Functional Assays by Flow Cytometry

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Cell function assays have been redefined over the past several years, largely because of technologies such as flow cytometry. The major difference between other techniques and those proposed using flow cytometry is the ability to analyze viable, single cells in a rapid and quantitative fashion. One of the advantages of flow cytometry methods lies in the ability to obtain population information. This means that what may appear to be a homogeneous population using conventional bulk assays may be demonstrated to be multiple populations by flow cytometry.

This chapter deals primarily with phagocytes—macrophages and polymorphonuclear cells. Many factors can cause alterations in either the number or function of phagocytic cells. Among these factors are a variety of drug interactions. For example, corticosteroids commonly cause a significant increase in the number of circulating leukocytes (primarily the result of accelerated neutrophil release from bone marrow stores), together with a decrease in neutrophil adherence to vascular endothelium, migration of cells out of the vasculature, and a slight increase in the neutrophil circulating half-life. Neutrophils are normally stored in the bone marrow for 5 to 7 days before being released into the blood. Granulocyte colony-stimulating factor and interleukin 1 play key roles in this release of neutrophils from the marrow. Neutrophils in the circulating pool have a half-life of about 7 h, after which they marginate and emigrate through tissue, where they remain functional for 1 to 2 days. The removal of neutrophils, irrespective of their state of activation, is primarily via phagocytosis by macrophages or by disposal through mucosal surfaces. Because neutrophils are short-lived reactive cells present in very high numbers, minor alterations in their function can have profound effects in microenvironments. The purpose of this chapter is to define some of the procedures that can be used to evaluate the function of neutrophils or macrophages by flow cytometry.

PHAGOCYTOSIS

Phagocytosis proceeds in several distinct stages. The first is recognition, which is followed by attachment or particle binding and, finally, ingestion. Without proper attachment of the microbe, the last stage will not take place. Abnormal phagocytosis can be due to a failure in the opsonization process or a defect in the ingestion capability of the phagocyte. The main cell surface receptors mediating phagocytosis are C3b (CR1) and FcR (Fc portion of immunoglobulin G). Phagocytosis of bacteria has been well characterized by Bassoe and Bjerknes using flow cytometry (4, 5). Two advantages of flow cytometry over other methods are the relatively small number of cells required and the significantly fewer preparative procedures for isolating leukocytes. These are especially important in the evaluation of pediatric patients.

The principle underlying studies of phagocytosis is that bacteria can be labeled with fluorescein isothiocyanate (FITC) and then phagocytosed by macrophages or neutrophils. Following this, a second dye, ethidium bromide (EB), which binds DNA, is added. The EB will stain only nonphagocytosed bacteria; internalized organisms are protected by the intact phagocyte membrane and are not exposed to the dye.

On the flow cytometer, light scatter can be used to isolate the neutrophil or macrophage populations. A combination of fluorescence versus scatter will identify labeled organisms, phagocytes, and phagocytes containing ingested organisms.

There are a number of protocols for performing phagocytosis assays and an almost endless number of particles to ingest. One simple and straightforward method that can easily be performed by most benchtop instruments uses killed, fluorescein-labeled *Candida albicans* as the target for ingestion; phagocytosis is completely inhibited in a control tube by the presence of cytochalasin B (5 μg/ml). After phagocytosis is complete, EB (50 μg/ml) is added to label the external organisms. On excitation at 488 nm, the internalized bacteria fluoresce green (fluorescein), while the surface-attached organisms are red (10). This occurs because of the phenomenon of resonance energy transfer between FITC and EB. The EB stains the nuclear material of the external bacteria; because these organisms are also labeled with FITC, the FITC excites the EB, resulting in red fluorescence. Internalized organisms are not exposed to EB and so cannot emit red fluorescence. While phagocytosis assays with C. *albicans* can be very informative, a more common method uses bacteria such as *Staphylococcus aureus* to measure predominantly neutrophil phagocytosis; this assay is described below.

Procedure

To prepare the bacteria, culture overnight on blood agar, and subculture to obtain discrete colonies which are then

cultured on brain heart infusion agar slopes. Transfer several colonies into culture broth, and culture overnight. Wash the bacteria in Hanks balanced salt solution (HBSS), add 0.01 mg of FITC in 500 μl of carbonate-bicarbonate buffer to the pellet, and rotate for 30 min at 37°C to incubate. Centrifuge the bacterial slurry in a high-speed benchtop centrifuge at 10,000 **X** g for 10 min, and wash the pellet at least two times with phosphate-buffered saline (PBS). Kill the bacteria by heating at 60°C for 60 min. Wash the bacterial slurry once more in PBS, and set the concentration so that the optical density of the suspension is approximately 0.35 at 620 nm. For future assays, aliquot 1 ml of bacteria into cryovials, add a drop of sterile glycerol to each vial, and freeze at 70°C. Before each assay, the thawed suspension must be gently sonicated for use. In addition, the bacteria must be opsonized by incubating 1 ml of bacteria with **4** ml of serum (or a 1: 2 dilution of serum in PBS) for 15 min at 37°C with rotation.

Prepare the neutrophils as follows. Collect 10 ml of fresh heparinized blood; do not use EDTA or citrate, because they markedly reduce phagocytosis. Dilute the blood with 20 ml of PBS, and overlay this 30 ml of diluted blood on 15 ml of Ficoll-Hypaque. Centrifuge for 30 min at 400 **X** g at room temperature. Discard all layers above the erythrocyte layer, and add 3 ml of 3% Dextran T500 (Pharmacia) in PBS to the tube together with 15 ml of warm (37°C) PBS. Mix and place the tubes in a 37°C water bath for 45 min to sediment the erythrocytes. Collect the buffy coat, which contains all the neutrophils. Centrifuge the buffy coat to a pellet (300 **x** g for 10 min), decant the supernatant, and mix the pellet. To lyse the small number of contaminating erythrocytes, add 3 ml of sterile water, mix gently for 20 s, and immediately add excess PBS (at least 30 ml). Centrifuge at 300 **X** g for 10 min, and resuspend in PBS-gel (PBS containing 2 mM disodium EDTA, 5 mM dextrose, and 0.1% gelatin). Count the cells, and adjust the concentration to 2 **X** 10^6/ml. Verify that the cells are predominantly neutrophils (>95%) and are viable (>95%) by trypan blue exclusion or propidium iodide exclusion on the flow cytometer. The cells are now ready for the phagocytosis assay.

For the assay, add 5 ml of neutrophil suspension at 2 **X** 10^6/ml to the bacteria-serum tube. Immediately remove 1 ml into a tube (12 by 75 mm) containing 1 ml of ice--cold 0.9% saline with 0.02% EDTA, and place the tube on ice. This is the zero control tube. Repeat this procedure every 15 min with a freshly withdrawn aliquot. This provides a survey of the kinetics of phagocytosis over a 1-h period.

Ingestion of bacteria is determined by measuring green fluorescence at 525 nm on the flow cytometer, using 488 nm excitation. To estimate extracellular fluorescence, immediately after running each tube add 1 ml of 3-mg/ml trypan blue, mix, and measure the same fluorescence signals again. Quenched fluorescence indicates extracellular organisms, since trypan blue is unable to penetrate the cell within the 2 to 5 min required to perform the measurement. It can safely be assumed that if the fluorescence is reduced, some organisms are attached to the outside of the cells.

The optimum ratio of bacteria to leukocytes is 20 : 1. This ratio can be achieved by adding different concentrations of bacteria to a fixed concentration of leukocytes, counting a standard number of leukocytes, and then estimating the number of bacteria from the fluorescence histogram of forward light scatter versus green fluorescence.

Phagocytosis kits are commercially available; they are very easy to use and very reproducible.

Clinical Considerations

Abnormal phagocytic function has been associated with a variety of clinical disorders, both inborn and acquired. The problem can be either with the neutrophil itself or with an opsonization or complement defect. Immature neutrophils released prematurely from the bone marrow show defective phagocytosis that may be related to a high negative surface charge. Abnormal phagocytosis has also been identified in the neonate and in juvenile periodontitis. Neutrophil phagocytic ability is enhanced by tuftsin, a tetrapeptide produced by the spleen. A deficiency of tuftsin is a familial disorder or can be acquired as a consequence of splenectomy and may result in increased susceptibility to infection due to defective neutrophil phagocytosis (20). Clinical findings include respiratory infections such as bronchitis and pneumonia and enlarged fluctuant lymph nodes (20). Actin polymerization defects may interfere with the phagocytic process, because normal actin polymerization and microfilament function are also necessary for phagocytosis. Complement receptor C3bi deficiency can also result in altered phagocytosis.

OXIDATIVE METABOLISM

Flow cytometry is uniquely suited for the measurement of oxidative burst in neutrophils, The major advantages of flow cytometry over more conventional techniques are that only very small volumes of blood are required for the assays and the results are objective and quantifiable. It is possible to perform such assays using as few as 5,000 cells per tube; however, the degree of difficulty also increases under these conditions. Using this technique to measure the oxidative burst in blood samples from neonates and small children is a very practical application of the method.

A terminal acceptor for electrons in oxidative phosphorylation, oxygen plays a crucial part in the reactions after neutrophil activation. A variety of reactive oxygen species, including O_2^-, H_2O_2, and OH- and its higher--energy singlet states, are formed very rapidly. Intracellular sources of reactive oxygen species in neutrophils include mitochondrial oxidation, the microsomal cytochrome P-450 system, and plasma membrane NADPH oxidases. The initial defense system against bacterial insult is afforded by modulating tissue concentrations of superoxide dismutase, catalase, glutathione (GPX) peroxidase, cytochrome c oxidase, glutathione (GSH), ascorbate (vitamin C), α-tocopherol (vitamin E), p-carotene (vitamin A), and polyunsaturated fatty acids. Uncoupling of the mitochondrial electron transport system of neutrophils leads to increased free radical generation, as can be demonstrated by the addition of cyanide, an inhibitor of cytochrome c oxidase, and diphenyliodonium, an inhibitor of the plasma membrane NADPH oxidase (14). Electron transport throughout the mitochondrial respiratory chain accounts for the bulk of O_2 consumption in resting neutrophils. Therefore, the ability to measure intracellular activity in some or all of these reactive stages of neutrophil activation can provide valuable insight into the cells', and ultimately the body's, capacity to respond in the inflammatory response.

HYDROGEN PEROXIDE PRODUCTION

The H_2O_2 production assay using flow cytometry has been available for many years (3), during which time it has been continually refined. The principle of the assay is that a

probe, the nonfluorescent molecule 2′,7′-dichlorofluorescin diacetate (DCFH-DA), is loaded into the cells to provide a readily oxidizable substrate. DCFH-DA is lipophilic and easily crosses cell membranes. Inside the cell, cytosolic enzymes (esterases) deacetylate the DCFH-DA to form polar, nonfluorescent 2′,7′-dichlorofluorescin (DCFH) which, due to its polarity, is trapped either within the cytoplasm or in myeloperoxidase (MPO)-positive intracellular granules. The oxidative potentials of H_2O_2 together with peroxidases are able to oxidize the trapped DCFH to 2′,7′-dichlorofluorescein (DCF), whose green fluorescence at 525 nm is easily measurable on the flow cytometer in a manner similar to the detection of fluorescein. The amount of DCF formed is proportional to the cellular oxidant production. The fluorescence intensity becomes a measure of the oxidants produced by the cells, in particular, H_2O_2. The technique is well accepted. Investigators have utilized this fluorochrome to measure intracellular H_2O_2 in HL-60 cells, endothelial cells, chondrocytes, renal epithelial cells, and neurons, as well as neutrophils and monocytes/macrophages (3, 22, 24, 25). Since this probe can be pumped out of the cell as well, it is unsuitable for use with some cells having very active P-glycoprotein systems. In any case, it is imperative to ensure that a nonstimulated control is available for comparison with stimulated cells.

Although the original measurements were shown to be H_2O_2 related, recent evidence indicates that the DCFH probe is not specific for H_2O_2; if nitric oxide is present, that will oxidize the DCFH to DCF as well, and this must be taken into consideration when performing the assay. However, it is reasonable to say that under normal stimulatory conditions of neutrophil activation, in the absence of the $NO\cdot$ substrate L-arginine, the great majority of the oxidation measured is H_2O_2 related. Specific conditions can be employed to shift the system toward $NO\cdot$ production, and together with appropriate metabolic blockers the assay can be employed to measure $NO\cdot$.

Procedure

Preparation of cells for this assay is relatively simple. Either whole blood or purified cell suspensions can be used. The most straightforward method is to use whole blood and lyse the erythrocytes. Obtain 5 to 10 ml of heparinized blood (use preservative-free heparin), although the assay can be done with far less if necessary. Put 3 ml of heparinized whole blood in a 50-ml conical tube, and add 47 ml of lysing solution consisting of 150 mM NH_4Cl, 10 mM $NaHCO_3$, and 1 mM EDTA. Rotate the tubes for 10 min at room temperature on a bench rocker, and then centrifuge at 400 × g for 10 min at 4°C. Decant the supernatant, and resuspend the pellet by drawing the tube gently across a test tube rack. Wash the cells with HBSS, centrifuge at 300 × g for 10 min at 4°C, and resuspend in 4 ml of HBSS. Count the cells, and adjust the concentration to 2.0×10^6 cells per ml.

To incorporate the probe into the cells, incubate the cell suspension for 15 min at 37°C with DCFH-DA (20 μM, final concentration). It is not necessary to wash the cells after loading. Some cells should be left unloaded to serve as negative controls. It is a good practice to load only the volume of cells required for the immediate assay. However, it is also important to bulk load the cells for a particular assay if at all possible. For example, if 15 to 20 tubes will be used for one assay, 3 to 4 ml of cells might be loaded with DCFH-DA and the cells dispensed after the 15-min loading time. Subsequent assays can be performed by loading cell suspensions 15 min in advance of the assay. The unloaded cell suspension should remain on ice at 4°C. The assay is designed to measure the rate of change and also the final activation state of the cell suspension. This is achieved by one of two means: either stimulating a bulk population and removing small aliquots for flow measurement at regular intervals or stimulating a series of tubes and taking periodic measurements for each tube at 10-min intervals over a total of 45 min. Phorbol myristate acetate (PMA, 10 to 100 ng/ml) stimulation can be used as a positive control to measure the maximal possible stimulation for human neutrophils. As shown in Fig. 1, there is a significant shift in green fluorescence intensity between the unstimulated and the stimulated cells. This shift can be as little as a few channels to more than a 10-fold increase in fluorescence. It is therefore necessary to take care in setting the unstimulated control fluorescence channel number so that a significant increase will not drive the fluorescence signal into the uppermost channel, making quantitation impossible. An alternative is to collect log fluorescence signals. Obtaining more specific information regarding the status of the cells is not generally necessary in the initial clinical situation. If a defect is detected, a more detailed analysis can be attained by using a variety of metabolic blockers.

O_2^- PRODUCTION

An assay can be performed to detect superoxide anion in a manner similar to that used for H_2O_2 measurements. There are some reports of flow cytometry-based measurements of intracellular superoxide anion (O_2^-) production in stimulated neutrophils using hydroethidine (HE) (7, 23, 24). In conjunction with DCFH-DA, the HE-based method may provide a more comprehensive assessment of the oxidative burst, or the assay can be performed simply to determine the amount of superoxide formed within the cells.

Procedure

Cells are prepared as described above for the hydrogen peroxide production assay. To incorporate the probe into the cells, incubate the cell suspension with HE (10 μM, final concentration) for 10 min at 37°C. HE is the sodium borohydride-reduced form of EB and was initially developed as a vital dye which would cross intact cell membranes and label DNA. It is possible to monitor HE emission through a 434-nm bandpass filter; however, this requires UV excitation at 353 nm and does not provide clinically useful information. HE freely enters cells, where it can be directly oxidized to EB by O_2^- produced by the cell. The intracellular EB is fluorescent (610 nm) when excited with 488-nm light, so this assay can be performed on any benchtop flow cytometer. After EB is formed, it rapidly intercalates with DNA, and thus the predominant fluorescent structures inside the cell are nuclear. The same assay setup already described for H_2O_2 can be utilized. HE does not "leak out" of cells like DCFH-DA, nor is it necessary to wash the cell suspension prior to stimulation. If the O_2^- and H_2O_2 are to be collected simultaneously, the DCF fluorescence is collected through a 525-nm bandpass filter (standard fluorescein filter).

Clinical Considerations

There are not many clinical situations where a total failure of the respiratory burst exists. One such instance is chronic granulomatous disease, where the neutrophil fails to produce H_2O_2 because of defects in the membrane oxidases required for the reduction of oxygen. It is possible to determine par-

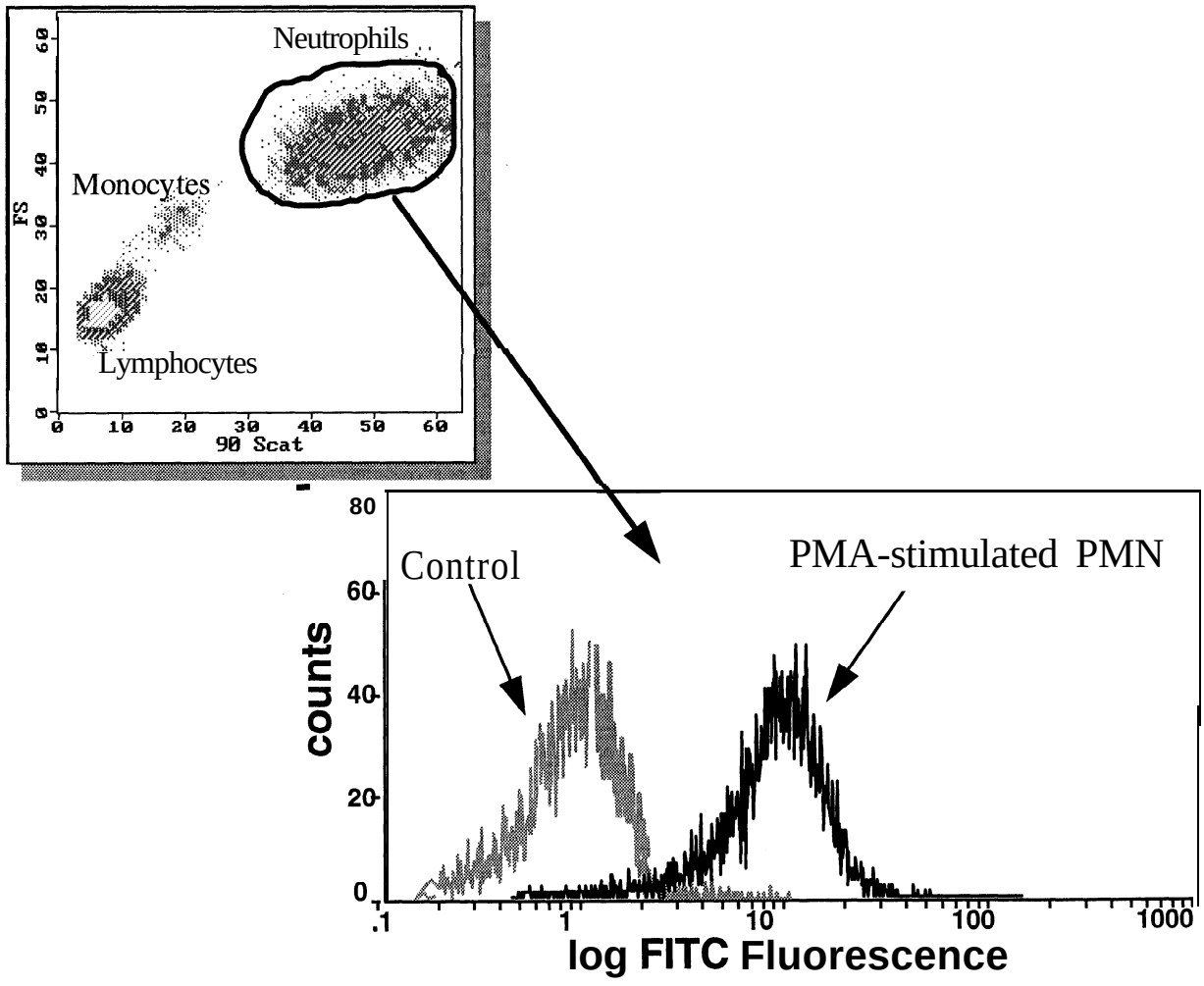

FIGURE 1 Neutrophil function can be determined from unseparated leukocytes by scatter gating. Once loaded with DCFH-DA, neutrophils can be stimulated with PMA **(10** ng/ml) to produce H_2O_2, which converts DCFH to DCF. As shown here, the PMA-stimulated cells are around 10 times brighter than the unstimulated cells. This is normal for human neutrophils after 30 min of stimulation. PMN, polymorphonuclear leukocytes.

tial abnormalities or carrier states by using the DCF assay. It is always vital to run unstimulated controls for this assay, as oxidation of the intracellular DCFH can occur through other oxidants in the system. The advantage of the DCF assay by flow cytometry is that it is possible to detect small populations of abnormal cells, because each cell is measured individually.

GSH LEVELS

It is usually thought that most or all of the toxicity of O_2^- and H_2O_2 involves their conversion into OH·, which is likely to be the major damaging species formed by the Haber-Weiss reaction under biologically relevant conditions (13). Hydroxyl radical can attack DNA to induce strand breaks, damage cellular proteins, and lead to peroxidation of lipids directly or via intermediate radicals with a longer half-life (2). This process is somewhat in balance with antioxidant defenses such as superoxide dismutase, catalase, and GSH. Catalase and the GSH cycle break down H_2O_2 to oxygen and water, preventing further damage to other tissue, including the producing cell. Thus, adequate amounts of GSH are required for protection against these damaging reactants. GSH therefore plays a key role in the antioxidant system.

The GSH cycle is a sequence of reactions by which the degradation of H_2O_2 is coupled to the increased activities of the hexose monophosphate shunt, GSH peroxidase, and GSH reductase. The enzymes of the hexose monophosphate shunt, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, reduce $NADP^+$ to NADPH, and the continued activity of the shunt is dependent upon the reoxidation of NADPH. This is accomplished by the GSH cycle, which is initiated by the GSH peroxidase-catalyzed oxidation of reduced GSH by H_2O_2. The oxidized GSH formed is then reduced by NADPH in the presence of GSH reductase with the subsequent formation of $NADP^+$.

Procedure

GSH content in neutrophils is estimated by using monobromobimane (mBrB) (Molecular Probes, Eugene, Oreg.), which combines with GSH nonenzymatically at low con-

centrations. The predominant transferase found in human cells, πGST, has both a low affinity for, and a low catalytic reactivity with, mBrB. mBrB is more specific for human cells than monochlorobimane, which combines enzymatically with GSH by means of glutathione S-transferase. mBrB reacts specifically with GSH at low concentrations in the first 10 min of reaction time. The stock solution for mBrB is 40 mM in absolute ethanol.

Neutrophils are stained with mBrB (40 μM, final concentration) (16) for 10 min at room temperature and run on the flow cytometer with excitation at 353 to 361 nm and emission at **450** nm. An increase in fluorescence is correlated with GSH content. A replicate sample should be depleted of GSH by treatment with 100 μM *N*-ethylmaleimide to give a measure of nonspecific binding of the mBrB probe. One major advantage of this assay is that it is a quick and simple measure of an important oxidant defense system which can be performed on a very small number of cells. Hydrogen peroxide and/or superoxide production can be determined simultaneously. The obvious disadvantage is the requirement for a UV light source.

DEGRANULATION ASSAYS

Neutrophils contain a large number of granules, including primary granules (MPO, acid hydrolyases, β-glucuronidase, α-mannosidase, lysozyme, neutrophil proteases, and cationic proteins) and secondary granules (lysozyme, lactoferrin, and collagenase). Additional granules known as tertiary granules also contain gelatinase. These cellular granule contents are generally bactericidal but are capable of causing significant tissue damage under inappropriate conditions. Release of granule contents from activated neutrophils is a vital normal function. Several assays can be performed to evaluate degranulation; one such assay measures lactoferrin. In this assay, cells are activated to release bound lactoferrin, fixed to allow penetration of an FITC-conjugated antilactoferrin antibody, fixed again, and run on the cytometer.

Procedure

As previously described, neutrophils are prepared by Ficoll separation followed by dextran sedimentation. The cells must be resuspended in HBSS containing 1% bovine serum albumin (BSA) at a concentration of 1 x 10^6 to 2 x 10^6 per ml. Prior to activation, the cell suspension and all stimulation reagents must be warmed to 37°C. Accurate timing is essential for this assay, as it is designed to provide kinetic data. Put 50 μl of HBSS control or 50 μl of activating agent (formyl-methionyl-leucyl-phenylalanine [fMLP], 1 x 10^{-7} M; PMA, 10 ng/ml) into small microcentrifuge tubes or test tubes (12 by 75 mm) and place them in a 37°C water bath. At zero time and at 10-min intervals, add 450 μl of cell suspension (500 μl of total reaction volume) to the appropriate tubes, mix, and incubate for 10 min at 37°C. To complete the reaction, add 500 μl of 4% paraformaldehyde at the same 10-min intervals, mix, and incubate for 30 min at room temperature. Wash two times at 250 x g for 10 min with ice-cold HBSS-BSA and finally with PBS containing 0.1% BSA. Resuspend the cells in 45 μl of ice-cold PBS-BSA. Add 5 μl of FITC-conjugated lactoferrin antibody (diluted 1: 5 with PBS-BSA), mix, and incubate at 4°C for 30 min. Wash twice in cold PBS-BSA, and resuspend the cells in 500 μl of PBS-BSA. Fix again by adding 160 μl of 4% paraformaldehyde (1%, final concentration) while vortexing, and run on the flow cytometer with excitation at 488 nm and emission at 525 nm. The spectrum should be similar to that of a traditional single-color antibody.

Other possible assays to perform are ones for MPO. These include the use of DCFH-DA described above, as MPO is required for hydrolysis of this probe. Failure of cells to load adequately with DCFH-DA may be related to a lack of MPO.

Clinical Considerations

MPO deficiency of neutrophils can be a complete or partial deficiency of the enzyme from primary granules (9). This autosomal recessive deficiency is relatively common (5 patients in 10,000). Monocytes from patients with MPO deficiency have increased respiratory burst duration with increased production of superoxide, which may partially compensate for the deficiency. The most pronounced expression of MPO deficiency clinically is an increase in susceptibility to and severity of *Candida* infections (2 1).

Congenital specific granule deficiencies have been reported, and some neonates have demonstrated deficiencies in specific granule formation, but the general result is relatively minor bactericidal abnormalities. Cord blood neutrophils have been shown to have reduced lactoferrin concentrations, and severe lactoferrin deficiencies are found in neutrophils from patients with chronic myelogenous leukemia (6).

MEMBRANE POTENTIAL CHANGES

One of the earliest signals of neutrophil activation is changes in membrane potential. This can be demonstrated in neutrophils undergoing a receptor-ligand interaction, where an increased permeability to ions and a subsequent reduction in transmembrane potential can be detected. A specific inhibitor of chymotrypsin-like enzymes has been shown to block the potential change, suggesting that after the ligand-receptor interaction a protease is required for the initial reaction. The carboxycyanine dye DiO-$C_5(3)$ or oxanol dyes reveal a loss of cell-associated fluorescence upon stimulation, indicating cell activation (8). The probe diffuses into the cells, equilibrates with the external medium and, upon stimulation of the cell, is displaced by the increased uptake of ions, resulting in a reduction in cellular fluorescence (depolarization). The depolarization may be reversible, as with fMLP stimulation; after 3 to 4 min, fluorescence intensity returns to its prior level as the membrane repolarizes. Neutrophils incapable of respiratory burst activation demonstrate no shift in fluorescence, suggesting a relationship between alterations in transmembrane potential and the generation of oxygen metabolites. Cells that have aged (24 h) may fail to undergo membrane depolarization although appearing normal in other functions (unpublished observations). This suggests that assays of membrane potential must be performed on fresh cells within 3 to 5 h of blood collection.

Procedure

The cells are prepared as described above by lysing whole blood, washing, and resuspending leukocytes at a concentration between 5 x 10^5 and 1 x 10^6/ml. Before collecting data, it is necessary to determine the optimal voltages on the photomultiplier tube. It is also valuable to collect a fluorescence-versus-time dot plot if possible. The cells are loaded with the DiO-$C_5(3)$ probe (1 μM, final concentration), gently mixed, and incubated for 3 min at 37°C, by which time they should have reached maximal fluorescence. This can easily be checked by measuring the loading kinetics of the dye. Set the fluorescence channel between the central channel and two-thirds of the maximum on a linear scale

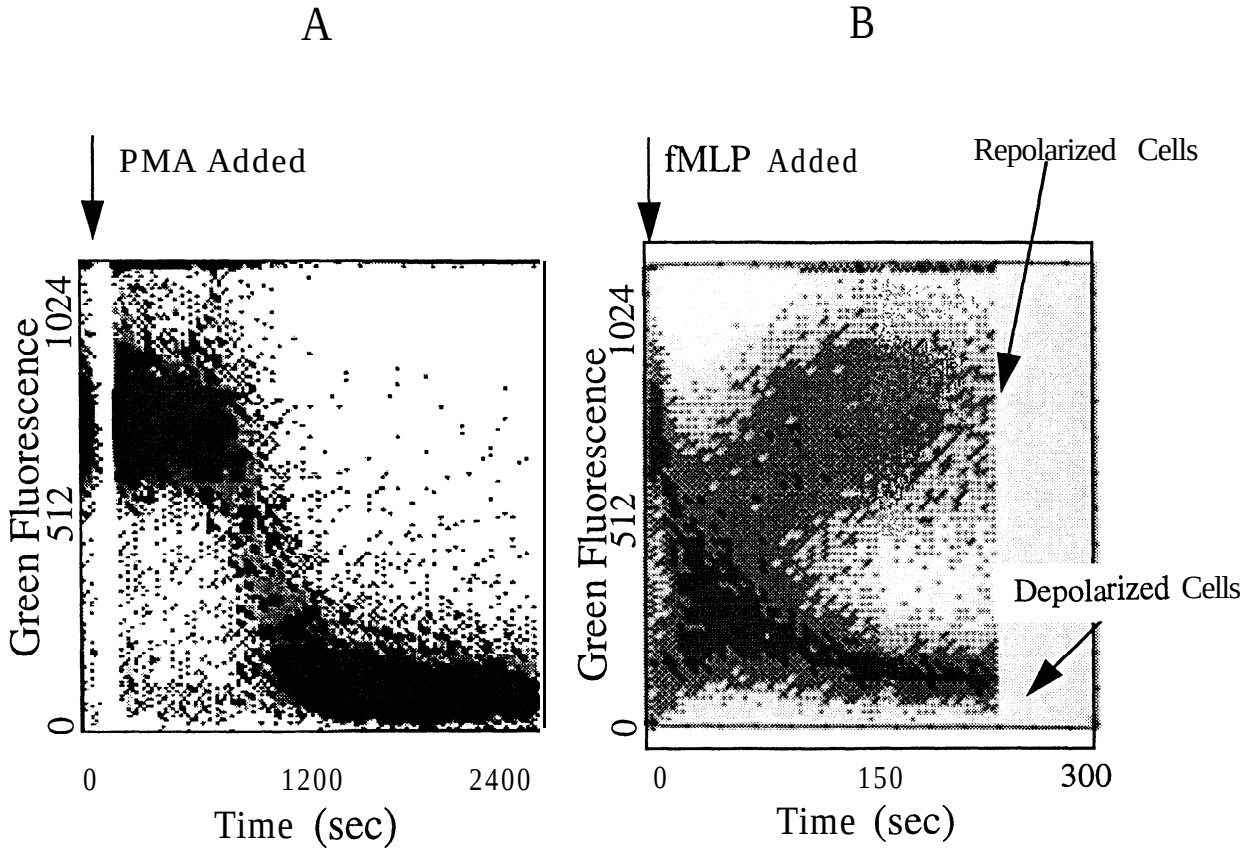

FIGURE 2 (A) This dot plot shows the rapid depolarization of neutrophils loaded with DiO-C_5(3) and stimulated with 100-ng/ml PMA. The assay is run at 37°C, and cells must be collected continuously for at least 2 to 3 min. Neutrophils stimulated with PMA do not normally show a repolarization within several minutes. (B) The same cells stimulated with 10^{-7} M fMLP depolarize and rapidly repolarize. However, if the blood is stored for 12 to 24 h before running the assay, a majority of the neutrophils fail to show the repolarization effect. Thus, a matched control taken at the same time as the patient sample is necessary to interpret abnormal membrane potential.

axis. This will allow for a major depolarization and subsequent loss of cell-associated fluorescence upon activation. Start collection of fluorescence versus time, pause collection just long enough to add the activating agent (10 to 100 ng of PMA per ml or 5 **X** 10^{-7} M fMLP), and immediately monitor the fluorescence signal. Continuous recordings collecting time versus fluorescence should be made.

Clinical Considerations

The assays described above are useful for determining the basic ability of a cell population to respond to stimulation. One advantage of flow cytometry is the possibility of identifying a heterogeneous response. If some cells respond rapidly, while others fail to respond in the same manner, this provides at least some information otherwise unobtainable by regular bulk assays. Care should be taken in interpretation of these data. Rapid depolarization occurs with freshly collected normal neutrophils. However, when fMLP is used, it is likely that blood collected more than 8 h prior to running the assay may show an inability to respond (unpublished observation). Therefore, it is crucial always to take a control sample at the same time as a patient sample. Figure 2 shows examples of the expected normal response of neutrophils to stimulation. In Fig. 2A, cells stimulated with PMA depolarize and fail to repolarize. In Fig. 2B, the blood had been stored for 24 h before cells were prepared for the assay. While essentially all the cells responded initially to stimulation with fMLP, only a small percentage demonstrated the expected normal repolarization. Clearly, when functional assays are to be performed on blood from patients, it is important to ensure that cells are isolated from fresh blood.

ION FLUX: MEASUREMENT OF CYTOSOLIC FREE Ca^{2+}

The activation of phospholipase c and membrane-bound phosphatidyl inositol is the initial step of signal transduction following receptor-ligand interaction. Subsequent release of inositol phosphates and fatty acids triggers activation of protein kinase C, followed by flux of calcium across the plasma membrane. The flow cytometer can monitor the major spectral change when indicators of Ca^{2+} penetrate cells and are excited at 350 nm (UV excitation) (12). This process can be monitored using Indo-1, an excellent dye for flow cytometric measurement of free intracellular calcium. When bound to calcium, Indo-1 undergoes a fluorescent emission shift to a shorter wavelength. This probe is an

acetoxymethyl ester which undergoes enzymatic hydrolysis (similar to DCFH-DA) within the cells; in this case the cleavage product is free and measurable dye. The ratio of the short (bound) wavelength fluorescence to the long (free) wavelength fluorescence reflects the calcium concentration independent of the loading concentration of the dye.

The main disadvantage in using Indo-l is the requirement for a UV excitation source, usually an expensive option on most flow cytometers. Alternative probes, such as Fluo-3, which are excited in the visible spectrum are available; however, the advantage of ratio measurements is lost with these probes to some extent.

Procedure

Either purified polymorphonuclear leukocytes or leukocytes obtained by ammonium chloride lysis can be used for this assay, since neutrophil populations can be gated by forward and 90" light scatter. Load the cells with Indo-l (final concentration, 3 μM) for 15 min at 37°C, and run on the flow cytometer immediately after the probe has equilibrated. Fluorescence emission must be collected at two emission wavelengths: 395 nm (bound calcium) and 525 nm (nonbound calcium). The ratio of these emissions is plotted against time to monitor the kinetics of the cellular response and determine the Ca^{2+} concentration independent of dye concentration. In addition, if a standard curve is developed by measuring the fluorescence of cells incubated in varying calcium concentrations, actual calcium concentrations are easily determined directly from the flow cytometer plots. A high 395/525-nm ratio indicates bound Ca^{2+}, and conversely, a low ratio indicates free calcium.

Ionomycin can be used as a positive control in measure-- ment of calcium flux. Ionomycin (3 to 5 μM) will cause an increase in the bound (short wavelength) fluorescence signal (i.e., an increase in bound $[Ca^{2+}]$ inside the cell). An example of this measurement is shown in Fig. 3.

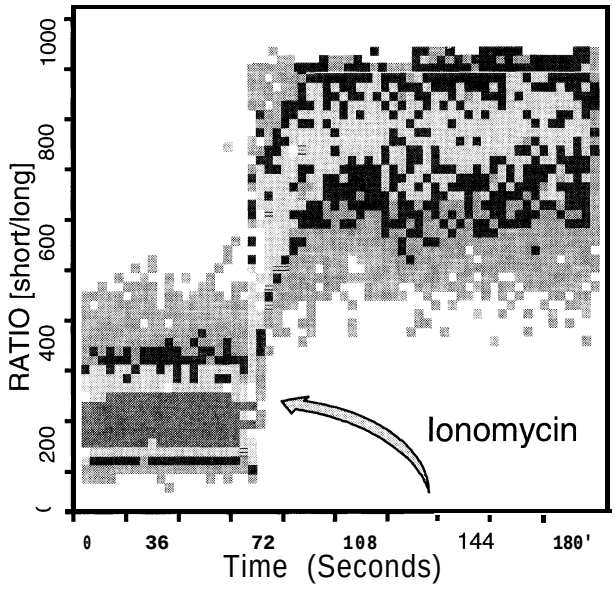

FIGURE 3 Cells loaded with Indo-1 can be stimulated to demonstrate normal signal transduction pathways. As an example, neutrophils were stimulated with ionomycin to demonstrate a rapid release of calcium from intracellular stores. Ratio measurements for each cell were collected, using flow cytometry, and were plotted against time.

CD1 1 b EXPRESSION

The link between leukocyte recruitment and inflammation was provided by the discovery of a number of glycoproteins (CD1 1/CD18 complex) whose primary purpose is to promote adhesion to vessel walls. Early studies of C3-coated particle adherence to cell membranes were facilitated by CD11b/CD18 adhesion molecules commonly called integrins (17), although this was not originally recognized. Upregulation of these molecules can be monitored relatively easily by flow cytometry in either single-, dual-, or three-color assays. The absence of some of these molecules can account for a small number of patients with recurrent infections, and a deficiency in surface glycoproteins CD1 1a/CD18 (LFA-1), CD11b/CD18 (CR3), or CD1 1c/CD18 (p150, 95) has been shown to be associated with chemotactic defects. This syndrome, called leukocyte adhesion deficiency (1), is an autosomal recessive disease characterized by recurrent bacterial and fungal infections, impaired pus formation, and poor wound healing.

Increased adherence of circulating neutrophils to the microvascular endothelium is an essential early event in the initiation of the acute inflammatory response, regularly preceding neutrophil migration through vessel walls and accumulation at site of tissue injury (27). Leukocyte (β_2) integrins, especially CD11b/CD18 (Mac-l) and CD1 1a/CD18 (LFA- 1), have been shown both in vitro and in vivo to be important in inflammation. Adherence of either stimulated or unstimulated human neutrophils to human umbilical vein endothelial cells is diminished significantly by antibodies to CD11b or CD18 (19).

The assay requires care in the preparation of the cell suspension, as sudden changes in temperature can induce increased expression of CD1 lb receptors on the surface of human neutrophils, rendering the results of the assay of little value.

Procedure

Take 10 ml of heparinized blood, and lyse the erythrocytes as previously described for measurement of H_2O_2. Again, it is unnecessary to purify neutrophils because of the ability to gate the appropriate population by flow cytometry. The leukocyte suspension should be at a concentration of 1 **X** 10^6/ml. Aliquots (100 μl) are placed into several test: tubes (12 by 75 mm) and exposed to one of several stimulants such as 10-ng/ml PMA or controls such as HBSS. After 30 min, 10 μl of appropriately titrated antibodies is added to identify expression of the receptors of interest. These antibodies include CD1 1 b (Mo- 1-FITC; Coulter Corporation, Miami, Fla.) and Leu 15-PE (Becton Dickinson Immunocytometry Systems, San Jose, Calif.). Other monoclonal anti-- bodies are available from a number of manufacturers. Optimal dilutions are 1: 8 for Mo-1 (10 μl), 1 : 50 for anti-CD18 antibody, and 1: 100 for human anti-CD1 la (DAKO-CD18 and DAKO-CD1 1 a; Dako Corporation, Carpinteria, Calif.). The tubes containing the leukocytes plus monoclonal antibodies are incubated at 4°C for 30 min. One milliliter of PBS buffer is added to each tube, and the tubes are centrifuged at 250 **X** g, 4°C, for 10 min. The supernatant is removed, and the cell pellet is loosened by dragging the tubes along the top of a test tube rack. The wash with PBS is repeated, and FITC-conjugated goat antimouse immunoglobulin G1 (1: 8 final dilution) (Caltag, San Francisco, Calif.) is added to the leukocytes tagged with appropriate monoclonal antibodies (CD18/CD1 la). The negative control tube contains 20 μl of pooled human serum added to the human leukocytes. The secondary antibody must be in-

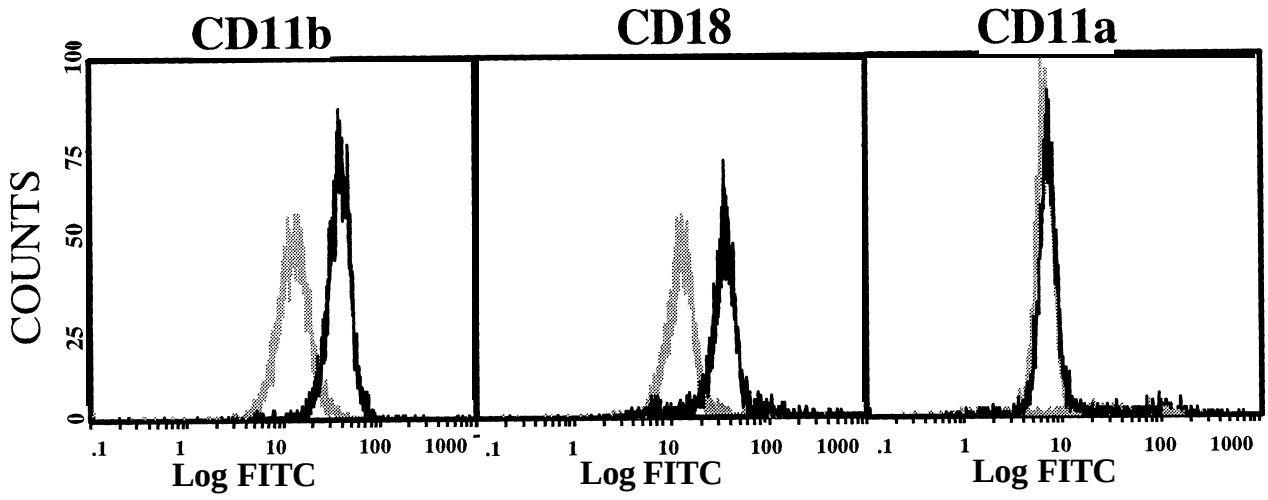

FIGURE 4 Histograms showing neutrophils labeled with primary antibodies to neutrophil adhesion markers: CD1 lb (Mo-1-FITC) at a dilution of 1: 8, CD18 (DAKO-CD18) at a dilution of 1: 50, and CD1 la (DAKO-CD1 la) at a dilution of 1: 100. The gray peaks show the expression after 30 min at 37°C, while the black peaks show the expression on neutrophils stimulated with 10-ng/ml PMA for 30 min at 37°C.

cubated with the cells at 4°C for 30 min and washed two times with PBS as described above. The cells are resuspended in 100 μl of PBS, fixed using an equal volume of 2% paraformaldehyde while vortexing, and kept in the dark at 4°C until analyzed on the flow cytometer.

The normal expression patterns need to be appreciated in evaluating the results of these studies. As mentioned above, the CD1 l/CD18 leukocyte adhesion molecules (Leu-CAM) consist of three surface membrane heterodimeric glycoproteins named LFA-1 (CD11a/CD18), Mo-1 or Mac-l (CD11b/CD18), and LeuM5 or p150,95 (CD11c/CD18). LFA- 1 is normally expressed on all leukocytes and serves as a general adhesion molecule involved in leukocyte homotypic and heterotypic interactions. Mo-1 and p150,95 are selectively expressed on granulocytes, monocytes, and NK cells. Since activated granulocytes express far more CD1 1 b/CD18 than the other two antigens, this is clearly the easiest one to monitor after activation. Figure 4 provides an example of the type of expression one can expect from human neutrophils.

Clinical Considerations

Neutrophil adherence to endothelial cells and subsequent diapedesis into tissue involve the CD1 lb/CD18 complex and the leukocyte selectin L-selectin (LAM-I). Both CD1 la/CD18 and CD1 lb/CD18 exhibit transient changes in the ability to bind endothelial cells after stimulation, and each contributes equally to mediate the adhesion. In contrast, CD1 1c/CD18 makes little apparent contribution (18).

FCγRIIIb RECEPTOR EXPRESSION

Apoptosis is not the same as necrosis. The latter term refers to the morphology most often seen when cells die from severe and sudden injury, such as ischemia, sustained hyperthermia, or physical or chemical trauma (26). In necrosis, the plasma membrane loses its ability to regulate osmotic pressure and the cell swells and ruptures. The contents are spilled into the surrounding tissue space and provoke an inflammatory response.

In apoptosis, the cell breaks up into apoptotic bodies without the spilling of intracellular contents in the absence of inflammation. Electron micrographs of cells undergoing apoptosis show an extremely condensed cytoplasm with normal-appearing organelles. The nucleus, which is the center of activity of many of the events associated with apoptosis, undergoes shrinkage, and its chromatin becomes very dense, collapsing into patches, then into crescents in tight apposition to the nuclear envelope, and finally in many cells into one or more spheres.

The appearance of DNA strand breaks during apoptosis in individual neutrophils can be detected by labeling 3′-OH termini with biotinylated deoxyuridine triphosphate (b-dUTP) using exogenous terminal transferase. This methodology enables early in situ detection of DNA strand breaks caused by the activation of a serine protease which hydrolyses a protein(s) associated with the internucleosomal linker DNA sections, thus increasing accessibility of linker DNA to the apoptosis-associated endonucleases (11). This proteolytic step precedes initiation of DNA degradation during apoptosis. Neutrophils are removed from inflammatory sites via an apoptotic recognition process, whereby macrophages determine by an as yet poorly defined mechanism which neutrophils to remove.

It is possible to detect apoptosis in neutrophils by incubating them with exogenous terminal deoxynucleotidyl transferase and b-dUTP and detecting the incorporated b-dUTP by flow cytometry after labeling with streptavidin conjugated to allophycocyanine (1: 32). Neutrophils at zero time will generally show a minimal incorporation of b-dUTP; however, when cells are cultured for 8 h and beyond, a population of cells (around 25%) incorporating the nucleotide becomes apparent. In addition to these, there is a relatively simple and straightforward method that can be used routinely-the expression of FCγRIIIb or CD16 receptors on the neutrophil surface.

Neutrophils manifest a homogeneous staining of a single-

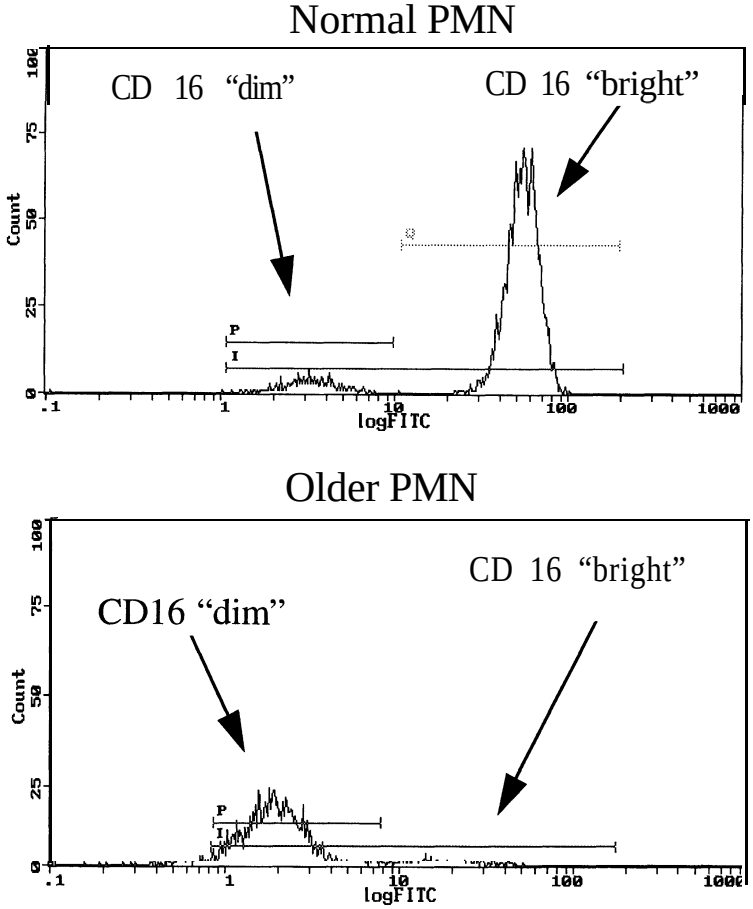

FIGURE 5 CD16 expression on neutrophils. Comparison of fresh, normal neutrophils with 24-h-old neutrophils. The CD16 "bright" population is severely reduced in older neutrophils. PMN, polymorphonuclear leukocytes.

cell population of FCγRIIIb (CD16) receptors at zero time (nearly 100%). In contrast, when neutrophils are cultured for 12 h at 37°C, a distinct bimodal distribution of CD16 surface expression can be observed. As shown in Fig. 5, approximately 35% of the total cell population are CD16 low-expressing neutrophils and 45% are CD16 high-expressing neutrophils. It can be shown that as the neutrophils age, nearly all of the high-expression CD16 is lost and the cells become almost entirely low-expression CD16. It is thought that this loss of high CD16 expression might well relate to one of the signalling mechanisms that macrophages require in order to phagocytose and remove exhausted but still viable neutrophils before these neutrophils undergo any necrotic change that would result in loss of granule contents.

The concept that aging neutrophils undergo apoptosis evolved recently (15). Neutrophils undergo apoptosis in a manner similar to that observed in many other cell types: condensation of nuclear chromatin, compaction of cytoplasmic organelles, cell shrinkage, and changes at the cell surface. Human neutrophils shed surface FCγRIIIb (CD16) and acquire annexin V binding sites during apoptosis in vitro. These changes take place without loss of membrane integrity, as shown by propidium iodide exclusion.

Procedure

This assay requires a population of cells as described for H_2O_2 production above. The measurement can be performed routinely on whole blood as well as on purified cells in culture. To determine the normal changes that take place over 48 h of incubation, each well of a 24-well culture plate (Costar) is seeded with 1 ml of neutrophil suspension at 2 x 10^6/ml (see above section on the preparation of purified neutrophil suspension). Cells are cultured for up to 48 h at 37°C (5% CO_2), with cells removed from wells at 0, 12, 24, and 48 h. After incubation, the contents of the wells are transferred to test tubes (12 by 75 mm) (or any other tube suitable for the flow cytometer), centrifuged at 250 x g for 10 min at room temperature, resuspended in PBS, and counted so that the concentration can be adjusted to 10^6/ml. One hundred microliters of cells is aliquoted into each of two tubes (12 by 75 mm), and 10 μl of 3G8(CD16)-FITC (1: 10 dilution) is added. The tubes are capped, gently vortexed, and incubated for 30 min on ice in the dark. The cells are then washed with 1 to 2 ml of PBS and centrifuged at 250 x g for 10 min at 4°C. This is repeated once, the supernatant is aspirated, and the cells are resuspended in 250 μl of 1% paraformaldehyde (final concentration) while vortexing. Cells labeled with 10 μl of mouse immunoglobulin G 1 -FITC are used as the negative control.

Fluorescence histograms will show a biphasic response as shown in Fig. 5. As the cells age, there should be a noticeable change in the CD16 high expression. Freshly collected cells from peripheral blood should express almost entirely

the CD16 bright peak. Reductions in this expression may be indicative of premature aging of neutrophils.

Clinical Considerations

While there are as yet no studies defining the clinical significance of the early manifestation of neutrophil apoptosis, it is reasonable to consider that under certain conditions, neutrophils may be inappropriately driven into apoptosis and become functionally incompetent. The observations that a large bright and small dim population with CD16 expression is observed in normal cells can be used as an indicator of the cells' functional capability. However, it should be noted that further studies must be performed to better understand the implications of these measurements from a clinical perspective.

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