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New Technologies for Use in Toxicology Studies: Monitoring the Effects of Xenobiotics on Immune Function

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ABSTRACT

New developments in flow cytometry are now being applied in toxicology studies. There are several reasons for using this technology. First, techniques are well characterized to measure functional parameters of single cells. Such measurements can be directly related to perturbations by xenobiotics, cell-mediated immune responses, or trauma. Second, there is a clear indication for movement toward in vitro systems as highly objective assessments of toxicologic interactions. By measuring specific cell functions at the single cell level, it is possible to define a range of normal responses. More importantly, a multiparametric analysis can be performed with flow cytometry and parameters can be directly related to one another. Furthermore, kinetic measurements can be made, providing vital clues to the mechanisms of actions of drugs or chemicals on functions of specific cell populations. Major advantages of this approach are that studies can be performed on very small volumes of blood, body fluid, or cell culture lines and it is not necessary to isolate pure populations of cells to perform these assays. We believe that this alternative approach in toxicology will provide valuable information unobtainable by traditional means.

INTRODUCTION

RECENT TRENDS TOWARD A REDUCTION OF THE USE of animal models for experimentation together with the necessity to design more comprehensive in vitro toxicological assessments have led to more dependence upon automated technology. It has also become apparent that some of the current in vivo models have no direct comparison to the in vitro test armament. Therefore, it is necessary to assess comprehensive alternative methods. One aspect of this approach is the emergence of flow cytometry in the field of toxicology. Probably the most common use of flow cytometry is the phenotypic analysis of cell populations and the determination of cell cycle by measurement of DNA content. The former procedure allows the identification of cells with specifically identifiable surface or intracellular antigens, while cell cycle analysis is a method for observing changes in growth kinetics of a population. Flow cytometry has a number of advantages over other technologies, specifically the need for fewer cells and the ease of the measurement process.

Major advantages of flow cytometry include the ability to make functional measurements of cells independently of, or simultaneously with, identification of the cell phenotype or cell cycle status. We discuss the applications and capabilities of flow cytometry and relate these to potentially useful assessments in toxicology studies.

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OVERVIEW AND DESCRIPTION OF FLOW CYTOMETRY

What is flow cytometry?

Flow cytometry is a technique whereby a coherent light source (usually a laser beam) is used to intersect a stream of fluid containing cells or particles. As the cells pass through the laser beam, several types of measurable light emissions are produced. These emissions are translated into electronic signals and transmitted to a computer processor which correlates the parameters into a picture or histogram of the cell characteristics. Table 1 describes the types of signals that can be generated by the typical flow cytometer.

There are several unique features about flow cytometers that make them particularly useful for biological studies. The instruments can identify pure populations of cells within a heterogeneous population without the time-consuming and often cumbersome process of physically separating the cells. Next, the flow cytometer can analyze the individual populations based upon the characteristics in Table 1. Because the cells are in the laser beam for such a short time, no damage occurs due to the detection system, and intact cells can be recovered. A subpopulation of cells can be identified electronically and physically separated using the sorting capabilities of the instrument. In this way, several thousand or several million cells can be collected for further analysis by microscopy, or another traditional assay. This feature could be used to sort out and identify cells which are reactive in a particular way, or fail to respond to stimulation. By collecting all the available data on a cell population using the "listmode" capabilities of the flow cytometer, later analysis is possible allowing correlations of parameters initially thought to be of no interest. For instance, one may be interested in the appearance or reduction in a specific subpopulation of lymphocytes by use of a fluorescent-labeled monoclonal antibody. Subsequently, it may be observed that other changes occurred in the target population such as a reduction in size or granularity. Both these parameters are routinely collected by flow cytometers, and so a reanalysis of the population of interest could involve monitoring the size and granularity without any further experimental work.

Why use flow cytometry?

There are several basic reasons why flow cytometric measurements are desirable for use on toxicological-related studies. These are:

1. Flow cytometric observations are made on single cells
2. Information about each cell contains correlated multiple parameters
3. Small numbers of cells are required for each assay
4. Large numbers of replicates or tests can be performed
5. Observations and measurements are objective

TABLE 1. SOME PARAMETERS AND DEFINITIONS OF MEASUREMENT AVAILABLE USING FLOW CYTOMETRY[a]

Parameter	*Measurement*
Forward angle scatter	Size
90° Scatter	Granularity
Impedance	Cell volume
Fluorescence	Autofluorescence
Fluorescence	Fluorescence
Time	Time of each event

[a] Most flow cytometers are capable of providing at least 3 or 4 of these parameters. This is usually sufficient to perform many of the studies we have discussed.

Because observations are made on single cells, it is possible to observe the complete cells in a multiparameter fashion by observing the intracellular components, extracellular antigens, DNA, RNA, enzymes, and physical components such as size, granularity, and cell volume. It is not necessary to have vast quantities of cells for these measurements, because the flow cytometer is capable of making each of these measurements simultaneously on every cell. Thus, a histogram can be built describing the biological response of a cell in a particular environment. Further, because of the ease with which replicate samples can be run on a flow cytometer, it is possible to run many replicates of an assay and correlate the results in an objective manner.

HOW CAN THESE QUALITIES BE USEFUL IN TOXICOLOGY?

There are at least three advantages offered by this technology. First, the observations made are restricted to the cellular level. By considering the cell as a "biochemical factory" we are making highly correlated measurements at the primary site of physiological function and control. Second, we are able to monitor direct alterations in function and structure such as changes in membrane fluidity[1,2] or expression of receptors.[3–7] Since these measurements are on single cells, we can also be sure of making observations on the exact cell of interest in situations where heterogeneity may exist. Third, the measurements are objective and reproducible by nature of the detection system.

PRIMARY ADVANTAGES OF FLOW CYTOMETRY

Flow cytometry allows one to make measurements on individual cells by examining a number of different parameters on each cell. This multiparametric approach makes it possible to identify populations of cells with varying degrees of functional activity within a heterogeneous population and make measurements on a specific population without isolating that population. For example, if it were observed that there were a small and a large population of cells in a sample of interest, the flow cytometer could electronically gate out either (or both) populations and make independent measurements of any available parameter (Fig. 1). Using a bulk measurement system (e.g., measuring superoxide of neutrophils using superoxide dismutase (SOD) inhibitable cytochrome C

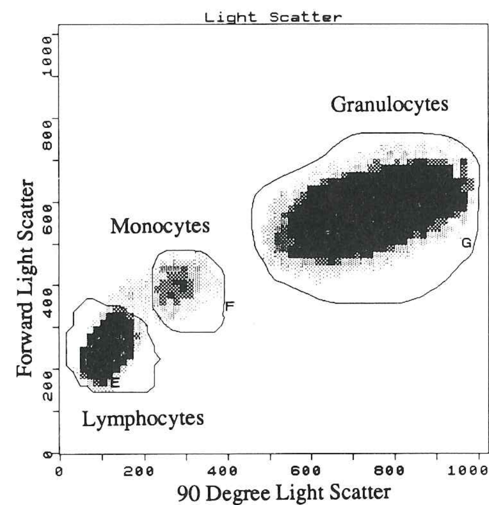

FIG. 1. This figure is a flow cytometric histogram of normal leukocytes showing three populations of cells: lymphocytes, monocytes, and granulocytes. Better separation of cell populations can be achieved by using a multiparameter approach. Each population can be defined electronically and the cells within that area can be analyzed independently of cells outside that area.

reduction) there is no way to account for dead cells, poorly or highly reactive cells, or non-neutrophils which may have been included in the cell suspension.[8,9] These cells can be excluded from calculations in flow cytometry by a process known as selective gating. This process uses a multiparametric approach whereby unwanted cells can be electronically marked and excluded from the analysis.

Therefore, one can isolate a cell using a physical parameter and relate a functional parameter to that cell or perform the reverse; observe an effect (e.g., H_2O_2 production) and backgate to the particular cells that produced the H_2O_2.

Another major advantage of flow cytometry is the possibility for measuring large numbers of samples in a highly controlled and potentially automated way. Because relatively few cells are required for each tube (from 1×10^4 to 1×10^6), multiple samples can be generated with a small volume of blood, lavage fluid, or cell culture sample.

WHY MONITOR IMMUNE CELL FUNCTION?

There are a number of well-defined effects of xenobiotics on immune function. Heavy metals are known to alter both humoral and cell-mediated immunity and in particular have effects on lymphocyte proliferation.[10] Altered immune function, particularly reduced percentages of circulating T and B cells and reduced numbers of helper and suppressor cells, has been documented in cases of exposure to halogenated aromatic hydrocarbons.[11] Studies involving reductions in the number and function of circulating T and B cells have also been reported after exposure to noxious gases. While the numbers of cells remained constant after exposure in these studies, the function of the cells was significantly reduced as measured by lymphocyte proliferation assays.[12]

Immune cells [e.g., lymphocytes, macrophages (MPs)], and polymorphonuclear leukocytes (PMNs) are not only important targets in immunotoxic responses, they also represent valuable tools for the study of biochemical mechanisms by which xenobiotics alter cell function. Much is known about the molecular events associated with the activation and differentiation of immune cells into effector cells performing specialized functions. Recognition events involving specific receptors (e.g., for antigens, mitogens, inducing agents) trigger changes in the membrane structure and function (e.g., alterations in ion fluxes and fluidity; activation of enzymes such as phospholipases) and the transduction of signals via second messenger pathways (e.g., intracellular increases in Ca^{2+}, inositol phosphates, and products of the arachidonic acid cascade). Protein kinases are induced to phosphorylate key substrates in the cell which play a role in regulating gene expression. Finally, the cells undergo terminal differentiation into specialized cells whose soluble mediators (e.g., cytokines, antibody, etc.) or effector function (e.g., tumor cell cytotoxicity) can then be quantitated.

Second, immune cells can be conveniently obtained, separated, and cultured (including the ready availability of such cells from the peripheral blood of human subjects), which makes these cells ideal for toxicological analysis after in vitro or in vivo exposures. Third, lymphoid/myeloid organs contain rapidly proliferating cells and therefore constitute sensitive target organs for detection of exposure to xenobiotics. Finally, the commercial availability of monoclonal reagents for phenotyping of specific cell populations in mice, rats, and, in particular, the cluster differentiation antigen (CD) markers on leukocytes, affords the opportunity to investigate biochemical changes occurring in separate cell subpopulations at a level of sophistication previously unavailable.

We believe that there are five major reasons that suggest value in monitoring immune cell function as a primary toxicological measurement. These are:

1. Measurements are directly related to immune status
2. Immune cells are sensitive to environmental influences
3. These cells produce rapid responses to many stimuli
4. Events can be measured in real-time
5. Evaluations are conducted at the cellular level

Immunotoxicology is the study of adverse effects of chemicals, drugs, and biologics on the immune system. The National Toxicology Program (NTP) organizes and conducts a comprehensive testing and research program to

determine potential human health risks in this area. A comprehensive testing panel has been suggested in animal studies.[13] Preliminary analysis of xenobiotic-induced immunotoxicity includes changes in splenic/thymic histopathology, a hematological profile, and organ/body weight ratios.

Subsequently, a battery of specific (cell-mediated and humoral) and nonspecific [macrophage (MP) and natural killer (NK) activity] immune function tests are proposed for further analysis of dysfunction in chemically exposed animals. The sensitivity of these tests is superior to the histopathology/cellularity studies. Many laboratories have correlated chemically induced suppression of immune function with decreased host resistance to infectious agents and inoculated tumor cells.

Cell-mediated immune functions include sensitization of chemically treated animals to T-cell-dependent antigens (e.g., keyhole limpet hemocyanin) and subsequent quantitation of a delayed hypersensitivity response by monitoring footpad/ear swelling or by radiometric means. In addition, in vitro proliferative responses of splenic lymphocytes to T-cell mitogens (concanavalin A and phytohemagglutinin) are also routinely determined. The mixed lymphocyte reaction (MLR) may be monitored in mice [rat spleen cells contain suppressor cells (MPs) which can inhibit proliferative responses of T cells to alloantigens in vitro].

The assessment of humoral functions in chemically treated animals routinely involves determination of IgM-specific antibody plaque-forming cell responses to T-cell-dependent (e.g., sheep red blood cell) antigens. In vitro proliferative responses to the B-cell mitogen lipopolysaccharide may be evaluated in mice (rat lymphocytes respond poorly to this mitogen).

Lymphoproliferation assays are laborious procedures that require the use of expensive reagents (e.g., radioisotopes, fetal bovine serum, tissue culture medium, etc.) and personnel trained in proper aseptic technique. In addition, one has to wait several days to harvest the cells in order to determine functional activity. The application of flow cytometric analysis to early cell activation events such as intracellular Ca^{2+} fluxes (see below), pH changes, or the appearance of interleukin-2 (IL-2) receptor, Ia, or other markers (e.g., adhesion proteins such as LFA-1) on activated lymphocytes, has not as yet been considered for interlaboratory validation in the NTP testing protocols. However, many of these functional indicators of cell activation may serve as convenient and possibly more sensitive endpoints for immunotoxicity testing relative to the more traditional assays.

Activated MPs generate a wide variety of secretory products (e.g., proteases, arachidonic acid metabolites, cytokines, etc.) indicative of their biochemical integrity; however, NTP testing protocols generally only measure bulk phagocytosis and/or the cytostatic (growth inhibiting) ability of MPs against tumor cells in culture (an assay which is subject to significant sources of variability). Significantly more sensitive and quantitative approaches could include analysis of the phagocytosis of fluorescent latex microspheres by individual cells via flow cytometry[14] and/or the generation of intracellular H_2O_2 using the fluorescent probe DCFH-DA.[15] These approaches would also more satisfactorily address the functional heterogeneity known to exist within MP populations.

NK cells may also be important in host defense against certain types of tumors and viral infections. NK function in chemically dosed animals is classically determined from the in vitro cytotoxicity mediated by nonadherent lymphoid cells against the YAC-1 lymphoma cell line, an NK-sensitive target cell. The release of ^{51}chromium (^{51}Cr), a gamma emitter, from radiolabeled tumor target cells over a short-term (4 h) incubation period, is a measure of the lymphocyte-mediated cytotoxicity (LMC).

The use of fluorescent monoclonal antibodies to 5-bromodeoxyuridine (BrdUdr)-labeled DNA to label tumor target cells (as recently reported by Ackermann et al.[16] for flow cytometric analysis of tumor cell cytostasis by activated murine PMNs) may allow for kinetic analysis of NK cell-mediated cytostasis of YAC-1 lymphoma cells in S phase obviating the need for gamma emitters to measure cytotoxicity. (The cytostasis would include cytotoxicity as well as the growth inhibitory capacity of the effector cells.) The inconveniences associated with the use of ^{51}Cr (e.g., the safety factor, the relatively short half-life, the expense, etc.) can then be eliminated. Assessment of cytotoxic T-lymphocyte capabilities may be performed in a similar manner.

Immunotoxicity profiles in chemically dosed animals routinely include surface marker phenotyping of leukocytes by flow cytometry in conjunction with evaluations of immune function. Such studies are important because they confirm whether chemically induced immune suppression (or stimulation) is the result of functional changes in individual cells and/or the result of alterations in the proportions of subpopulations in a given lymphoid organ.

CHEMICAL-INDUCED INFLAMMATION AND ACUTE TARGET ORGAN TOXICITY

The primary concern in toxicology relative to understanding target organ damage involves the metabolism of xenobiotics and generation of tissue destructive reactive intermediates. Recently, however, the possibility that some chemicals produce acute organ toxicity indirectly by stimulating blood PMNs/monocytes to release oxygen radicals or other injurious substances is becoming of great interest.

Recent studies with 12-0-tetradecanoylphorbol-13-acetate (TPA, a phorbol ester tumor promoter) administered IV to rats demonstrate that, although the ensuing pulmonary toxicity is, in part, due to the direct toxicity of the chemical, accumulating PMNs contribute significantly to the tissue damage.[17] Similarly, studies in TPA-treated dogs suggest that significant increases of PMNs in nonpulmonary organs may occur as well, particularly in the liver.[18] Activated MPs have recently been shown to enhance acetaminophen-induced hepatocellular necrosis as a consequence of bioactivation.[19]

PMNs may be activated in a variety of ways after chemical exposure. As a result of chemically injured tissue, factors chemotactic for PMNs may be generated, including complement fragments such as C5a or leukotriene B4. Chemicals may also directly activate PMNs. However, it is also possible that activated MPs in target organs (and/or the target organ cells per se) may release small molecular weight cytokines such as interleukin-1 (IL-1), tumor necrosis factor (TNF), and/or colony-stimulating factor (CSF) which may activate and mobilize PMNs and induce the proliferation of hemopoietic precursor cells. Recent work in Dr. Pfeifer's laboratory[20,21] has focused on the possibility that strain-dependent differences in the local production of such mediators in response to topical application of TPA may account for the increased skin inflammation (and tumor promotion) in SENCAR mice relative to TPA-resistant strains (e.g., C57B1/6, B6C3F1).

THE INFLAMMATORY RESPONSE AND CHEMICALLY INDUCED CARCINOGENESIS

The development of tumors has long been associated with chronic inflammatory processes, particularly in the skin.[22] Lewis and Adams[23] have demonstrated histopathologically that SENCAR mice, outbred for sensitivity to phorbol ester tumor promoters in two-stage chemically induced carcinogenesis protocols, develop more edema, vascular permeability, and a greater influx of acute inflammatory cells (i.e., PMNs) in skin after topical application of TPA relative to the TPA-resistant C57B1/6 inbred strain. Also, both resident and inflammatory peritoneal MPs isolated from SENCAR mice generated more H_2O_2 (see following section) and arachidonic acid metabolites in response to in vitro exposure to TPA relative to those obtained from the B/6 mice.[24]

Local production of prostaglandins, leukotrienes, and reactive oxygen intermediates (ROIs) has long been associated with skin tumor promotion in TPA-dosed mice; in addition, anti-inflammatory agents such as corticosteroids and NSAIDs can also act as antipromoters.[25] TPA-activated inflammatory cells have been hypothesized to contribute to the genetic damage incurred in chemically initiated epidermal cells through the generation of ROIs and, in so doing, facilitate tumor progression.[26] Furthermore, activated human PMNs have been shown to induce malignant transformation in murine C3H 10T1/2 fibroblasts.[27]

IMMUNOTOXICITY AS A CONSEQUENCE OF CHEMICALLY INDUCED INFLAMMATION

The MP plays a central role in regulation of nonspecific and specific host defense processes, i.e., the inflammatory response and antigen-driven immune functioning. Cytokines (e.g., IL-1 and TNF) released by activated MPs play an important role in inflammatory processes by activating and mobilizing PMNs to the site of tissue injury or infection; they also induce the proliferation of granulocyte/MP precursor cells.[28,29]

These cytokines also modulate immune responses, for instance, a membrane-bound form of MP IL-1 is important in activating T and B cells. Alpha-interferon produced by activated MPs can induce NK activity.[30] Activated MPs also negatively regulate immune functioning by suppressing T- and B-cell proliferation and NK

activity.[31,32] The synthesis and release of prostaglandins and ROIs may be important in some of these suppressive regulatory activities.

Although some European groups[33] have been interested in systemic immunomodulatory changes as a result of local inflammation (e.g., Ca^{2+} pyrophosphate crystals inoculated into the pleural cavity), the concept of a *chemical* inducing *inflammation* which then contributes to local and/or systemic *immune suppression* is an area which is relatively unexplored by immunotoxicologists. Most studies assume chemically induced immunotoxicity is the result of a direct suppressive interaction of the chemical with the immune system.

OTHER APPROACHES TO FUNCTIONAL CELL MEASUREMENTS

Several categories of measurements can be classified as functional in nature. For instance, the ability to mark a cell type with a surface marker does not indicate the viability of the cell. Similarly, a viable cell may not be metabolically responsive. Thus, there are many different types of functional studies that can be evaluated. Many functional tests have been developed for flow cytometric application using phagocytic cells such as neutrophils or monocytes. The following section will therefore describe these functional measurements, many of which would be suitable for use in a battery of tests for effects of xenobiotics.

One approach that we are developing is to use cells such as neutrophils or mononuclear phagocytes and observe the effects of addition of xenobiotics on their normal function. An example of this is shown in Figure 2 where the relationships between volatile organic chemicals (e.g.) may affect a number of immune-related phenomena in inflammatory cells such as neutrophils or monocytes. Products from these interactions may then interact with lymphocytes and subsequent alterations in antigenic expression or cellular immune response. Such interactions are further characterized in Figure 3, where two primary reactions could occur. These divisions are based upon the effects observed on normal immune function of the cell as opposed to the effects observed after further stimulation of that same cell. This interaction requires induction, the normal result of which is activation of the cell. Any xenobiotic-mediated adverse effect could be well characterized using some of the methods described herein. Several of these techniques have been developed for other applications, and with minor modification could be used efficiently for these studies. In such situations it would be necessary to monitor both normal cell function as well

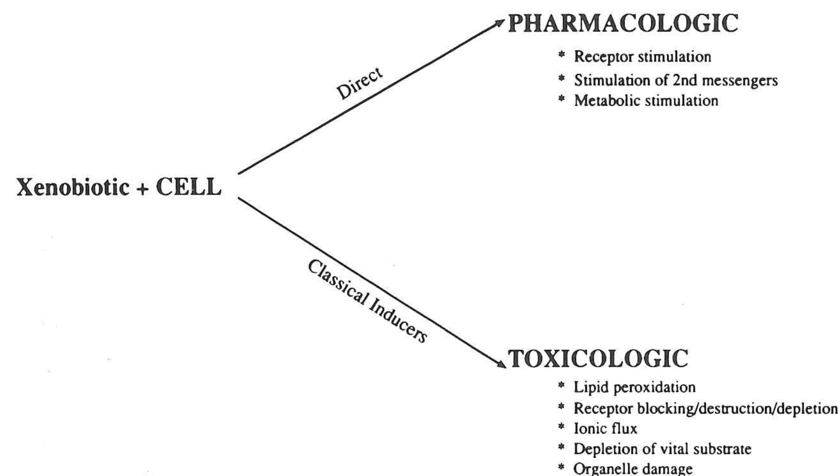

FIG. 2. The relationships between some xenobiotics may affect a number of immune-related phenomena in inflammatory cells such as neutrophils or monocytes/macrophages. Products from these interactions may then interact with lymphocytes causing subsequent alterations in antigenic expression or cellular immune response. The xenobiotics may cause direct production of oxidants or cytokines or may provide the stimulus for one cell type (such as the macrophage) to produce organ damage or genetic damage directly.

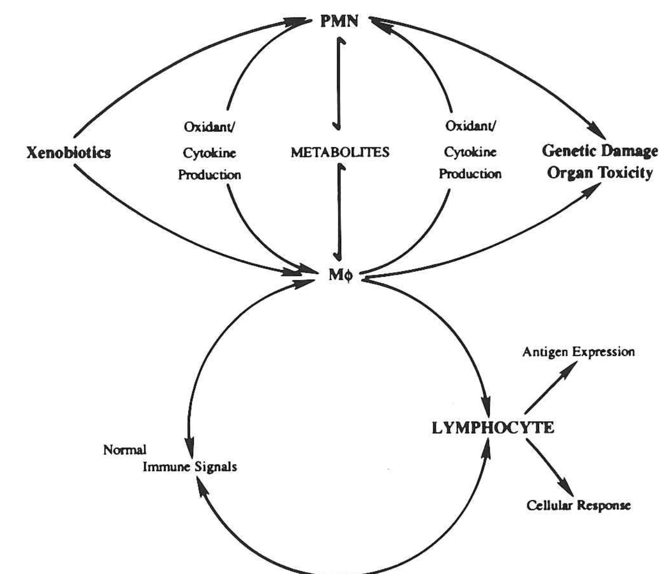

FIG. 3. Two primary reactions could occur after xenobiotic–cell interactions. This division is based upon the effects observed on normal immune function of the cell as opposed to the effects observed after the cell is further stimulated with a stimulant. This interaction requires induction, the normal result of which is activation of the cell. Any xenobiotic-mediated adverse effect could be well characterized using some of the functional methods described.

as the "stimulated" cell function. Further, as described in Figure 4, antagonistic and synergistic effects must also be considered. In this example, two specific measurements of cell function are indicated; however, these represent a number of available measurements. Measurements such as those described below can be applied to a variety of cell types. Table 2 provides an overview of how the assays proposed might be used with different cell types. All of the methods can be performed with flow cytometry.

The following section describes in more detail several of the assays discussed above.

Membrane integrity (viability)

Probably the most commonly used function assay in flow cytometry is measurement of cell viability using dyes such as propidium iodide or fluorescein diacetate. There are definite advantages in being able to determine viability directly in assay samples immediately after making functional measurements. Further, often nonviability is used to gate out dead cells from analysis[34] particularly in functional assays. Such measurements can be performed rapidly and objectively and have been used successfully in toxicologic applications.[35] As a simple measure of effect of a xenobiotic on a cell, viability is probably one of the most straightforward measurements available with flow cytometry.

Enzyme content/activity

Dolbeare, Smith, and Phares[36,37] demonstrated the presence of phosphatases and glucuronidases using naphthol derivatives as fluorogenic substrates, Similarly, cellular enzyme activity can be measured by flow cytometry,[38,39] as can esterase activity using dyes such as dichlorofluorescin diacetate.[15,40,41] These latter assays are more useful as indicators of the presence of esterases. Some use can be made of this activity to differentiate cell populations as well as indications of metabolic normal function. Since active metabolism is involved in these hydrolytic reactions, alterations induced by chemical modulators could be observable.

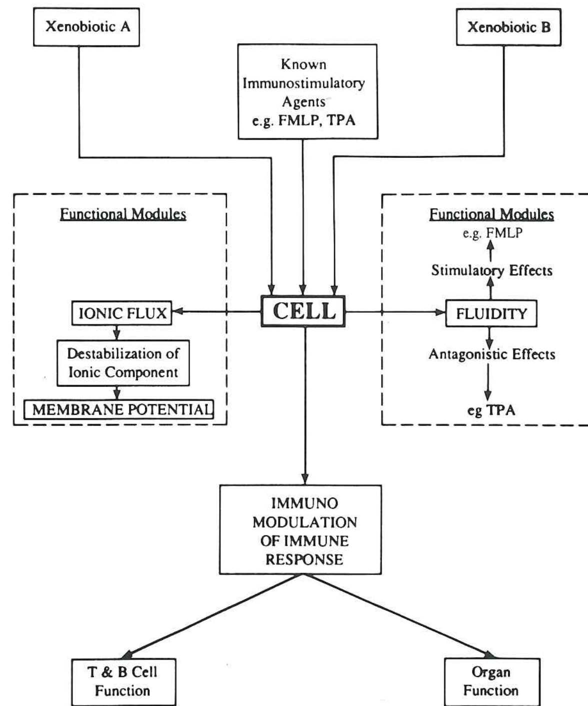

FIG. 4. This figure demonstrated an overall interaction between xenobiotic and cell. Both antagonistic and synergistic effects must be considered. In this example, two specific measurements of cell function (neutrophils or macrophages in this instance) are indicated; however, these represent just two from a number of available measurements. This figure demonstrates the necessity for measuring the resting or unstimulated state of the cell as well as the actively stimulated state. This allows assessment of the xenobiotic interaction as stimulatory or inhibitory in nature.

TABLE 2. OVERVIEW OF THE APPLICATIONS OF FUNCTIONAL STUDIES TO SEVERAL CELL TYPES[a]

Cell type	*Oxidative burst*	*Phagocytosis*	*Membrane ion flux*	*Membrane potential*	*Membrane fluidity*	*Receptors*
Lymphocytes	−/?	−	+	+	+	+
Monocytes	+	+	+	+	+	+
Neutrophils	+	+	+	+	+	+
Erythrocytes	+/?	−	+	+	+	+/?
Macrophages	+	+	+	+	+	+
Endothelial	+/?	?	+	+	+	?
Epithelial	?	−	+	+	+	?
Fibroblasts	−	−	+	+	+	+
Platelets	+	−	+	+	+	+/?
Chondrocytes	−	−/?	+	+	+	?
Cell lines	+/−	+/−	+	+	+	?
Hepatocytes	+/−	−/?	+	+	+	?

[a]While this is not meant to be an exhaustive list, it provides a background for development of applicable studies.

Carboxyfluorescein diacetate is useful in this regard since it can be hydrolysed directly in a cell to a fluorescent compound, carboxyfluorescein. This is a rapid process in most cells (1–5 min). Blair et al.[42,43] demonstrated the presence of oxidative burst enzymes in HL-60 cells using nitroblue tetrazolium using a series of experiments whereby simultaneous measurement of NBT reduction, cell cycle phase and phagocytosis were made. Several publications attest to the efficacy of measurement of other enzymes by flow cytometry.[38,39]

Membrane fluidity

The use of diphenyl hexatriene (DPH) or related probes has been described as a useful tool in measuring alterations in membrane fluidity.[44–46] DPH is a polyene hydrocarbon which is uncharged and hydrophobic and therefore partitions into lipid, primarily in the plasma membrane,[47] but also in most lipid-containing structures.[45,48] When excited by ultraviolet (UV) light, DPH emits fluorescence measurable at 430 nm. The basis of the polarization measurements is that the dye is taken into the more hydrophobic regions of the cells, particularly the lipid bilayer in the cell membrane. By measuring light in different degrees of polarization, variations can be observed in the structural integrity of the membranes. Possible uses could be for identifying old, deformed, or degenerated cells since cell rigidity increases with age,[49] relationship to cell function,[50] and other functions.[51] We recently studied the utility of measuring membrane fluidity on human neutrophils.[50] Such measurements may reflect variations in signal transduction through neutrophil membranes after perturbation. These initial studies were performed on bulk cell populations and required very large numbers of cells. Neutrophils (1×10^6/ml) were loaded with diphenylhexatriene (DPH) (1 mm final concentration) for 60 min at 37°C followed by two washes in phosphate-buffered saline (PBS). Resuspended cells were placed in a T optic spectrofluorometer (excitation 351 nm, emission 429 nm) so that both the vertically and horizontally polarized signals were recorded simultaneously. After background readings were taken, agonist (FMLP, 1×10^{-7} M) was added using a 100 μl Hamilton syringe. Calculations were made of polarization, emission anisotropy, and lipid order.[49] More recently an alternative probe, trimethylammonium diphenylhexatriene (TMA-DPH)[52–56] has been described which has an excellent application to the methodology of flow cytometry because it loads into the cells within 3 minutes and is excited by the UV line of the argon laser (357 nm).

The use of measurements of membrane fluidity or microviscosity as a measure of cell status or function is becoming more prevalent with the availability of good fluorescent dyes such as TMA-DPH.

Weber[57] and Shinitzky[49,58] have previously described in detail the theory for determination of membrane fluidity in biological membranes. There are a number of reports of the use of flow cytometry and the DPH probe for fluidity determinations.[46,59,60]

A number of studies have demonstrated that an important relationship exists between the microviscosity of neutrophil membranes and the affinity and number of some membrane receptors such as those for formyl peptides.[61,62] Tomonaga et al.[62] clearly demonstrated that formyl peptide receptor expression increased as neutrophil membranes were fluidized with aliphatic alcohols of *cis*-vaccenic acid.[62] Other studies have indicated that human peripheral blood monocytes demonstrated an increased fluidity during culture concomitant with increasing phagocytic ability.[63] It has also been demonstrated that events such as superoxide production are strongly related to the physical restructuring of the cell membrane upon activation since O_2^- was measured only at chemotactic peptide concentrations which induced fluidity changes.[50] This suggests that production of neutrophil oxidative products may be closely related or dependent upon a conformational alteration in the membrane structure.

A recent study of spleen B cells has demonstrated that endocytosis of membrane IgG is inhibited by increasing rigidity but not increasing fluidity.[64] While this study was primarily concerned with the effect of addition of rigidifying or fluidizing agents per se, the study provides support for the concept that cellular activation is related to the membrane structural status. Measurements of membrane fluidity may therfore be predictive of cellular activation status. A clear relationship has been established between phagocytosis and the organization of membrane lipids by demonstrating a fluidizing change during phagocytosis.[65–67]

The use of membrane fluidity measurements as an indicator of cell function may well become a method of significance since the use of flow cytometry provides an ideal technology. With the improvement to the methodology now available for flow cytometry, this sensitive measurement of cell perturbation should be of value in determining effects of xenobiotics on a variety of different cells.

Measurement of cytosolic free Ca^{2+}

Since calcium plays a critical role in cell function, it is desirable that we be able to determine the extent to which chemical interactions affect the redistribution of this divalent cation. A major spectral change can be measured when indicators of Ca^{2+} penetrate cells and are excited at 357 nm.[68] A new generation of Ca^{2+} indicators has been synthesized by Grynkiewicz and co-workers.[69] Cells can be loaded with Indo-1 (final concentration 3 μm) for 15 min at 37°C. These cells are then immediately run on the flow cytometer (UV excitation 357 nm) to obtain fluorescence histograms at two emission wavelengths; 395 nm (bound Ca^{2+}) and 525 nm (nonbound calcium). The Ca^{2+} concentration of cells can be determined independent of dye concentration by evaluating the ratios of the two fluorescent emissions. Thus a high 395/525 nm ratio would indicate bound Ca^{2+}. There is ample evidence to suggest that such a sensitive measure of the cell's metabolic pathways can be employed to determine the effects of xenobiotics. This measurement is a very rapid event which can be observed on the cytometer in real time. Alterations in calcium flux may be therefore directly attributable to the presence of xenobiotics.

Oxidative burst

An excellent method for the measurement of H_2O_2 is using the dichlorofluorescin diacetate (CDFH-DA) probe. The assay depends upon the incorporation of 2′-7′,dichlorofluorescin diacetate (DCFH-DA) into the hydrophobic lipid regions of the cell, where the acetate moieties are cleaved by hydrolytic enzymes to a nonfluorescent molecule 2′-7′,dichlorofluorescin (DCFH). This is 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 is able to oxidize the trapped DCFH to 2′-7′,dichlorofluorescin (DCF), which is fluorescent (530 nm). 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. We have used this assay to compare the activation states of resting and stimulated neutrophils from a number of animals including rats, mice, and humans.[15,70,71] Conversion of the fluorescence as measured on the flow cytometer in mean channel number for each histogram, is accomplished by use of a calibration curve which we have generated based upon data obtained from spectrophotometric and flow cytometric measurements according to the methods of Bass et al.[40] This test is a very sensitive measure of a cell capacity to undergo a respiratory burst in response to a stimulus. An alternative measurement is that of the amount of fluorescence (therefore H_2O_2) produced by the cell without active stimulation. Thus a xenobiotic may induce the cells to become immunologically activated or, alternatively it may turn off the cell altogether. A simple measurement of cell-related fluorescence is all that is required to observe oxidative changes in phagocytic cells. By adding appropriate concentrations of a compound to a preparation of neutrophils, the effects on both unstimulated and stimulated oxidative burst could easily be determined.

Cell cycle analysis

In vitro lymphoproliferative response to mitogen stimulation using measurements of DNA is a common functional assay for lymphocytes.[72] This measurement allows identification of some functional subsets of lymphocytes. Because only small numbers of cells are required, many replicates and stimulation conditions can be monitored. The traditional method for determination of lymphocyte proliferation is by incorporation of tritiated thymidine. It takes from 30–36 h after stimulation for DNA synthesis to begin before measurements can be made. Flow cytometry allows identification of specific subpopulations and the use of less cells overall. By measurement of RNA, some workers have shown by flow cytometry that it is possible to measure lymphocyte proliferation within 24 h of stimulation.[73,74] Since the ability of a cell population to proliferate upon stimulation is an important functional property, the ability to accurately monitor this function using flow cytometry provides advantages as opposed to radio isotopic methods.

Alteration to cellular organelles

As reviewed by Pfeifer and Irons,[75] the sensitivities of a variety of immune cell functions (e.g., lectin-induced lymphocyte blastogenesis and LMC; MP-mediated phagocytosis) are dependent upon subtle cell shape changes (and therefore dependent upon functioning microtubules and microfilaments) and are also sensitive to membrane-penetrating sulfhydryl (SH)-alkylating agents such as cytochalasin A and *N*-ethylmaleimide (NEM). Functional suppression does not involve inhibition of lectin-binding to the cell surface, decreases in intracellular reduced

glutathione, inhibition of ATP production, nor loss of membrane integrity as measured by trypan blue exclusion. In addition, these functions are less sensitive to SH reagents impermeable to the cell membrane, including 5,5′-dithio-*bis*(2-nitrobenzoic acid) (DTNB) and *p*-chloromercuribenzene sulfonate (PCMBS).

Studies with *p*-benzoquinone,[75,76] the ultimate toxic metabolite of benzene, catechol estrogen metabolites[77] and other potentially more potent immunotoxic compounds such as the epipolythiodioxopiperazines[78] suggest that particularly reactive SH groups, presumably on cytoskeletal proteins such as tubulin and actin, may constitute an intracellular target critical to the growth response of lymphocytes and the functioning of phagocytic cells.

After exposure of immune cells to oxidants, heavy metals, quinones, or other xenobiotics, the availability of fluorescent maleimide derivatives and Ca^{2+} indicators such as Indo-1 should allow for an increased understanding of the role of Ca^{2+} fluxes and/or the inactivation of SH groups in the events leading up to cell death.[79,80] Cytometric studies may be invaluable in understanding how these changes lead to necrosis in other cell types as well, including hepatocytes.[81]

CONCLUSIONS

The use of single cell measurements provides an attractive capability in the assessment of the effects of xenobiotics from an immunotoxicity perspective. Flow cytometry has become a standard technique in clinical pathology laboratories throughout the world. The availability of small, inexpensive benchtop instruments has provided a major technological advance for what was previously restricted to research applications. The combination of flow measurements with the more traditional assays in toxicology makes a powerful addition to the tools available to the toxicologist. Expansion of the monoclonal antibody technology and flow cytometry will be important in the future development of "toxicological" markers in immunotoxicology. Flow cytometry may also become an important adjunct to the development of alternative in vitro assays for industrial toxicology. Flow cytometry is state-of-the-art technology and has become firmly established in many fields of science. There are definite applications for flow cytometry in immunotoxicology already and flow cytometry could become a preferred technique in toxicology.

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