accomplished by observing the

light scatter at 90° under polarizing conditions. Many of the studies of neutrophil function evaluated using flow cytometry have taken advantage of the ability to selectively gate the neutrophil population without physical separation of the cells. This is a major advantage over some of the more conventional techniques used in studying neutrophils for several reasons. Firstly, there is less handling of the cells, which reduces activation of various metabolic pathways. Secondly, functional evaluations can be performed faster and in the presence of other cells that can be used as internal controls. Thirdly, properties of other leukocytes can be evaluated as part of a protocol, resulting in a significant time and sample volume reduction. Finally, far fewer cells are usually required when using flow cytometry, so a smaller volume of blood or other tissue is needed from a patient.

Table 24.2
Pharmacologic Alterations of Neutrophil Physiology

Chemical	Chemotaxis	Adherence	Degranulation	Microbicidal Killing	Phagocytosis
Alcohol	(367–369)	(367–369)			
Aminoglycosides				(370)	
Amphotericin B	(73, 371, 372)			(372)	(372, 373)
Aspirin	(367–369)	(367–369)			
Auranofin	(374)			(374, 375)	
Azelastine				(376)	
Chloroamphenicol	(377)				
Clindamycin	(378)				
Colchicine	(368)	(367–369)	(72)		
Cyclophosphamide				(379, 380)	
Dapsone				(381)	
Epirubicin				(382)	
Erythromycin	(378)				
γ-Interferon				(282)	(282)
Gentamycin	(383, 384)				
Ibuprofen	(367–369)	(367–369)		(385)	
Idarubicine				(382)	
Indomethacin	(367)				
Ketoconazole	(372)			(372)	
Naproxin	(367)				
Oxatomide				(386)	
Pentoxifylline	(387)	(387)		(387)	
Phenylbutazone	(367)				
Piroxicam	(367–369)	(367–369)			
Polymixin B				(388)	(389)
Rifampin	(377, 390)				
Steroids	(367)	(367–369)			
Sulphonamides				(356)	
Tetracyclines	(377, 378, 391, 392)			(393)	(394–397)

The specific property of 90° light scatter is thought to be related to refractive properties of the nucleus and cytoplasmic granules and is one of the most useful in the evaluation of neutrophils by flow cytometry. Other methods have been proposed for discrimination of different cell populations by flow cytometry. One in particular has been the use of the metachromatic dye acridine orange (AO), which has been shown to be useful for flow cytometric determinations of differential cell counts, among other things. Acridine orange intercalates into DNA and RNA as well as into the lysosomal granules. This property can be used to accurately discriminate between lymphocytes, monocytes, neutrophils, and eosinophils, although some caution should be used since AO-fluorescence wavelength is altered by changes in pH (20).

Bassoé and coworkers have made a significant contribution to many of the leukocyte quantitation techniques necessary for accurate determination of phagocytosis (21–24). Other methods for establishing a differential cell count using flow cytometry, such as esterase activity, are also useful and rapid. For instance, carboxyfluorescein diacetate (CF-DA), when in the presence of the cell, is rapidly hydrolyzed by cellular esterases to a highly fluorescent molecule, carboxyfluorescein. Figure 24.2 shows the two-parameter representation of leukocytes using 90° light scatter versus fluorescence before and after addition of CF-DA. This useful technique provides another means of identification of neutrophils, thereby also providing some information about the metabolic status of these cells.

PREPARATIVE PROCEDURES FOR EVALUATION OF NEUTROPHIL FUNCTION

Methods for neutrophil preparation vary considerably according to the isolation site and the number of cells required. It is important not to unduly "activate" neutrophils, since their ability to undergo stimulation may provide critically important information. Several preparation methods for functional evaluation have been used, such as Ficoll-Hypaque separation using the methods of Boyum (25), percol density gradient separation technique (26), dextran sedimentation of buffy coats (27, 28), erythrocyte lysis using ammonium chloride (20), and many variations of these. One preparation technique that we have found particularly useful for clinical evaluations is a simple technique that we have termed the **overlay** method (29). This method uses only 500 µl of blood, requires little equipment, and is very fast.

Overlay Method: 500 µl of undiluted blood is carefully overlaid onto 1 ml of ficoll in a 2-ml plastic centrifuge bullet. The bullet is left motionless for 20 minutes on the bench at room temperature (Fig. 24.3). The top 250 µl of buffy coat is very carefully removed and washed with PBS.

The resultant leukocyte-rich suspension contains a large number of neutrophils useful for functional analysis. The primary value in using this technique would be the need for a

Table 24.3
Methods for Functional Assessment

Function	Traditional Method	Flow Methods (general reference (398))
Chemotaxis	Boyden chamber (12)	None
	Under agarose (399)	None
H_2O_2 production		DCFH-DA assay (56, 400)
O_2- production	Cytochrome c reduction	Hydroethidine
		Dihydrorhodamine 123 (401)
Respiratory burst	Chemiluminescence (285, 350, 402–407)	
Bactericidal	S.aureus killing assay (302)	FITC-labeled S. aureus (172, 408, 409)
	AO Uptake (302)	DCF-Texas red (199)
Membrane potential	Spectrofluorometry (105, 106, 108, 110, 410–412)	DiOC6(5) (56, 104, 191, 413–417)
Viability	Trypan blue exclusion	PI exclusion
		Ethidium monoazide
Membrane fluidity	DPH assay (418–420)	
Adhesion glycoproteins	Fluoresceinated receptor (31, 259, 264, 421)	
	³H-fMLP receptors (422–424)	FITC-fMLP (425–427)
Microtubule disruption	Phalacidin	Phalacidin (85)
Membrane structure	NBD phalacidin (428, 429)	
Degranulation	B-glucuronidase (430–432)	AS-B1 (52, 53)
		Lactoferrin (433)
Enzyme activity		LAP assay (434–438)
		Esterase activity (54, 439)
		mCIB
		ADB activity (60)
pH measurement		FITC quenching (171)
Calcium flux	Quin 2 spectrofluorometry (440)	
	Fura-2 (441, 442)	
	Indo-1 spectro	Indo-1 flow (99, 101, 443–446)
Phagocytosis	Colony counts, S. aureus	FITC-labeled orgs. (172, 176, 447)
	Fluorescence microscopy (302)	3-color flow assay (199)
	Latex phagocytosis (448, 449)	2-color methods (264, 421)
		Latex phagocytosis (450–452)
Pinocytosis		Fluid pinocytosis (187)
Bacterial degradation		AO fluorescence (60)
		DNA measurements (170)

population of minimally-activated neutrophils or for a very rapid technique when a limited cell number is required.

An important consideration when using neutrophils in a flow cytometer is their adhesive properties. Since most functional assays involve lengthy incubations, it is necessary to ensure that neutrophils remain in suspension during the duration of the experiment. Once neutrophils clump, there is little that can be done to separate them without significantly activating them further or damaging them. Neutrophils can be maintained for several hours at 4°C in PBS buffer containing EDTA, glucose, and gelatin (or bovine serum albumin) in the absence of calcium. It is important to understand, however, that this treatment can alter the antigenic expression of adhesion glycoproteins, in particular CD11b. This is discussed in more detail in a later section on these adhesion molecules.

In situations where few cells are available, flow cytometry can be used where other techniques might be less attractive. One such example is the collection of leukocytes from extremely small microenvironments, such as subgingival pockets that exist in periodontal disease. We have used flow cytometry to determine cell function capabilities of neutrophils isolated from a single diseased subgingival pocket (30). In these studies, it was necessary to study neutrophils derived from individual pockets and only a few microliters

were available. Clearly the ability to measure functional and phenotypic characteristics by flow cytometry was important in these studies.

POTENTIAL BENEFITS OF USING FLOW CYTOMETRY

An additional benefit of using flow cytometric techniques can be demonstrated in situations where simultaneous measurements of multiple functions are desired. Examples of such combinations include measurements of phagocytosis and bacterial killing, phagocytosis and calcium flux, adhesion glycoproteins and phagocytosis, and many others. A specific study where several neutrophil functions were evaluated concurrently demonstrated that tumor necrosis factor (TNF) caused dose-dependent PMN activations, thereby stimulating phagocytosis, respiratory burst, and C3b expression (31). Each of these functions was evaluated using flow cytometric techniques. Some of these techniques will be discussed later in this chapter. Additionally, there may be situations where functionally distinct subpopulations of neutrophils may exist (32, 33). Few other techniques would be capable of identifying such populations or making simultaneous functional evaluations. A number of fluorescent probes are required to undertake many of these measurements. Most
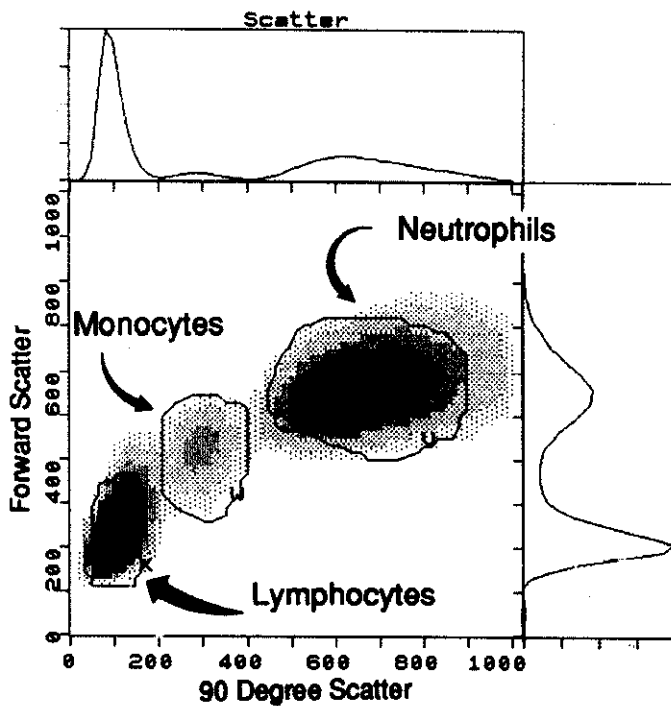
Figure 24.1. Neutrophils can be separated from other leukocytes using dual-parameter light scatter. The abscissa shows the 90° light scatter (90 LS) usually associated with "granularity." The ordinate shows forward-angle light scatter (FALS) normally associated with size. The neutrophils are clearly separated from the monocytes and lymphocytes. The single-parameter "projections" are shown for each parameter.

Figure 24.2. CF-DA (1 mM final) was added to a suspension of leukocytes and the increasing fluorescence of a gated neutrophil population was monitored for 5 min (300 seconds). Shown is the change in fluorescence of the cells during that incubation. Viable functional cells immediately hydrolyzed the CF-DA to carboxyfluorescein, which emits a strong green fluorescence when excited by 488 emission (Argon laser).

of the currently available fluorescent probes used for cell function studies are listed in Table 24.4.

EVALUATIONS OF NEUTROPHIL FUNCTION

Neutrophil Mobility

The ability to observe the in vivo mobilization of neutrophils in response to a chemotactic stimulus can only be performed by a test known as the **skin window** assay. An abrasion is made on the skin over which a glass coverslip or, alternatively, a small chamber is placed (34, 35). At several intervals, the coverslip is either removed or the chamber flushed with fresh buffer and leukocyte numbers are evaluated. This is a very difficult test to standardize, particularly in relation to the formation of the skin abrasion, and is considered to be of limited value in evaluating neutrophil function abnormalities, except, perhaps, for severe chemotactic deficiencies. Other in vitro methods are better suited to the determination of chemotactic deficiency and are discussed below.

Granule Development and Function

Neutrophil granules are synthesized at different stages during the maturation period. The release of granule contents from neutrophils is a critical function of the normal neutrophil carrying out its role in the immune response. Primary
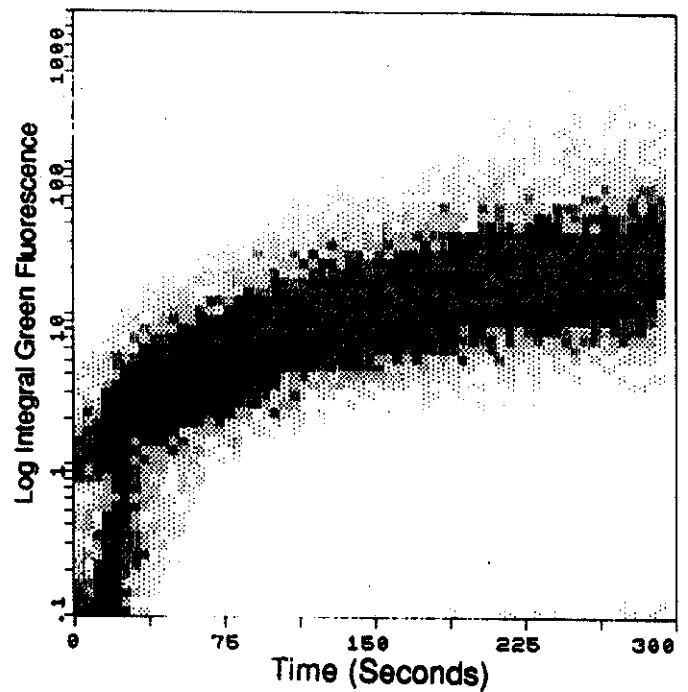
(azurophilic) granules develop following the myeloblast stage, whereupon they become promyelocytes, which are essentially incompetent neutrophils (36). As the cell further develops into the myelocyte stage, secondary (specific) granules are manufactured, a process that is complete at the metamyelocyte stage, when the immature neutrophil acquires some functional capabilities. In addition to these two well-differentiated granule types, two other types have been proposed and are known as tertiary granules and secretory granules.

The primary granules contain myeloperoxidase (MPO), required for respiratory burst function; and nonoxidative enzymes, including acid hydrolases such as B-glucuronidase, α-mannosidase, and 5'-nucleosidase; lysozyme; neutral proteases, such as cathepsin G and elastase; and cationic proteins (37–40). Further, an increasing number of small peptides, of approximately 30 amino acids in length, known as *defensins*, have been identified that also have important nonoxidative antibactericidal activity in humans (41, 42), rabbits (43) and rats (44).

The secondary granules are formed later in the maturation of the neutrophil and, thus, conditions that result in the release of immature neutrophils may cause a degradation in function related to secondary granules. Contained within these granules are lysozyme, lactoferrin, collagenase, vitamin B_{12} binding protein, cytochrome b, and possibly some of the adhesion glycoproteins (39, 45, 46).
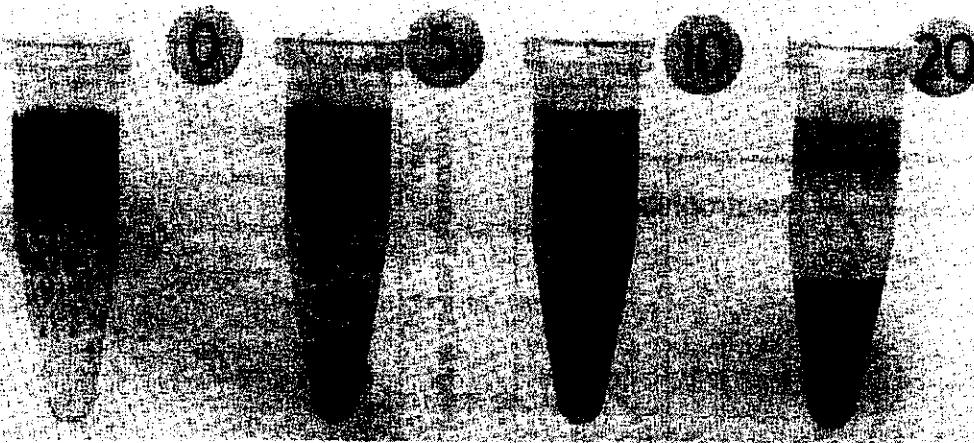
Figure 24.3. A rapid neutrophil isolation technique that we have termed the overlay method is shown. The overlay method is particularly useful in the clinical laboratory as a rapid means of isolating leukocytes for flow cytometric analysis. Heparinized blood (undiluted) is overlayed carefully onto Ficoll-Hypaque and left stationary on the bench at room temperature for 20 min. By carefully removing the top layer of suspension, a red-cell depleted suspension can be achieved. The suspension also contains platelets that can be removed by a gentle centrifugation step. Cells isolated using this technique are the least "activated" of any separation technique.

Tertiary granules contain gelatinase (a metalloproteinase that acts on protein constituents of extracellular matrices (47)), CD11b (MAC-1) receptors, and cytochrome b (47–49). Secretory granules (50, 51) contain gelatinase, although evidence and function for these granules is not well elucidated.

Enzyme Content/Activity

Dolbeare et al. (52, 53) initially demonstrated the presence of phosphatases and glucuronidases by flow cytometry using naphthol derivatives as fluorogenic substrates. Similarly, cellular enzyme activity can be measured by flow cytometry (54, 55) as can esterase activity, using dyes such as dichlorofluorescin diacetate and several others that are listed in Table 24.3 (56–60). The latter assay is more useful as an indicator of the presence of esterase. Because of the variation in the rates of hydrolysis for cellular enzymes, this activity can be used to differentiate cell populations as well as to indicate normal metabolic function. Active metabolism is involved in these hydrolytic reactions, and alterations induced by immunochemical modulators are observable. Some dyes can be very rapidly hydrolyzed to fluorescent compounds directly within a cell. Carboxyfluorescein diacetate is useful in this regard since it is hydrolyzed to the fluorescent carboxyfluorescein (530 nm) very rapidly. The process is complete within a few seconds to a maximum of 5 min in most cells, allowing very rapid evaluation. Several publications attest to the efficacy of the measurement of other enzymes by flow cytometry (54, 55).

Clinical Evaluation

The most common granule deficiency of neutrophils is a myeloperoxidase (MPO) deficiency in which there is a complete or partial deficiency of MPO from the primary granules (61–63). The deficiency is relatively common with an incidence of 5 patients in 10,000 subjects and is characterized by autosomal recessive genetics (63). It has been reported that monocytes from patients with MPO deficiency have increased respiratory burst duration with increased production of superoxide, which may partially compensate for the deficiency (64). Myeloperoxidase deficiency is almost silent clinically except for an increase in susceptibility and severity of *Candida* infections (63). Functionally, neutrophils demonstrate normal chemotaxis, phagocytosis, and degranulation, but a prolonged respiratory burst. The diagnosis is easily made by peroxidase stain of a blood smear. Such deficiencies may become more significant in patients who have another primary condition or receive therapy that may leave them with a reduced immune function.

Congenital specific granule deficiencies have been reported and some neonates have demonstrated deficiencies in specific granule formation (65–71). However, these deficiencies usually result in relatively minor microbial killing abnormalities. Severe recurrent bacterial infections can occur but are the exception.

Microtubule disorders can adversely affect the ability of a neutrophil to degranulate, as demonstrated by colchicine, which interferes with microtubule formation (72). Clinically, patients with microtubule dysfunction, such as Kartagener's syndrome, have recurrent sinus, middle ear, and respiratory infections, but this is more directly related to dysfunctioning cilia and impairment of leukocyte migration and chemotaxis (73–75), as discussed below. Abnormal microtubule metabolism has also been identified in chronic granulomatous disease (76).

Cytoskeleton Function

After activation of neutrophils, extensive movement of the receptor-ligand complexes within the membrane has been demonstrated (77–79), resulting in characteristic shape

Table 24.4
Fluorescence Probes for Neutrophil Studies

Function	Probe	Excitation	Emission	References	Considerations
H_2O_2	DCFH-DA	488	515–575	(56)	Broad emission spectra
O_2^-	HE	488	575–590		
	Bodippy	352			UV Laser required
Calcium	Indo-1	352	420; 525	(453)	UV Laser required
Calcium	fura 2	340	520	(191, 453, 454)	Dual Laser excitation required
Calcium	fura 3				
Calcium	Quin-2	352; 420	525	(440)	UV Laser required
Markers	FITC	488	525		
Markers	PE	488	575		
Markers	Texas Red	610	630		
Markers	APC	532	650		
Esterase	CFDA	488	525	(59)	
Viability	FDA	488	525		
Esterase	Decanoyl Fluor	488	525	(455)	
Esterase	CDF	488	525	(59)	
Esterase	CDF DA	488	525	(59)	
Esterase	CDMDF-DA	488	525	(59)	
Esterase	ADB[a]	488		(60)	
pH	ADB	488		(60)	
Phosphatases	MFP	488			
GSH	mCIB	352	460–510		UV Laser required
Enzymes	AS-B1			(52, 53)	
Fluidity	DPH	352	420	(418, 419, 456–459)	UV Laser required
Fluidity	TMA-DPH	352	420	(458, 460, 461)	UV Laser required
Fluidity	Pyrenedecanoic acid	360	400/450	(462)	
Actin	NBD-Phallacidin	488	520	(86, 428, 429, 463)	UV Laser required
Actin	PE-Phalloidin	488			
Membrane Potential	$DiOC_5(3)'$	488	505–560		
Membrane Potential	$DiOC_6(3)$	488	505–560	(110)	
Membrane Potential	Rhodamine 123	488		(111)	
Cell Tracking	PKH1	488	525		
Cell Tracking	PKH2	488	575		
pH	AO	488			
Viability[b]	AO	488		(60)	
pH	FDA	488			
pH	DA-dicyanobenz	352	460; 525		UV laser required
Killing	AO	488			Emission is pH dependent
Viability	PI	488	>630		
Viability	EMA	488			
Peptidase	leucyl aminopeptidase	352	525	(438)	UV laser required

[a]ADB: 1,4-diacetoxy-2,3-dicanobenzene
[b]Used to measure bacterial degradation (red DNA)

changes (77). It is likely that this redistribution of the complex occurs within the plane of the plasma membrane utilizing the microfilaments. Evidence for this hypothesis is based partially upon the role of cytoskeletal disruptors, such as cytochalasin B (a fungal metabolite) and chloropromazine. It is thought that cytochalasin B binds to the free ends of the F-actin molecule and thus inhibits fMLP-induced (formyl-methionyl-leucyl-phenylalanine) polymerization of actin in neutrophils (80–82). Evidence of the involvement of regulatory G proteins in the transmembrane signaling after fMLP activation in human neutrophils has recently been demonstrated (83). GP 140, which interacts with the cytoskeleton during activation by wheat germ agglutinin (WGA), has also been implicated as playing a role in neutrophil activation (84). No clear evidence exists as to effective flow cytometric methods for detection of cytoskeletal defects. However, there are well-developed methods for measurement of actin polymerization by flow cytometry (85, 86).

Clinical Evaluation

As discussed above, Kartagener's syndrome, and more specifically immotile cilia syndrome, is an autosomal recessive disorder resulting in a microtubule defect that impairs leukocyte migration and chemotaxis (73, 75). The clinical manifestations of immotile cilia syndrome include recurrent sinusitis, otitis media, and respiratory infections due to the cilia and leukocyte dysfunctions (75). In a separate report, a patient with recurrent bacterial infections and abnormal chemotaxis had excessive neutrophil microtubule assembly (87).

Microfilament disorders inhibit leukocyte locomotion, as demonstrated by in vitro treatment of neutrophils with cytochalasin B, which disrupts actin filaments (88, 89). A clinical report of abnormal actin polymerization involving an infant with impaired chemotaxis has been reported (90).
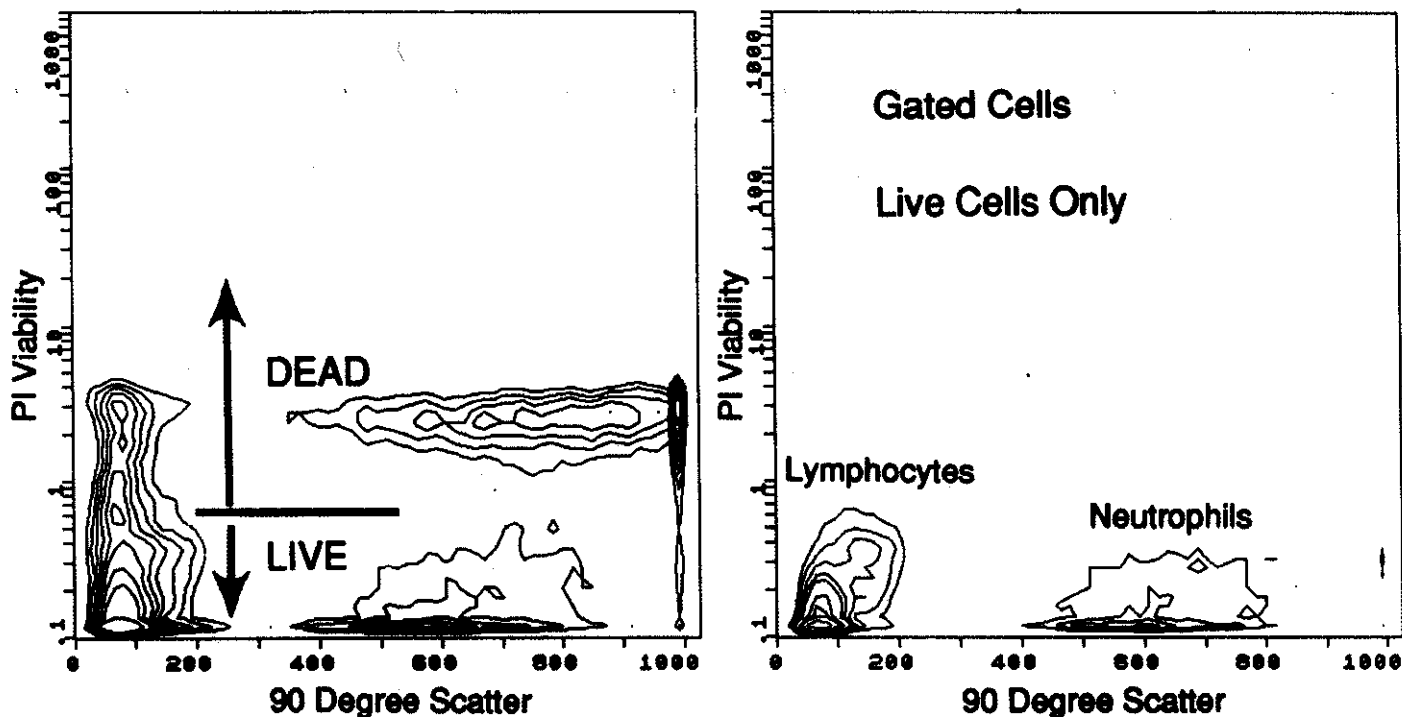
Figure 24.4. Demonstration of the use of *PI* to identify viable and nonviable cells by flow cytometry. *PI* was added to a suspension of cells with a suspected number of dead cells. Using a 2-parameter contour display that shows each population of cells, the lymphocytes and neutrophils can be identified. Cells that take up the *PI* dye are considered nonviable. The histogram on the right shows the viable cells selectively gated from the left histogram.

Membrane Integrity (Viability)

Measurement of cell viability using dyes, such as propidium iodide or fluorescein diacetate, should also be considered functional tests. Failure to evaluate viability in an assay of neutrophil function can lead to erroneous data, since a nonviable population will significantly influence the results. The advantage of flow cytometry is that determinations are made during or immediately after functional measurements. Nonviable cells can be gated out of the analyses (91) by either backgating on the fluorescent population of viable cells or observing scatter alterations of dead cells. An example of this is shown in Figure 24.4, which shows the changing fluorescence of cells that take up the PI stain (dead cells).

Such measurements can be performed rapidly and objectively and have been used successfully in toxicology applications (92) as well as in routine clinical assays. As a simple measure of the effects of xenobiotics on a cell, viability is certainly one of the most straight-forward measurements available using flow cytometry.

METABOLIC FUNCTIONS

Measurement of Cytosolic Free Ca^{2+}

The initial steps of signal transduction following receptor-ligand interaction involve activation of phospholipase c and membrane-bound glycophotidyl inositol. This leads to the release of inositol phosphates and fatty acids, which trigger activation of protein kinase C and subsequent flux of calcium across the plasma membrane. Thus, a direct cellular response can be measured if alterations of calcium concentration can be monitored.

Since calcium plays a critical role in cell function, it is important to be able to determine the extent to which chemical interactions affect the redistribution of this divalent cation. The area is not without controversy, however, since there is not complete agreement on the role of calcium in neutrophil activation during phagocytosis. There are at least two schools of thought on the role of calcium in neutrophil activation. Of importance, is the type and number of different ligand interactions involved. Essentially, when a single ligand-receptor interaction occurs, such as phagocytosis of yeast (via C3b), calcium is not required for respiratory burst (93). However, others have shown a lack of respiratory burst in the absence of calcium (94–96).

Indo-1 is an excellent dye for flow cytometric measurement of free intracellular calcium. This dye has the ability to undergo a fluorescent wavelength emission shift when bound to calcium. Indo-1 is introduced to the cells as an acetoxymethyl ester that undergoes enzymatic hydrolysis in cells to yield free dye. Flow cytometry has proved to be a valuable resource in the evaluation of the role of calcium in neutrophil function. A major spectral change can be measured when indicators of Ca^{2+} penetrate cells and are excited at 357 nm (ultra-violet excitation). Several different Ca^{2+} indicators are now available for use (97–100).
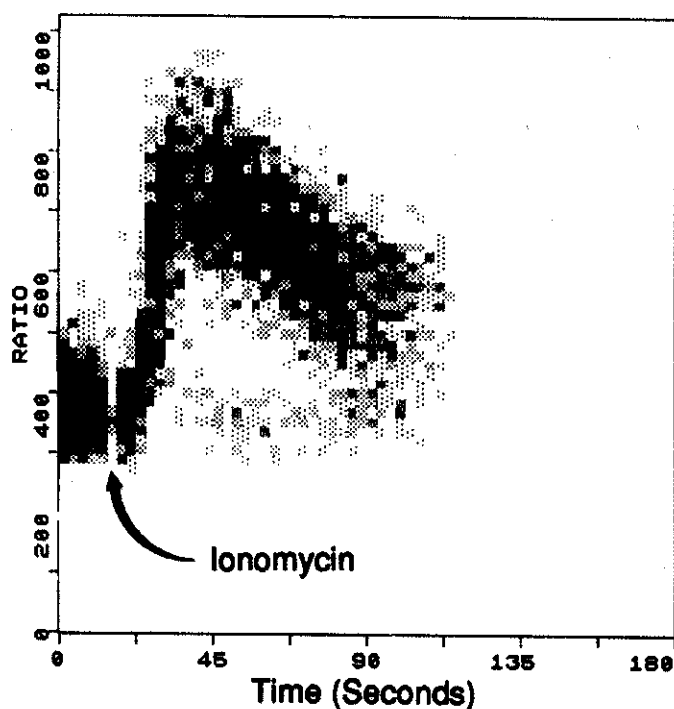
Figure 24.5. A population of Indo-1-loaded cells stimulated by ionomycin to demonstrate the rapid response of viable functional cells. By calibrating the flow cytometer with known concentrations of calcium, accurate measurements of intracellular free calcium can be determined.

Method: Cells are loaded with Indo-1 (final concentration 3 μM) for 15 min at 37°C and then immediately run on the flow cytometer to obtain fluorescence histograms at two emission wavelengths; 395 nm (bound Ca^{2+}) and 525 nm (non-bound calcium). The Ca^{2+} concentration of cells can be determined independently of dye concentration by evaluating the ratios of the two fluorescent emissions. Thus, a high 395/525 nm ratio would indicate bound Ca^{2+}. Ionomycin is used as a positive control for measurement of calcium flux. Ionomycin (3–5 mM) will cause an increase in the BOUND (long wavelength) fluorescence signal (i.e., increase in BOUND $[Ca^{2+}]$ inside the cell).

This measurement is a vary rapid event that can be observed on the flow cytometer in real time given appropriate instrumentation. Accurate determination of intracellular calcium concentration can be made if aliquots of Indo-1 loaded cells are placed in solutions of various known calcium concentrations and treated with an ionophore such as ionomycin. The properties of Indo-1 are well-described in the literature (101). Observations of the real-time alteration in $[Ca^{2+}]$ can be performed using list mode on the flow cytometer. Figure 24.5 shows an example of cells that have been stimulated by ionomycin, demonstrating the rapid alteration in calcium flux as measured by the flow cytometer.

One example of a calcium–requiring activation of human neutrophils is after stimulation by fMLP. In this interaction, fMLP binds to its receptor initiating a G-protein regulated activation of phospholipase C that then hydrolyzes a membrane-bound phospholipid (4,5 bisphosphate) to form both 1,2-diacylglycerol (DAG) and 1,4,5-triphosphate (IP3), which release calcium from intracellular stores primarily in the endoplasmic reticulum (102).

Less-recognized clinical abnormalities include glycogen storage disease type 1b, which is characterized by recurrent bacterial infections. Reduced phagocytic function appears to be related to diminished calcium mobilization and defective calcium stores; it is recognized by decreased elevation of cytosolic calcium to fMLP and decreased mobilization of calcium in response to ionomycin (103).

Membrane Potential

Neutrophils undergoing a receptor-ligand interaction show an increased permeability to ions and a subsequent reduction in transmembrane potential (104–107). A specific inhibitor of chymotrypsin-like enzymes can block the potential change, suggesting that, after the receptor-ligand interaction, a protease is required for the initial reaction (108). By use of the carboxycyanine dye [diO-C5-(3)], a loss of cell-associated fluorescence is demonstrated, indicative of cell activation and, therefore, a change in resting transmembrane potential (108). Neutrophils that are incapable of responding oxidatively to stimulation do not show this fluorescence shift (109), suggesting a relationship between alterations in transmembrane potential and the generation of oxygen metabolites (108). The dye diffuses into the cells and equilibrates with the external medium. Upon stimulation of the cell, the intracellular dye is displaced by increased uptake of ions, resulting in a reduction of cellular fluorescence (depolarization). With some activating agents (fMLP), the cell will reequilibrate after a short period and repolarization will be demonstrated by a return to the previous fluorescence intensity.

Using flow cytometry, individual cells can be monitored kinetically, allowing a determination of the rates of polarization. Variations in these rates could be a significant factor in observing alterations in cellular function. This is shown kinetically in Figure 24.6, where a population of neutrophils was stimulated with 100 ng/ml of PMA after being loaded with the carboxycyanine dye diO-C5-(3), as described above. Response is rapid and definitive. Defects are easily observed using this method. However there are a number of problems associated with membrane potential measurements that make this a difficult test to use clinically. Measurements in mitochondrial membrane potential can also be measured directly. This has been demonstrated by Korchak et al. (110) using DioC6(3) and also by Darzynkiewicz et al. with the mitochondrial-specific dye Rhodamine 123 (111).

Clinical Evaluation

One of the first functional abnormalities to be lost in neutrophils is the normal capabilities of alteration in membrane potential. Our laboratory has observed that neutrophils isolated from blood after 18–24 hours will produce a significant respiratory burst (H_2O_2) or chemiluminescence response but
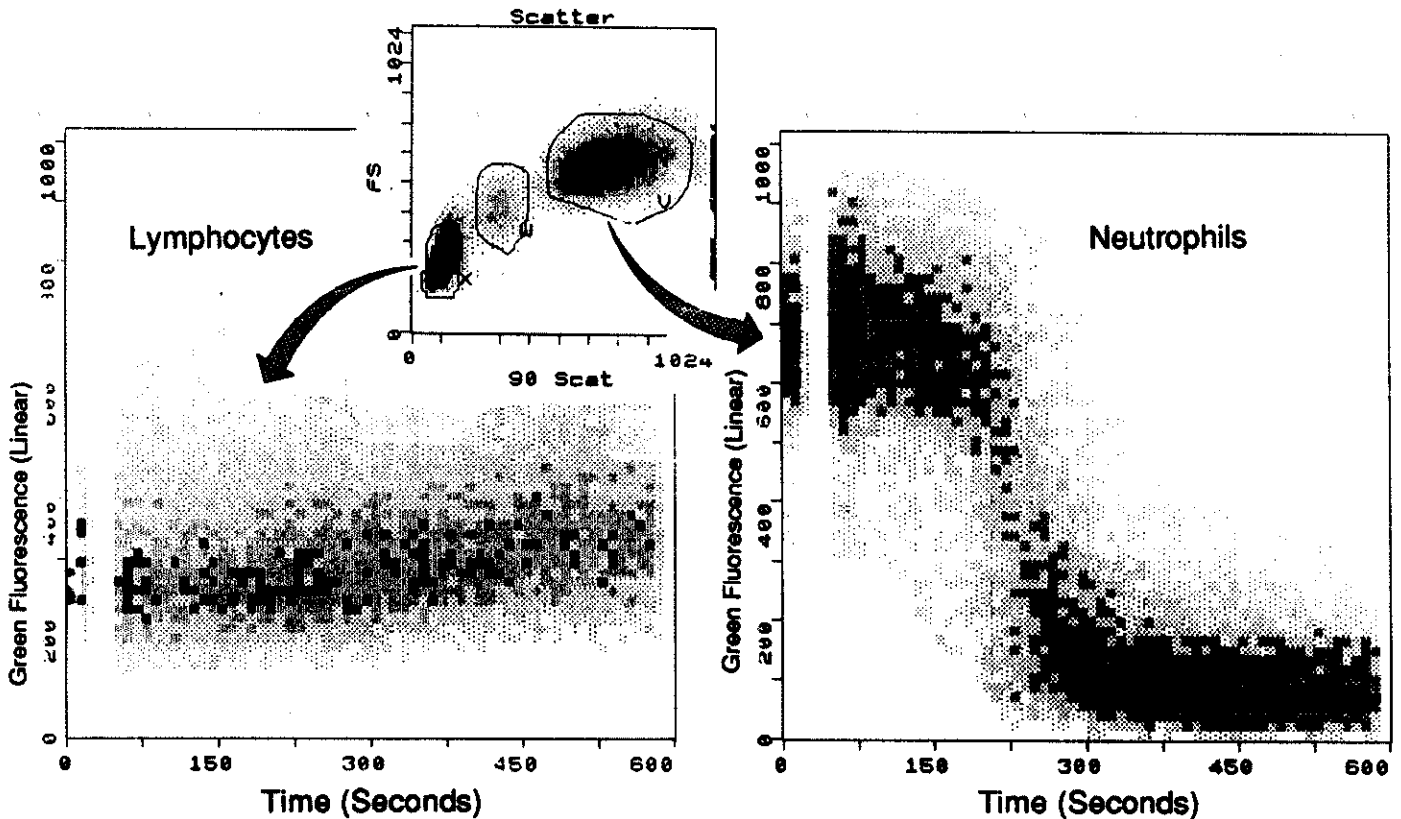
Figure 24.6. A population of neutrophils undergoing a rapid alteration in membrane potential after stimulation by PMA (100 ng/ml). Cells were loaded with DiOC5 (3) for 5 min, placed into the flow cytometer, and stimulated with the PMA. Measurements were begun immediately upon stimulation and maintained for 10 min. The two histograms shown demonstrate the changes in membrane potential of both lymphocytes (left) and neutrophils (right) from the same suspension of leukocytes. Each population was selectively gated and time versus fluorescence was recorded simultaneously. No alteration in the lymphocyte membrane potential of the lymphocyte population to the PMA was recorded. The neutrophil population, however, demonstrated a remarkable and rapid depolarization, consistent with the ionic flux expected with mebrane activation. Neutrophils normally do not repolarize after stimulation with PMA; however, with other activators such as fMLP, repolarization is observed after 4–7 min.

that the membrane potential response is severely deficient (unpublished observations). However, there are no reports of distinct clinical syndromes where abnormal membrane potential has been shown to be of particular importance as an individual phenomenon. Presently no data are available to demonstrate clinical utility.

Chemotaxis

The chemotactic response of the neutrophil is based upon the ability of these cells to determine a gradient of a chemoattractant substance and to direct the movement of the cell toward the source of the attractant. In order to accomplish this, neutrophils have a complex cytoskeletal mechanism involving both microtubules, used to directionally polarize the cell, and microfilaments for cell movement. The process of movement involves a constant attachment and detachment of the neutrophil to a substrate. Chemotactic function is an important neutrophil function and several excellent reviews have been written on the subject (112–116). Chemotaxis, like most neutrophil functions, is not an isolated function, but is part of a complex series of events that occurs during and after activation of the cells. The process of chemotaxis

itself cannot presently be measured satisfactorily using flow cytometric technology; however, the ability of a neutrophil population to become activated by common chemotactic agents, such as C5a and formyl methionyl leucyl phenylalanine (fMLP), can be easily evaluated. One useful method is the determination of alterations in fMLP receptors on neutrophils via fluoresceinated fMLP using fluorescein isothiocyanate (FITC). Upon activation of the cells, the appearance of available receptors can be evaluated by measuring the green-associated FITC fluorescence of the neutrophils. GM-CSF has been shown to increase the binding of fMLP to PMN (1).

Neutrophils contain storage pools of fMLP receptors within the specific granules and these receptors are transported to the surface in response to stimulatory signals (117). FMLP binding is saturable with an estimated 50,000 sites/neutrophil and a $K_D = 10^{-14}$ nM (118). The receptor-ligand (radiolabelled) complex has a molecular weight of 55–70 kD (119). The receptor is distinct from C5a (120) or LTB_4 (121). Flow cytometric studies have identified high- and low-affinity binding sites as well as neutrophil subpopulations with varying numbers of receptors (122, 123). Response to fMLP by neutrophils is down-regulated through internalization of
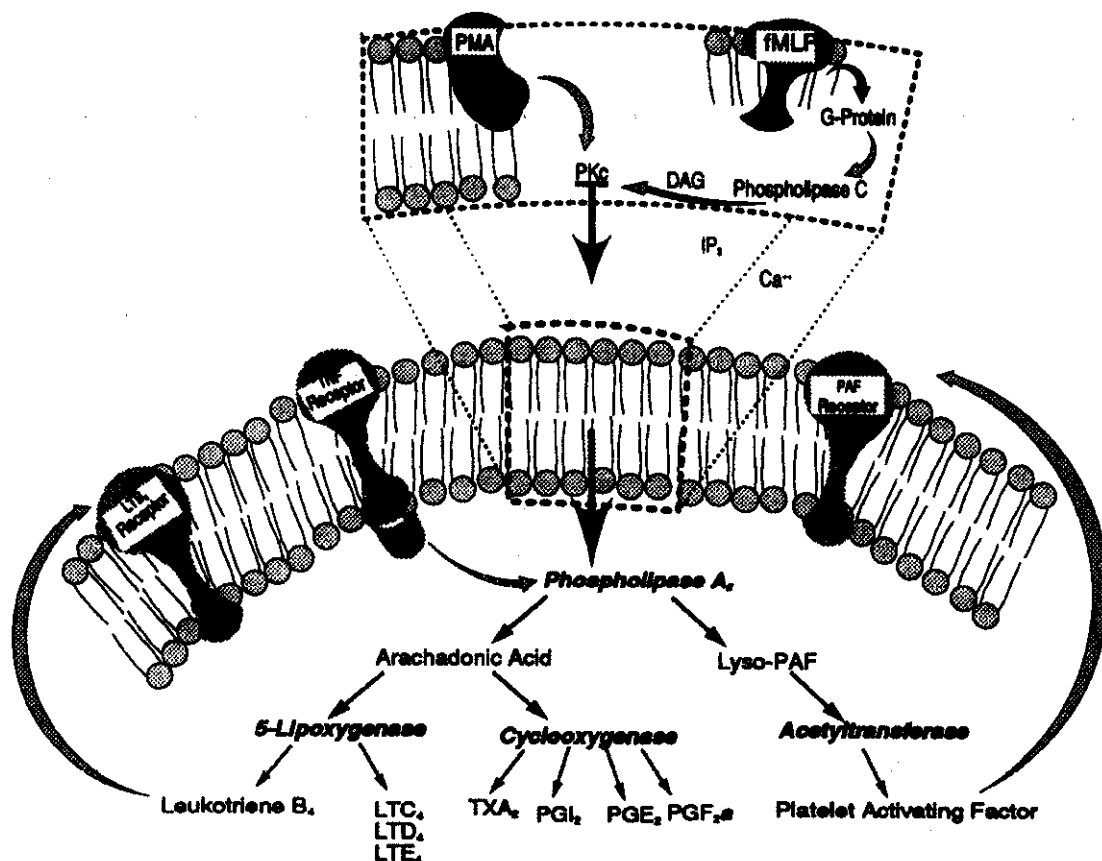
Figure 24.7. A schematic guide for the interaction of some of the neutrophil activation mechanisms and their relationship to cytokines and lipid mediators. Shown are several important receptor-mediated activation mechanisms associated with various inflammatory processes, including endotoxemia or thermal injury.

the receptor-ligand complex; however, recovery from this down-regulation is rapid (20 minutes at 37°C (124, 125). At low concentrations, fMPL will cause the release of vitamin B_{12} binding protein, and B-glucuronidase and lysozyme (47, 126) from secondary granules. However, at higher concentrations, it is known to stimulate the release of most primary and secondary granule contents (127). Since bacterially-derived peptides are remarkably similar to fMLP, the continued presence of fMLP or related molecules could cause a continued stimulation of enzyme release from stimulated neutrophils.

A specific C5a receptor has been identified on human neutrophils with a density of 100,000–300,000 sites/cell (120) and a molecular weight of approximately 44kD (128). Adherent neutrophils undergo exocytosis in the presence of C5a and both specific and azurophilic granules release their contents (129). In vitro, in the absence of cytochalasin B (and adhesion), very high concentrations of C5a are required to stimulate neutrophils (130). Human neutrophils have been shown to be able to activate (131) and inactivate C5a via a specific granule factor (132), thus providing a closed-loop control of responsiveness to this activator.

LTB$_4$ receptors occur in a low affinity form ($k_D = 3.9 \times 10^{10}$M, ~4400 sites/cell) and in a high affinity form ($K_D = 6 \times 10^{-8}$M, ~270,000 sites/cell) and are quite independent of the previously mentioned receptors (121,133). Neutrophils can be both stimulated by LTB$_4$ and produce LTB$_4$ as a product of arachidonic acid metabolism via the lipoxygenase pathway. Several physiological effects have been demonstrated on neutrophils by LTB$_4$, such as chemotaxis (134), aggregation (135), and degranulation (136). Further, LTB$_4$ has proved important in the mechanism of neutrophil adhesion to endothelial cells. As noted previously, increased adhesiveness (aggregation) due to LTB$_4$ has been shown, but these adhesion-promoting properties have also been reversed in the presence of an anti-CD18 monoclonal antibody (137). Figure 24.7 provides a schematic guide for the interaction of some of the neutrophil activation mechanisms and the relationship to cytokines and lipid mediators.

Interleukin 8 (IL-8) is an important chemotactic factor. This factor has numerous nomenclatures including the following: MDNCF–Monocyte-derived neutrophil chemotactic factor) (138); MONAP (139); NAP-1 (140); NAF; and IL-8 (141). The major form of IL-8 is a 72-amino-acid protein, but it has not shown significant homology with other cytokines including, IL-1, TNF, or IFNs (138, 142). IL-8 is capable of stimulating most activation pathways in neutrophils, such as degranulation (139), directional migration, expression of adhesion molecules, activation of the respiratory burst (143), enhanced *Candida* killing (144), and general ac-

tivation (143, 145). There are specific IL-8 receptors on PMN (146) with about 20,000 high affinity binding sites ($K_d = 8 \times 10^{-10}$). IL-8 also very rapidly regulates its own receptor expression associated with ligand internalization. Further, this down-regulated receptor was shown to be rapidly recycled to the surface of the neutrophil (147).

Products of arachidonic acid metabolism are also well-known chemoattractants. Products of the lipoxygenase pathway, such as 5-HPETE, are converted to 5-HETE and leukotrienes, most of which are known to exert strong chemotactic activity upon neutrophils (148). Regulation of chemotaxis is modulated by the concentration of the chemoattractant. Several chemicals are known to exert enhancement (alcohols, degranulation) or depression (polyene antibiotics) of chemotactic function.

Clinical Evaluation

Defects in neutrophil chemotaxis are usually accompanied by recurrent infections of the skin or respiratory tract. Clinical syndromes with infections usually begin in the infant and are characterized by severe infections caused by organisms normally considered to be of relatively low pathogenicity.

A deficiency in the surface glycoproteins CD11a/CD18 (LFA-1), CD11b/CD18 (CR3), or CD11c/CD18 (p150,95) can lead to chemotactic defects (149). This syndrome, called Leukocyte Adhesion Deficiency (LAD) has been identified by Anderson et al. (149), Arnaout et al. (150), and Ross (151). This is an autosomal recessive disease characterized by recurrent bacterial and fungal infections, impaired pus formation, and poor wound healing (149). The genetic abnormality is caused by defective biosynthesis of the B subunit of the heterodimer complex associated with each glycoprotein adhesion molecule (149, 152–154). Lazy leukocyte syndrome was first identified in children and characterized by gingivitis, stomatitis, and recurrent upper respiratory infections (155). Severe circulating neutropenia was identified, but normal myeloid precursors and mature neutrophils were evident in the bone marrow (155).

Lazy leukocyte syndrome is now recognized to have several variants, including a range in severity of neutropenia, variation in age of onset, and cases with defective phagocytosis and microbial killing. However, all possess a basic functional defect in locomotion (156).

Hyperimmunoglobulin E, or Job's syndrome, is manifested clinically as dermatitis, recurrent staphylococcal skin infections, and elevated serum concentrations of IgE (157). Recurrent staphylococcal pneumonia, otitis externa and media, sinusitis, mucocutaneous candidiasis, and eczema are also seen (157, 158). Neutrophil chemotaxis abnormalities are common, although variable (75).

Chediak-Higashi syndrome is an autosomal recessive disease characterized by severe recurrent pyogenic infections (159). Many cells, including the neutrophils, display abnormal granule formation with giant cytoplasmic lysosomal granules. Neutrophils display abnormal chemotaxis and oc-

casionally decreased numbers of centriole-associated microtubules (75, 160, 161). Other clinical manifestations are granule-related and include partial albinism, associated with a melanocyte dysfunction, and bleeding tendencies due to platelet defects (159).

Several diseases have neutrophil chemotaxis disorders that may be associated with a circulating inhibitor of locomotion or an inhibitor of a serum chemotactic factor. Associated diseases include Hodgkin's disease (162), hepatic cirrhosis (163), thermal injury (164), and severe inflammation (165). Circulating immune complexes in rheumatoid arthritis can act on neutrophils to inhibit chemotaxis (166, 167). Disorders of the microtubule or microfilament system (discussed above) can also inhibit chemotaxis.

Phagocytosis

The phagocytic process can be divided up into a number of clearly defined stages, each of which can fail. These stages are broadly defined as attachment or particle binding and ingestion. Unless phagocytes are able to bind to the microbe, phagocytosis will not take place. By utilizing both opsonized and nonopsonized organisms, both opsonic capacity and phagocytosis can be measured at the same time. Thus, it is important to determine whether abnormal phagocytosis is due to a failure in the opsonization process or to a defect in the ingestion capability of the phagocyte. Since the main cell receptors for phagocytosis are C3b (CR1) and FcR (Fc portion of IgG), it is also possible to evaluate these functional receptors as discussed earlier. An example of a neutrophil phagocytosis of *S. aureus* is shown in Fig. 24.8.[1]

Assays of phagocytosis of bacteria have been developed for flow cytometry by Bassoe et al. and other investigators (20, 21, 168–174). These measurements can be valuable in trauma, such as thermal injury, or in recurrent infections where specific bacteria can be used for assessment of immune function. Immune complexes can also be measured with methods similar to those for bacteria (175).

Several innovative methods for determining phagocytic capacity using flow cytometry have been demonstrated. A major advantage of flow cytometry over other methods is the relatively small number of cells required and the significantly fewer preparative procedures for isolating leukocytes. These are important when using small animals or when evaluating pediatric patients.

The availability of a number of fluorescent probes has increased the number of methods available. One useful method described uses fluorescein heat-killed *Candida albicans* and, after phagocytosis is complete, ethidium bromide (EB) (50 ug/ml) is added. Analysis using ultraviolet (UV) excitation with red and green emissions reveals green internalized organisms, while surface attached, but noninternalized, organisms are red (176). This procedure utilizes the phenomenon of resonance energy transfer between FITC and

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[1]See Color Plate II between pages 432 and 433.

ethidium bromide. Since EB does not penetrate the cell membrane, only the external organisms are affected by the EB. This provides good discrimination between internal and external organisms. One major advantage of this test is the use of an inexpensive clinical analyzer flow cytometer that does not require expensive lasers and complex optical configurations.

Another interesting use of flow cytometry in evaluating neutrophil function is in the evaluation of phagocytosis of fluorescent-labeled viruses. In this study FITC-labeled *Herpes simplex* viruses (HSV) were phagocytosed by human neutrophils and both internalization and surface binding were determined by flow cytometry. Surface bound virus fluorescence was quenched using a trypan blue quenching procedure (177, 178).

Clinical Evaluation

Abnormal phagocytosis can occur with a variety of clinical disorders. The defect can be associated with the neutrophil itself or with an immunoglobulin or complement defect. Immature neutrophils released from the bone marrow have a defective phagocytosis that may be related to a high negative surface charge (179, 180). Abnormal phagocytosis has also been identified in the neonate and in juvenile periodontitis (181, 182). Tuftsin deficiency is either a familial disorder or is acquired as a consequence of splenectomy; it results in increased susceptibility to infection due to defective neutrophil phagocytosis (183). Tuftsin is a tetrapeptide produced by the spleen that enhances the neutrophil phagocytic ability (184). Clinical findings include respiratory infections such as bronchitis and pneumonia, and enlarged fluctuant lymph nodes (183, 185). Normal actin polymerization and microfilament function are also necessary for phagocytosis and, therefore, actin polymerization defects may interfere with the phagocytic process (90). Complement receptor C3bi deficiency can also result in altered phagocytosis (186).

Pinocytosis

Pinocytosis can also be a useful measure of cell function and several well-defined assays have been developed for flow cytometry (187, 188). fMLP-stimulated pinocytosis studies have demonstrated a linkage between the initial phase of pinocytosis and the characteristic shape changes observed in activated neutrophils. The assay used for these studies uses FITC-dextran and is relatively simple to establish, considering the availability of a flow cytometer. One of the most useful aspects of this assay is the ability to evaluate a large number of concommitant effects such as pH changes, kinetics of the responses, and temperature and ionic concentration effects (187).

Neutrophil Defense Mechanisms

Traditional descriptions of neutrophil defense mechanisms include both oxidative and non-oxidative mechanisms. Rare clinical syndromes may selectively deplete one major component of one or more pathways but, by and large the neutrophil activates many of its defense mechanisms concurrently and often the clinical manifestation of defects in specific components is minimized. One such case would be in MPO deficiency, which is characterized by a reduced bactericidal rate but ultimately has a normal killing capacity of neutrophils.

Oxidative Systems

Oxidative mechanisms require oxygen in significantly larger amounts than resting neutrophils require. A variety of toxic oxygen species is produced both inside and outside the cell. The associated activity is known as the respiratory burst, which results from activation of NADPH oxidases via an electron transfer reaction involving 2 electrons from NADPH through an FAD-flavoprotein utilizing cytochrome b^{245} to oxygen. The superoxide anion produced in this reaction can be converted to H_2O_2 by superoxide dismutase and, in concert with myeloperoxidase (MPO) and a halide (primarily chloride), hypochlorous acid can be produced. Each of these species is capable of damaging ingested bacteria, external microbes, the neutrophil itself, or closely situated tissue.

C-reactive protein has also been proposed to have a modulatory role in neutrophil oxidative burst, inhibiting superoxide release, chemotaxis, degranulation, and phagocytosis of activated neutrophils (189). C-reactive protein has also been demonstrated to bind to the surface of PMA-activated neutrophils (190).

By far the most useful measurement of intracellular H_2O_2 estimation is the dichlorofluorescin diacetate (DCFH-DA) probe technique as proposed by Bass et al. (56). The assay depends upon the incorporation of 2'-7',dichlorofluorescein diacetate (DCFH-DA) into the hydrophobic lipid regions of the cell, where the acetate moieties are cleaved by hydrolytic enzymes to the nonfluorescent molecule 2'-7',dichlorofluorescein (DCFH), which becomes trapped within the cell due to its polarity. Upon cell activation, NADPH oxidase catalyzes the reduction of O_2 to O_2^-, which is further reduced to H_2O_2. The oxidative potential of H_2O_2 and peroxidases are able to oxidize the trapped DCFH to 2'-7',dichlorofluorescein (DCF), which is characteristically fluorescent at 530 nm (the same emission as FITC). Since the green fluorescence produced is proportional to the amount of H_2O_2 generated, it is possible to calibrate this assay to allow the expression of the intracellular production of H_2O_2 in neutrophils in terms of attomoles/cell (56). There are many examples of use of this assay in the literature, including studies on various animals such as rats, mice, and humans (30, 56, 58, 191–199).

A calibration curve can be generated based on data obtained from spectrophotometric and flow cytometric measurements that allows conversion of the fluorescence histograms on the flow cytometer into quantitative estimations of
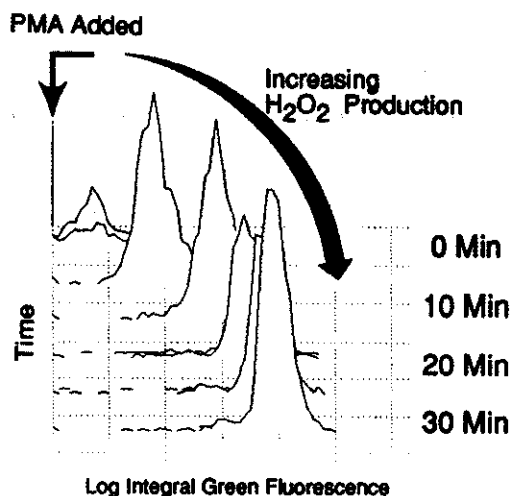
Figure 24.9. A histogram showing the kinetic response of neutrophils stimulated with *PMA* as measured by the H_2O_2 assay (described in the text). As the neutrophils respond to the *PMA*, respiratory burst activation results in production of H_2O_2. Cells had been previously "loaded" with DCFH-DA which becomes intracellularly hydrolysed to DCFH by cellular esterases. Oxidizing conditions (H_2O_2) caused oxidation of the nonfluorescent DCFH to fluorescent DCF, which was measured on the flow cytometer as green fluorescence. Each single-parameter histogram is from measurements taken from a tube sampled seven times over the 30-min reaction period.

H_2O_2 production (56). The assay is a very sensitive measure of a cell's capacity to undergo a respiratory burst in response to a variety of stimuli. While it may be of interest to determine the exact amount of H_2O_2 per cell, it is unnecessary in most clinical situations and an alternative measurement is the relative amount of fluorescence (therefore H_2O_2) produced by the cell before and after stimulation. Figure 24.9 shows the kinetics of the neutrophil response to PMA (100 ng/ml) over a 30-min period. It is not always necessary to measure the kinetics of the entire response. For most clinical evaluations, a beginning and ending measurement at 0 and 30 min is adequate. Figure 24.9 displays the difference in the fluorescence histograms of unstimulated and stimulated cells after a 30-min incubation at 37°C. The fluorescence histograms represent the amount of H_2O_2 (proportional to the amount of green fluorescence) produced by the cell, as described above.

Before the development of the DCF assay for flow cytometry, accurate estimations of intracellular H_2O_2 were very difficult. Several laboratories, including our own, have further developed the assay of Bass et al. for use with micro-quantities of blood. This is particularly valuable for the evaluation of cell function in experimental models using small animals (mice and rats) and also for pediatric evaluations of cell-function studies. In this respect, several innovative developments have been reported whereby several functions can be determined in μ-quantities of whole blood. Trinkle et al. (199) have used a combination of DCF, phagocytosis, and killing in a few hundred μ-liters of blood. This assay is rapid and comprehensive and would be particularly useful

for pediatric patients. The major disadvantage of the procedure however, is the relatively complicated setup required for the flow cytometry since dual-laser excitation is necessary.

Superoxide–Nitroblue tetrazolium (NBT) reduction has been measured flow cytometrically by Blair et al. (200). The method is a variation of a traditional method using a microscope and a glass slide. The presence of oxidative burst enzymes in HL-60 (human leukemia) cells has been demonstrated using NBT using a series of experiments whereby simultaneous measurement of NBT reduction, cell cycle phase, and phagocytosis were made (200, 201). Because of the problems of interpretation of this technique, it is not widely used in the routine clinical laboratory as a flow cytometric assay.

Clinical Evaluation

Chronic granulomatous disease (CGD) is an inherited disorder in which phagocytes have a defective oxidative metabolism and an inability to produce hydrogen peroxide. The clinical picture usually starts with staphylococcal dermatitis and enlarged lymph nodes (202). Pulmonary changes are prominent with bronchopneumonia, hilar lymphadenopathy, and lung abscess formation (202, 203). The inflammatory reactions are usually excessive and often develop into granuloma formation despite appropriate antibiotic treatment. Infections usually begin in infancy or early childhood, however several reports describe the neutrophil abnormality discovered in adults (204–206). The disease is probably a family of diseases with similar clinical manifestations and different defective enzyme systems. Various enzyme deficiencies that have been associated with CGD include cytochrome b_{558} (207–211), glucose-6-phosphate dehydrogenase (212, 213), flavoprotein (214), and a defect in protein or protein phosphorylation p47 or p67 (211, 215). Neonates can also have defective bactericidal activity (216), which may be related to impaired production of the hydroxyl radical from superoxide (67, 216).

Neutrophils isolated from CGD patients fail to produce any fluorescence in the DCF assay. It is important to be able to determine that there is a significant difference between a possible CGD and a normal. Since availability of CGD cells is probably unlikely in most clinical laboratories, the usual procedure is to utilize both stimulated and unstimulated normal cells. The unstimulated normal can serve as a satisfactory control for a possible CGD patient. Thus, by always running an unstimulated normal with a stimulated normal any defect or partial defect should be identified. Several reports demonstrate the detection of possible carriers of CGD using non-flow-cytometry (217) and flow-cytometry-based methods (194, 195). These reports demonstrate the use of the DCF assay whereby neutrophils from heterozygous carriers of CGD produced histograms midway between the unstimulated and stimulated controls (195).

Nonoxidative Bactericidal Mechanisms

Neutrophil cytoplasmic granules contain a variety of different enzymes that do not require oxidative metabolism. These enzymes are released into phagolysosomes and include proteases, such as cathepsin D,E, and G (218), and elastase (219); hydrolytic enzymes, such as phospholipase A_2 (220) and lysozyme (221); bacterial permeability increasing protein (222, 223); lactoferrin (218); and defensins (41, 42).

Cathepsin G, also known as chymotrypsin-like cationic protein, is present in azurophilic granules and has both microbicidal and cytotoxic properties (224, 225). Cathepsin G is a protease that inhibits bacterial oxygen consumption and has microbicidal activity that does not depend on a primary proteolytic attack (226–228).

Elastase is another azurophilic granule component that is important for the degradation of the structural protein elastin (219). Elastase is also capable of degrading bacterial cell wall protein (229) and of potentiating the activity of cathepsin G (230) as well as the lytic activity of lysozyme (229). Elastase is cytotoxic to endothelial cells in culture and may be associated with neutrophil-mediated lung injury in emphysema (231, 232).

Lysozyme is present in both azurophilic and specific granules and acts by hydrolysis of the bacterial cell wall (233). The hydrolytic activity is directed at the B-1-4 glycosidic bond between N-acetylglucosamine and N-acetylmuramic acid and thus is only effective against selected gram-positive bacteria (228). Lysozyme is capable of killing gram-negative bacteria if the bacteria are first acted on by toxic oxygen products that damage the protective lipid envelope (234).

Bacterial-permeability-increasing protein (BPI) is a cationic protein present in azurophilic granules. BPI contributes to the ability of neutrophils to kill gram-negative bacteria, especially *E. coli* (222, 235, 236). BPI binds to and permeabilizes the bacterial envelope (237). Elsbach and Weiss proposed an initial ionic interaction that eventually activates bacterial phospholipases (235). Neutrophilic phospholipase A_2 in specific granules also increases bacterial envelope permeability and exerts a potent bactericidal effect (220). Another cationic protein with optimal activity at low pH is 37kD cationic protein. It is similar to BPI and is active against several gram-negative bacteria (238).

Lactoferrin is a glycoprotein from specific granules with binding sites for ferric iron (239, 240). Lactoferrin is a member of the iron-binding transferrin family and can also be found in tears, semen, and human milk (241). Lactoferrin exhibits bacteriostatic activity against gram-negative and gram-positive bacteria due to its ability to chelate iron (242, 243). Bactericidal activity of lactoferrin has also been reported against both gram-positive and gram-negative bacteria (244). Lactoferrin deficiency due to specific granule deficiency has been reported in human patients with recurrent infections (223).

Defensins are another group of cationic proteins and are the major protein constituent of azurophilic granules. Defensins have a broad spectrum of activity in vitro against gram-positive and gram-negative bacteria, fungi, and certain enveloped viruses (41, 44, 245–247). Defensins have also demonstrated cytotoxicity of mammalian cells in culture (248).

Despite countless studies and many different probes, a clear consensus about the microenvironment of the phagolysosome is lacking. Studies with fluorescent pH probes indicate the pH rises to 8.0 within a few minutes of phagolysosome formation (249). Within 15 minutes, the pH is neutral and then continues to decrease to 5.5–6.0 within 1–2 hours (249). A lower pH in phagolysosomes of thermal injury patients may contribute to reduced neutrophil intracellular killing due to a lack of initial alkalinization (250).

Due to the complexity of the phagolysosome environment, it is often difficult to separate oxygen-dependent mechanisms from oxygen-independent mechanisms. Considerable synergy also exists between the two mechanisms, which further hinders investigations to differentiate their effects.

Adhesion, Binding to Neutrophil Receptors

Integrins are a superfamily of heterodimeric, transmembrane glycoproteins that act as receptors on leukocyte surfaces (251) promoting several important cellular functions, such as adhesion of phagocytes to surfaces, phagocytosis, and diapedesis. Several related molecules have been identified notably CD11a (LFA-1), CD11b (also called Mol, OKM-1, CR3, Mac-1), and CD11c (p150,95) (252, 253). The three heterodimers share a common B-unit of 95 kDa which is known as CD18. Each has distinct α-subunits of 177 kDa (LFA-1, CD11a); 165 kDa, (Mac-1, CD11b), and 150 kDa (p150,95, CD11c). The primary role of these molecules is in adhesion-dependent functions and therefore they are found on lymphocytes and monocytes as well as neutrophils. The importance of integrins in normal immune function has been demonstrated in patients with deficiencies in mRNA for CD18 (154). Several reviews of the structure and function of this family of molecules (254, 255) as well as of the related clinical deficiency syndromes (256) have been written.

While the overall function of integrins is to regulate granulocyte diapedesis and migration into inflammatory sites, the mechanism of action is less certain. It is hypothesized that a primary role for the CD11/CD18 complex on phagocytes is the regulation of secretion of toxic oxygen mediators, enzymes, and other secreted products of activation. Thus, if the CD11/CD18 complex is involved in the regulation of these cells, it may be one of the mechanisms that only allows for the secretory response in appropriate circumstances such as adhered or aggregated neutrophils.
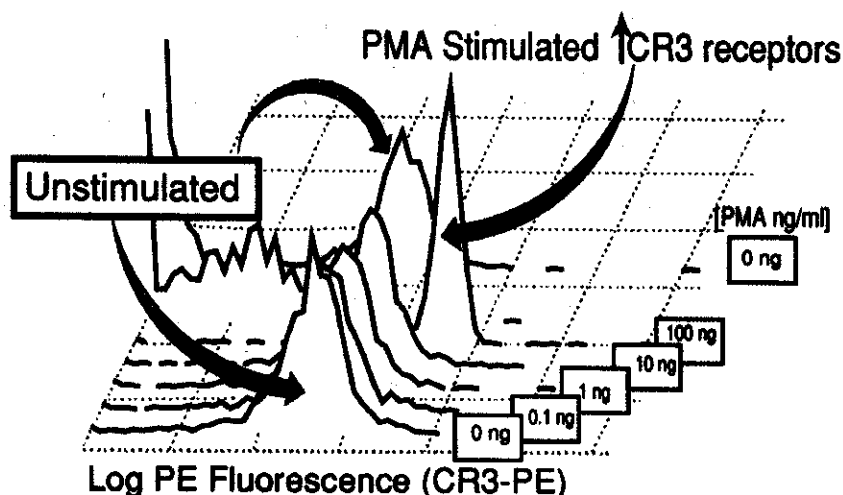
Figure 24.10. Neutrophils stimulated by *PMA* demonstrate an increase in the number of CD11b (*CR3*) receptors as shown in this figure. Shown is a dose response of *PMA* and the resultant increase in the fluorescence from CR3-FITC-labeled antibody bound to the cells.

The CD11/CD18 complex is not essential for neutrophil activation by lipopolysaccharide (LPS) since CD18-deficient patients show normal priming for enhanced release of superoxide anion in response to LPS (257). However, antibodies to CD18 were capable of inducing a defect similar to that described in children with a genetic deficiency of the CD11/CD18 integrins (258). Another report describes profound chemotactic defects in patients suffering from dysmyelopoietic syndromes (DMPS), refractory anemia with excessive blasts (RAEB), and acute nonlymphoblastic leukemia (ANLL). However, in all of the cases reported, no abnormality was detected by flow cytometry for granulocyte integrins (259). Thus, the chemotactic defects observed were unrelated to the integrins in these patients.

Stimulation of neutrophils with PMA directly down-regulated the CD11/CD18 independent mechanism of neutrophil adherence to interleukin-1 (IL-1), tumor necrosis factor (TNF), or LPS pretreated human endothelial cells (260). IL-1 stimulated PMN-endothelial adhesion (261) but IL-1 did not alter PMN degranulation or chemotaxis (262).

CD11b—(CR3; Mac-1) Expression

This receptor is the primary glycoprotein associated with cell adhesion found on neutrophils and regulates the adherence and phagocytosis of particles opsonized with C3bi. Arnaout et al. (263) demonstrated the activation of neutrophils (via increased CD11b expression) by complement fragments generated by new hemodialysis membranes.

CD11b (CR3) has been shown to be critical in the phagocytosis of *S. aureus* and *E. coli*. In one study, *E. coli* enhanced CD11b expression despite not being phagocytosed. This study further demonstrated an initial fall in CD11b expression despite not being phagocytosed. This study further demonstrated an initial fall in CD11b after addition of bacteria to the neutrophils, but a subsequent enhancement of expression after 5 to 10 min (264). In terms of oxidative response, opsonized *E. coli* and *S. aureus* stimulated H_2O_2 production measured by flow cytometry. Nonopsonised *E. coli* did not stimulate H_2O_2 production despite an increase in CD11b. As an example, Figure 24.10 shows the flow cytometric histograms of neutrophil CD11b expression before and after stimulation by PMA (0.1–100 ng/ml). The increased fluorescence indicates increased expression of the CD11b receptor on the neutrophil surface (above normal expression found on unstimulated neutrophils).

Absence of the B-subunits will cause a profound and debilitating deficiency in neutrophil function. An inherited syndrome known as Leukocyte Adhesion Deficiency exists where none of the B-subunits are synthesized (149, 153, 154, 265) (described above). Absence of these adhesion glycoproteins will prevent the chemotaxis and subsequent diapedesis of neutrophils at inflammatory foci. Partial or incomplete B-subunit deficiencies result in less severe functional defects. Several chemotactic stimuli including C5a, LTB₄, and fMLP have been shown to rapidly increase the number of CD11b receptors on neutrophils (266, 267) and, in particular, activation of C5a increases the adhesive characteristic of the neutrophil to the capillary endothelium. Storage pools of CD11b receptors are contained in neutrophils, most likely in granule membranes (50, 268).

Other Functional Neutrophil Receptors

A recent report has described an alteration in the number of CD16-positive neutrophils in HIV-I-infected individuals (269). CD16 (Fc$_\gamma$RIII) is a relatively late differentiation antigen on neutrophils, natural killer (NK) cells, and a subset of T-cells, and is a low-affinity receptor for IgG (270, 271). Fc$_\gamma$RIII is anchored to the plasma membrane of neutrophils via a phosphatidyl inositol glycan moiety (272, 273) that can be released by chemotactic activation (274). One consistent finding in HIV-infected patients is the frequent observation of neutrophil defects in terms of chemotaxis, phagocytosis,

microbicidal killing, and respiratory burst activity (275–277). Other Fc receptors such as Fc$_\gamma$I (CD64) on monocytes and neutrophils and Fc$_\gamma$II (CD32) found on monocytes, neutrophils, eosinophils, basophils, B-cells, and platelets are also important. Some studies have demonstrated that CD32, but not CD64, can trigger activation of the respiratory burst of neutrophils (278–280), whereas others have shown that the glycosylphosphatidylinositol-linked CD16 receptor can also trigger respiratory burst activity (281). Upregulation of CD64 by γ-interferon on neutrophils results in cells that can be activated to trigger an oxidative burst through that receptor (282). Thus, with the possible therapeutic use of γ-interferon for CGD patients, monitoring of upregulation of these receptors on peripheral blood neutrophils may become important. A recent flow cytometry method for quantitation of receptor numbers in peripheral blood neutrophils has been reported (283). Thus, the evaluation of neutrophils, including CD16 receptor expression, may be useful in certain patient groups. An excellent review of Fc receptors has recently been published by van de Winkel and Anderson (284).

Other Neutrophil Dysfunctions

A recent study by Lawton et al. (285) demonstrates the effectiveness of preparations of human immunoglobulin for intravenous use in stimulating oxidative burst and chemiluminescence of isolated human neutrophils. These studies suggest there may be a beneficial therapeutic effect in severe, life-threatening infections. Clear evidence is shown as to the stimulation of oxidative burst but no consistent information can be derived from studies of chemotaxis. The mechanism of action is probably related to the passive immunization with preformed specific antibody. However, there is little doubt that this study demonstrates significant stimulation of neutrophil function directly by the intravenous immunoglobulin. Another recent study has confirmed the above observations of stimulation of oxidative burst in the presence of intravenous immunoglobulin (286). These authors have suggested that inflammatory reactions observed occasionally during infusions in hypogammaglobulinemic patients may well be related to neutrophil activation.

Clinical Prediction by Flow Cytometry

Several studies using flow cytometry have proved of value in the evaluation of trauma. A major study by Valet demonstrates that a multiparametric-multifunctional analysis of neutrophils could accurately predict the course of disease three days in advance of the clinical manifestation of pulmonary or cardiovascular organ failure in 92% of samples from 47 patients (60). Tests used in this study included measurements of phagocytosis and degradation, cell volume, intracellular pH, and esterase activity. A significant value to this study is the use of a small bench type mercury arc-based flow cytometer. Further, studies such as these demonstrate

the depth of knowledge that can be gained from a small number of cells when using a multiparametric approach.

Neonatal Infections and Neutrophil Function

An excellent review of the role of complement deficiencies and related neutrophil function abnormalities in neonates has been written by Berger (287). Important considerations in neonates are the combined effects of minor abnormalities of cellular function. Reduced levels of C3, low specific antibody titers, reduced mobility, or reduced numbers of neutrophils can combine to form a critical immune deficiency in these patients. Adhesion glycoproteins are present in significantly reduced numbers on stimulated neonate neutrophils compared to adult neutrophils (288, 289). Several studies have demonstrated an indication for granulocyte transfusions in septic neonatal patients (290–292).

Antibodies against Neutrophils

The normal physiologic removal or destruction of neutrophils occurs constantly; however, accelerated removal can be a result of an antineutrophil antibody. This immune neutropenia might be due to an auto- or alloantibody or may be related to anti-HLA antibodies. In infants, alloantibodies can result from maternal IgG antibodies that cross the placenta, causing severe neonatal neutropenia (293).

Antineutrophil antibody measurements by flow cytometry have become a useful clinical assay (294) because of the relatively small number of neutrophils required and because evaluations can be made of patient sera as well as of antibodies detected on patient neutrophils. The flow cytometric technique is well-suited for children with neutropenia since evaluations can be made using only 1–2 ml of blood. Usually, there are sufficient cells for a simple flow cytometric H$_2$O$_2$ screen (using the DCF assay described above) to be carried out on these neutrophils also.

Measurement of Neutrophil Antibodies

One important aspect in performing the antineutrophil assay is in the selection of the normal control neutrophils or neutrophil pool. Because of the possibility of damage to neutrophil surface antigens, the best method for preparation of neutrophils for this assay has proven to be one of the simplest and has already been described above (overlay method). Two washes in buffer remove serum proteins and platelets leaving a leukocyte-rich suspension that is highly suitable for evaluation of antineutrophil antibodies. The various cell populations can easily be light-scatter gated on the flow cytometer. Evaluation of histograms from patient and control sera can determine the presence or absence of antineutrophil antibodies (usually IgG). The histograms shown in Figure 24.11 are indicative of the presence of such antibodies in patient sera. Patient neutrophils can also be evaluated for the presence of surface-bound antibody if sufficient neutrophils can be obtained from the patient.
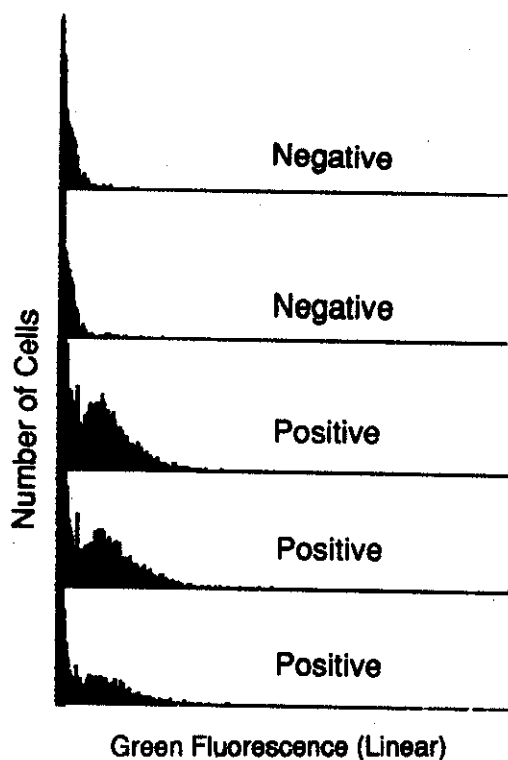
Figure 24.11. The presence of antineutrophil antibodies in patient sera is demonstrated by the increasing fluorescence of gated neutrophils. Control-negative sera demonstrate no fluorescence while the positive control sera are strongly positive.

Antineutrophil Cytoplasmic Antibodies (ANCA)

The presence of antineutrophil cytoplasmic antibodies has been reported in a number of vascular diseases, including uveitis (295) and glomerulonephritis (296, 297). Association of high titers of ANCA is consistent with the diagnosis of Wegener's granulomatosis and several reports have demonstrated positive correlations (295–298). ANCA have also been shown to stimulate oxidative bursts and degranulation in normal neutrophils, effects that were more pronounced after priming with TNF (299). The same study was able to demonstrate the presence of myeloperoxidase on the surface of neutrophils, which was interpreted as indicating that neutrophils have ANCA antigens on their surfaces to interact with ANCA (299). Measurement of ANCA by flow cytometry has been demonstrated (299a) and, in association with immunofluorescence. such measurements may have significant clinical utility.

CONCLUSION

The evaluation of neutrophil function can provide useful information on the capability of immune cells to perform normal operations. This information cannot be achieved by determinations of numbers of neutrophils, or even the presence of neutrophils, at a particular site. Specific defects of neutrophil function can become important, particularly when secondary to other underlying disease. The neutrophil interac-

tion with other cells, particularly endothelial cells, is becoming better defined at the present time. It is clear that the neutrophil is not an isolated, suicidal cell with little effect on other cells, tissues, or organs. It is these relationships that are currently being studied using tools such as specific monoclonal antibodies, a variety of valuable fluorescent dyes, and technologies such as flow cytometry. While many of the studies involving neutrophil function are of a research nature, there are several important evaluations of clinical importance. The neutrophil is the most prolifically produced cell in the human immune system. Future developments in understanding the relationships between neutrophils and other cells will be an important determining factor in the utility of neutrophil function methods for clinical use.

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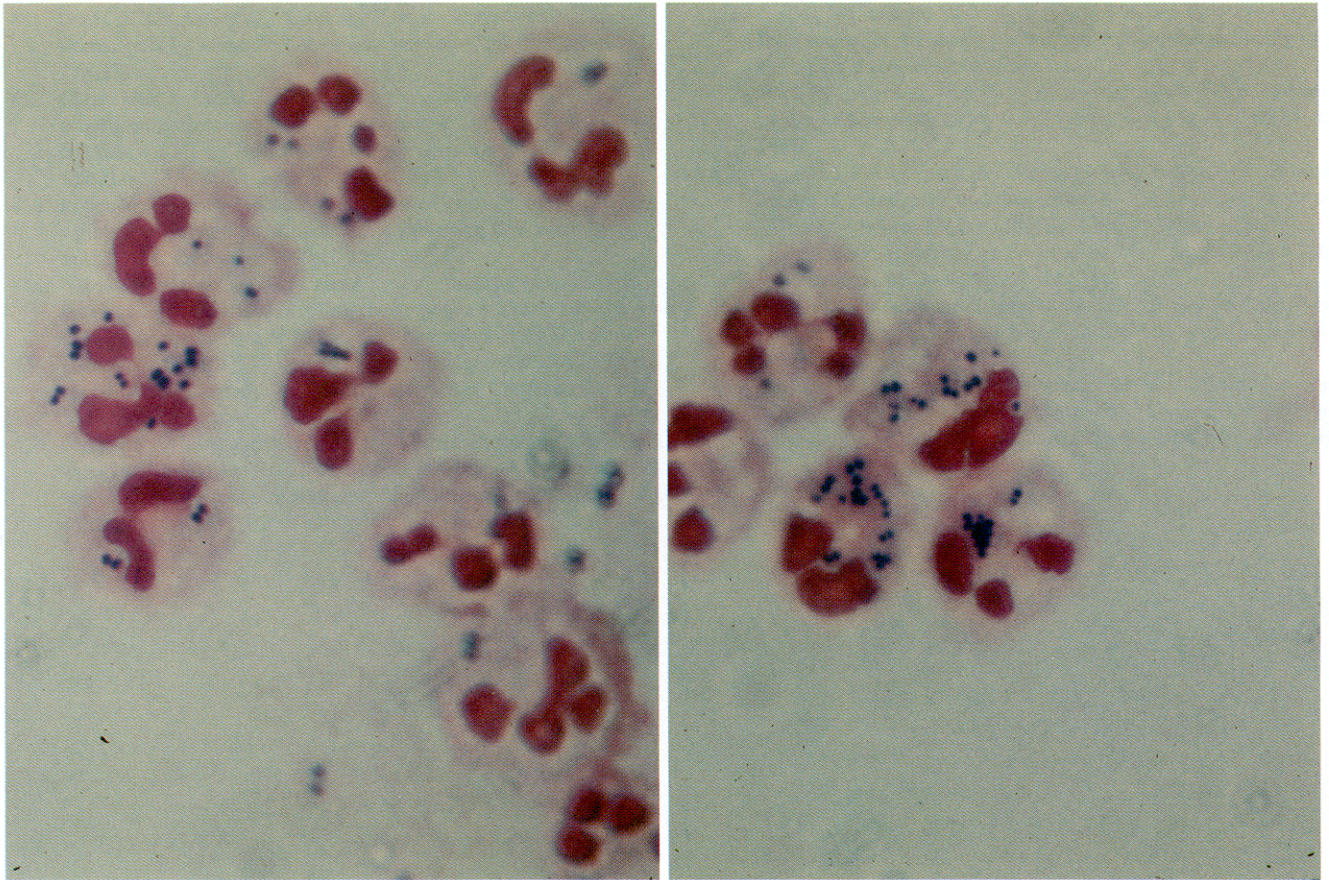
COLOR PLATE II

Figure 24.8. Neutrophils containing *S. aureus* (stained dark blue) after a 30-min incubation. Present are several neutrophils containing the organisms as well as some free organisms (Wright stain).

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