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REVIEW

Fluorescent probes to detect lymphocyte activation

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SUMMARY

Fluorescent probes can monitor events in lymphocytes stimulated by mitogens and antigens. Early activation is associated with conformational changes in membrane macromolecules, and has been studied by measurement of fluorescence intensity or polarization of the membrane-localizing probes ANS, NPN, DPH and TMRITC. Subsequent changes in cytoplasmic macromolecules have been detected by altered fluorescence polarization of intracellular fluorescein. Altered metabolic activity in the activated lymphocyte is also revealed by fluorescent probes: the increased red fluorescence of lysosomes seen by AO staining, is attributable to altered lysosome membrane permeability. AO fluorescence has also detected early changes in the nuclear nucleoprotein complex. The later synthesis of new DNA is readily demonstrated by increased staining with the nuclear probes AO, ethidium bromide, propidium iodide, mithramycin and the Hoechst dyes.

Adaptation of fluorescent probe analyses to the now rapidly developing flow microfluorimeters is providing rapid and sensitive assays of lymphocyte stimulation. Such methods will permit routine detection of lymphocyte response to particular antigens or mitogens, as well as identification of antigenic substances by their stimulation of known reactive lymphocytes. Last but not least, fluorescent probes are providing new understanding of the cellular events and regulatory mechanisms associated with lymphocyte activation.

INTRODUCTION

The binding of an antigen or mitogen to specific surface receptors on a responsive lymphocyte induces a complex sequence of cellular events, culminating in DNA synthesis and possible cell division (reviewed by Wedner & Parker, 1976). Some of the earliest events following lymphocyte activation involve conformational changes in macromolecules of the plasma membrane, cytoskeleton, organelles and nucleic acids. Fluorescent cell probes can be used as sensitive monitors of such cellular behaviour because their fluorescence emission is altered by subtle variations in their microenvironment (Ward & Fothergill, 1976). Many probes are available with different subcellular binding affinity and sensitivity to local stimuli; they provide an array of precision tools for detecting lymphocyte reactivity and studying its nature.

Triggering events for lymphocyte activation may be redistribution of surface receptors with disruption of microtubule associations (Edelman, 1976), increased membrane fluidity following fatty acid unsaturation (Resch & Ferber, 1975), or increased intracellular concentration of calcium ions (Freedman, Raff & Gomperts, 1975) or of cyclic nucleotides (Hadden *et al.*, 1972). Certainly, some cross-linking of surface receptors by the ligand is required (Gunther *et al.*, 1973), and this will be associated with changes in the ordering and state of aggregation of membrane lipids (Curtain *et al.*, 1978), and movement of cytoskeleton components (Bray, 1978) although this last may not be essential for triggering blastogenesis (Steen & Lindmo, 1978).

Biochemical events of lymphocyte activation appear closely dependent on changes in membrane

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fluidity. Within minutes of stimulation, there is an increased turn-over of phospholipid fatty acids with increased fatty acid unsaturation (Barnett *et al.*, 1974; Resch & Ferber, 1975). The consequent increased membrane fluidity is associated with activation of membrane-bound enzymes (Kaplan, 1978), and of active transport mechanisms for calcium, potassium and zinc ions (Kaplan, 1977; Ozato, Huang & Ebert, 1977). Increase in the uptake of amino acids and glucose also relates to the activation of cell surface carrier sites (Mendelsohn, Skinner & Kornfeld, 1971; Peters & Hausen, 1971). Intracellular concentrations of cyclic AMP and cyclic GMP are regulated by the membrane-bound $(Na^+ + K^+)$-ATPase and calcium-dependent adenyl and guanyl cyclases: in activated lymphocytes initial increase in levels has been observed (Wedner & Parker, 1976; Hadden, 1977). Activation of intracellular enzymes and of synthetic pathways have been detected some hours after stimulation (Mizoguchi *et al.*, 1975); lymphokine release is recognizable within a few hours (Valdimarsson, 1976), but DNA synthesis requires 48 to 72 hr (Ling, 1968). The earliest nuclear changes detected in an activated lymphocyte are acetylation of histones and phosphorylation of non-histone proteins (Kleinsmith, Allfrey & Mirsky, 1966; Pogo, Allfrey & Mirsky, 1966; Mukherjee & Cohen, 1969).

Fluorescent probes have been defined as organic molecules having fluorescence characteristics that are dependent on their environment (Azzi, 1975). Transitions between molecular ground and excited electronic states accompanying excitation and subsequent fluorescence emission are strongly influenced by properties of the solvent and immediate molecular environment (Ward & Fothergill, 1976). The binding of a fluorescent probe to cellular macromolecules or membranes and analysis of fluorescence behaviour can yield information on polarity, fluidity and availability of binding sites, molecular proximity of specific groups, rotational mobility, and local electric fields. Parameters of fluorescence include absorption and emission spectra, intensity and quantum yield, fluorescence lifetime, and polarization (Nairn, 1976). The range of fluorescent molecules that have been used as probes of protein structure and function has been reviewed by Chance, Lee & Blaisie (1971), Brand & Gohlke (1972), and Azzi (1975). Probes for studying changes in lymphocytes are so far few, and the parameters analysed restricted largely to intensity and polarization of fluorescence.

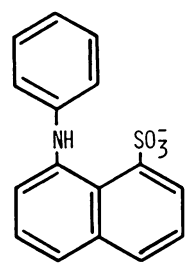
Probes of lymphocyte properties, reviewed here, will be classified according to their cellular localization. Those already in use and others which seem potentially useful for lymphocyte studies are discussed.

MEMBRANE PROBES

Fluorescent probes have been used extensively for studies of membrane structural changes related to function in several different tissue cells (Brand & Gohlke, 1972; Azzi, 1975; Radda, 1975). Analysis of discrete regions of the membrane is possible because of the different chemical characteristics of the probes, e.g. whether hydrophilic, lipophilic (hydrophobic), or amphiphilic. Consideration of data from artificial membranes has helped to determine probe localization and affinities (e.g. Thulborn & Sawyer, 1978), but should not be assumed to apply precisely to more complex biological membranes.

Cell membrane perturbations may be caused by introduction of the probe, and it is advisable to employ minimal concentration of probe and confirm retention of relevant normal cell functions after labelling. Probes are perhaps of more value for investigating membrane changes rather than their absolute properties.

1-Anilinonaphthalene-8-sulphonate (*ANS*)



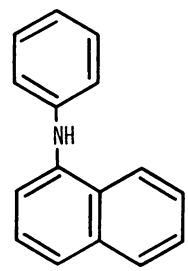
ANS is amphiphilic: its charged sulphonate group attached to the hydrophobic naphthalene-moiety dictates superficial membrane localization at polar–non-polar interfaces. The hydrophilic group binds to water or polar groups of phospholipids, and the non-polar component penetrates between the fatty acid chains of the hydrocarbon core. ANS is very sensitive to changes in polarity; its fluorescence is weak in aqueous solution, but there is an increased quantum yield and a blue-shift in emission maximum on binding to the more non-polar environment of cell membranes (reviewed by Brand & Gohlke, 1972; Azzi, 1975).

ANS has been used to study functional properties of biological membranes. Interesting applications include the detection of energization of membranes associated with electrical excitation of axons (Tasaki *et al.*, 1968), with addition of electron transport substrates to *E. coli* (Griniuviene, Dzheia & Grinius, 1975) and to plasma membranes of pig lymphocytes (Mani, Dornand & Mousseron-Canet, 1973), and with release of enzymes from isolated leucocyte lysosomes (Hawiger & Timmons, 1973). ANS has also been used to study membrane changes accompanying transport of salts and sugars into cells (Cheng & Levy, 1978; Ohyashiki & Mohri, 1979).

Detection of lymphocyte activation by mitogens has been reported by Akedo, Mori & Mukai (1976) and by ourselves (Nairn *et al.*, 1978). Our results showed a decrease in mean fluorescence intensity at 490 nm of lymphocytes stained with ANS after 3 hr incubation with pokeweed mitogen (PWM). Akedo *et al.* (1976) demonstrated increased fluorescence of ANS-stained rat thymocytes incubated for 5 min with concanavalin A (Con A). The explanation of the opposite responses to different mitogens, decreased fluorescence for PWM and increased for Con A, is not clear but we have observed similar behaviour with other probes. The phenomenon requires systematic study to establish whether opposite membrane structural changes are implicated, e.g. intermolecular relaxation and rigidity.

Fluorescence microscopy observations by ourselves and others (Dyckman & Weltman, 1970; Witz, Sivak & Van Duuren, 1973) suggest a substantial cytoplasmic localization of ANS. Vaillier & Vaillier (1977) also report binding to hydrophobic sites of the nuclear membrane and nucleoli. Fluorescence emission, either yellow-green or blue, from ANS-stained mouse thymocytes distinguished two cell populations, apparently correlating with DNA content. In a subsequent paper, Vaillier & Vaillier (1978) describe altered ANS fluorescence of spleen and thymus cells from mice immunized with bovine serum albumin or sheep erythrocytes, compared with control mouse cells.

N-phenyl-1-naphthylamine (NPN)

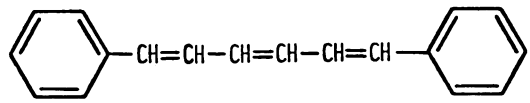


NPN is a neutral molecule, becoming fluorescent after binding deeply within the hydrophobic core of cell membranes (Sergeyev *et al.*, 1975). The fluorescence emission and its polarization can reveal changes in the polarity and rigidity of the probe's microenvironment (Radda, 1971; Edwards *et al.*, 1976). By such measurements, cell membrane changes have been demonstrated in viral transformation (Edwards *et al.*, 1976; Burleson *et al.*, 1978), after addition of colicin Ia and de-energizers such as electron transport inhibitors to *E. coli* (Nieva-Gomez, Konisky & Gennis, 1976), and in lipid phase transitions in phospholipid vesicles (Träuble & Overath, 1973; Ting & Solomon, 1975; Bashford, Morgan & Radda, 1976).

We ourselves have employed NPN for early detection of lymphocyte activation using microfluorimetry (Halliday *et al.*, 1979). The mean fluorescence intensity of NPN-labelled mouse thymocytes was con-

sistently increased 30 min after addition of Con A. Mixed incubation of BALB/c and C3H mouse spleen lymphoid cells for 30 min resulted in decreased fluorescence. This assay should provide a simple and rapid direct cross-match test of histocompatibility for tissue transplantation.

1,6-Diphenyl-1,3,5-hexatriene (DPH)



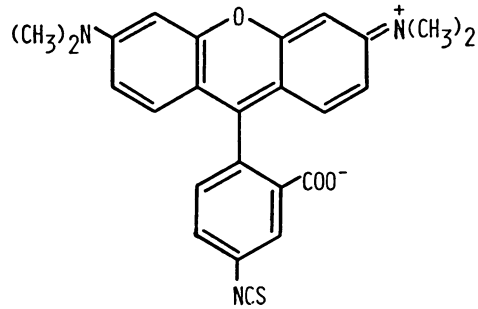
The fluorescent lipophilic probe, DPH, has been used to study hydrophobic regions of cell surface membranes, fluorescence polarization values providing an index of microviscosity (Rottem *et al.*, 1973; Shinitzky & Inbar, 1974). Studies by others using fluorescence microscopy (Collard & De Wildt, 1978) and electron microscope autoradiography (Pagano, Ozato & Ruysschaert, 1977) show that under similar staining conditions, the probe is also bound to internal membranes and lipid droplets, suggesting that fluorescence polarization data from intact cells may not be related exclusively to the plasma membrane. Isolated mitochondrial, microsomal and plasma membranes have been found to have different DPH fluorescence polarization (Gilmore, Cohn & Glaser, 1979). However, Inbar & Ben-Bassat (1976) report similar polarization data from isolated surface membranes and intact cells, and Inbar & Shinitzky (1975) describe the fluorescence microscopical appearance of DPH-labelled cells as 'a glowing ring around the cell periphery' in support of the surface localization of the probe, claimed in their experiments.

Change in DPH fluorescence polarization has been used to monitor cellular responses to many external stimuli (reviewed by Shinitzky & Barenholz, 1978). The membrane cholesterol/phospholipid ratio influences microviscosity and appears to play a role in cell growth control. Decreased viscosity preceded proliferation of cultured fibroblasts after disruption of a confluent layer (Collard *et al.*, 1977), heralded fusion of embryonic myoblasts to form multinucleated myotubes (Prives & Shinitzky, 1977), and followed treatment of rat embryo cells with tumour-promoting agents (Fisher *et al.*, 1979). When cell membrane cholesterol content was increased in Moloney virus-infected lymphoma cells, there was a decreased membrane microviscosity demonstrated by DPH; the cells when injected into mice were not as lethal as unmodified cells (Inbar & Shinitzky, 1974). Studies on cytotoxic lymphocyte–target cell interactions revealed initial exchange of DPH between the cells, suggesting that cellular lipid interchange may precede stable cell contact (Berke, Tzur & Inbar, 1978).

Con A-stimulated lymphocytes at 2 days have increased membrane lipid fluidity as shown by decreased polarization of fluorescence of cell-bound DPH (Inbar & Shinitzky, 1975; Stubbs *et al.*, 1978). The response to Con A by mouse spleen and thymus cells, assayed by ^{3}H-thymidine uptake, and the change in membrane fluidity could be inhibited by incorporating additional cholesterol into the cell membranes (Rivnay, Globerson & Shinitzky, 1978). Early changes in membrane microviscosity have been detected at 30 min using other plant mitogens. Kishiye, Toyoshima & Osawa (1974) detected increased fluidity with a mitogenic fragment of *Ricinus communis* lectin but not with a non-binding fragment. Toyoshima & Osawa (1975) reported similar findings with lectins from *Wistaria floribunda*, *Sophora japonica* and *Lens culinaris*, all of which bound to lymphocytes but not all caused transformation.

DPH has been used to discriminate between normal and malignant lymphocytes. Ben-Bassat *et al.* (1977) showed that leukaemic lymphocytes had abnormally high membrane lipid fluidity, which correlated with reduced mobility of Con A receptors. Remission of acute lymphatic leukaemia was accompanied by restoration of normal lymphocyte membrane viscosity (Inbar *et al.*, 1977). Investigation of DPH fluorescence polarization in the diagnosis of leukaemia showed reduced microviscosity in all cases of chronic lymphatic leukaemia tested and normal viscosity in other leukaemias (Blecher & Bisby, 1977). On the other hand, Johnson & Kramers (1978) observed no difference in DPH fluorescence polarization between human normal and leukaemic lymphocytes, and suggest that differences reported by others are attributable to platelet contamination of cell samples.

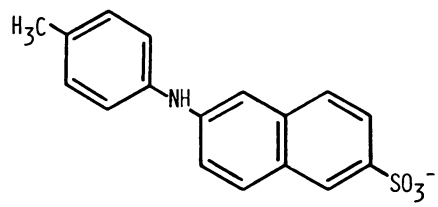
Tetramethylrhodamine isothiocyanate (TMRITC)



TMRITC is a hydrophilic probe that can be coupled by stable linkage to lymphocytes, which retain viability after labelling (Nairn *et al.*, 1979). Changes in fluorescence intensity of labelled cells have been detected 3 hr after stimulation with the mitogens PWM, Con A, and phytohaemagglutinin (PHA), and 30 min following 'mixed lymphocyte reaction'. Responses to PHA and to allogeneic cells were inhibited by addition of 5×10^{-3} M sodium azide to the incubation mixtures.

This probe seems very promising for automated cytopherometry: we now have corroborative results with mitogen stimulation by use of the Becton Dickinson Fluorescence Activated Cell Sorter (FACS II).

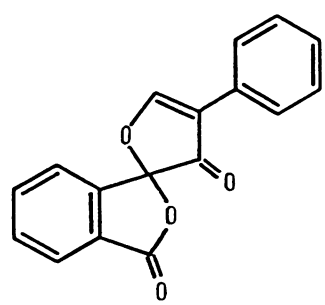
2-p-Toluidinonaphthalene-6-sulphonate (TNS)



Like ANS, TNS is practically non-fluorescent in aqueous solution, but fluoresces strongly when adsorbed to hydrophobic regions of proteins (Weber & Laurence, 1954). Studies with model lipid dispersions indicate that TNS binds at the interface between lipid polar groups and the aqueous phase (Huang & Charlton, 1972). Its superficial membrane localization in cells is also suggested by fluorescence polarization and spectra studies (Azzi, 1975) and by radioisotope labelling of the probe (Pagano, Ozato & Ruysschaert, 1977).

TNS has been used to monitor excitation of nerve cell membranes (Tasaki, Watanabe & Hallett, 1971) and the interaction of anaesthetics with human erythrocyte membranes (Kramer & Li, 1975).

Fluorescamine



Fluorescamine has been widely used as a sensitive reagent for the fluorimetric assay of primary amines, amino acids and proteins (Udenfriend *et al.*, 1972; Weigele *et al.*, 1972). It may be a useful cell probe because the unbound dye and its hydrolysis product are non-fluorescent (Puchinger, von Sengbusch & Sernetz, 1976); labelling is restricted to the outer surface of erythrocytes (Nakaya *et al.*, 1975)

and fibroblasts (Hawkes, Meehan & Bissell, 1976). Recently, fluorescamine was used to analyse surface membrane proteins of lymphocytes (Lambris, Papamichail & Fessas, 1979). Preservation of cell viability is a problem when labelling with this compound and the fluorescence depreciates within hours.

Other membrane probes

Fluorescent derivative of phosphatidylcholine. This has been used to detect changes in the membranes of PHA-stimulated human lymphocytes (Monti *et al.*, 1977). Fluorescence intensity was increased with a blue-shift in emission maximum wavelength and fluorescence polarization was increased.

F20C. F20C, a fluorescein derivative, in particulate suspension with A_2C (Kosower *et al.*, 1978) entered and dispersed more slowly in membranes of thymus cells cultured for 72 hr with staphylococcal enterotoxin B mitogen.

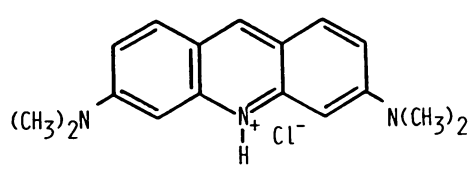
Other potentially useful probes. Other useful probes for studying membrane events in lymphocyte activation include those which monitor: membrane pH, viz. 1-dimethylaminonaphthalene-5-sulphonyl (dansyl) cephalin (Vaz, Nicksch & Jähnig, 1978), 9-aminoacridine (Elema, Michels & Konings, 1978); membrane permeability, viz. dansyl chloride (Schmidt-Ullrich, Knüfermann & Wallach, 1973); membrane-bound divalent cations, viz. chlorotetracycline (Chandler & Williams, 1977; Täljedal, 1978); transmembrane potential difference, viz. carbocyanines (Laris, Pershadsingh & Johnstone, 1976; Morgan *et al.*, 1976; Philo & Eddy, 1978), merocyanine 540 (Easton, Valinsky & Reich, 1978; Valinsky, Easton & Reich, 1978); membrane-bound $(Na^+ + K^+)$-ATPase activity, viz. formycin diphosphate and formycin triphosphate (Karlish, Yates & Glynn, 1976).

Probes reported to bind to different regions of the cell membrane include those binding to superficial protein sulphydryl groups, viz. maleimide dyes (Kanaoka *et al.*, 1968; Sekine *et al.*, 1972; Kanaoka *et al.*, 1973; Machida *et al.*, 1975; Kanaoka *et al.*, 1976; Mercado *et al.*, 1976), and other groups, viz. 4-acetamido, 4′-isothiocyanostilbene-2, 2′-disulphonic acid (SITS) (Maddy, 1964), fluorescein isothiocyanate (Roder & Kiessling, 1978); to hydrophobic areas, viz. dansyl cadaverine (Pincus *et al.*, 1975), 12-(9-anthroyloxy)-stearic acid and 2-(9-anthroyloxy)-palmitic acid (Bashford, Morgan & Radda, 1976), pyrene and pyrene butyric acid (Burleson *et al.*, 1978), perylene (Pagano, Ozato & Ruysschaert, 1977), and retinol (Radda & Vanderkooi, 1972).

CYTOPLASMIC PROBES

Fluorescent probes for monitoring cytoplasmic changes associated with lymphocyte activation include acridine orange (AO) to detect lysosomal activation (Rolland *et al.*, 1976), and fluorescein to detect 'structuredness of the cytoplasmic matrix' (Cercek & Cercek, 1977). Studies with other cell types suggest that metabolic activity may be detected by the blue fluorescence of the reduced coenzyme NADPH (Kohen *et al.*, 1979), or by the use of fluorogenic substrates (Marchesini *et al.*, 1973; Kaplow, Dauber & Lerner, 1976).

Acridine orange (AO ; 3,6-bis(dimethylamino)acridinium chloride)



Vital staining of cells with AO reveals red fluorescent lysosomal granules against green fluorescence of the cell body (Allison & Mallucci, 1964; Zelenin, 1966). We have found that stimulation of lymphocytes for 3 hr by antigen or mitogen results in an increased number of red lysosomes attributable to altered permeability of the lysosome membrane to AO (Rolland *et al.*, 1976). Analysis of cellular AO fluorescence by flow cytometry has also revealed increased red fluorescence in PHA-stimulated lymphocytes at 4 hr,

reaching a plateau between 20 and 24 hr (Braunstein *et al.*, 1979). This effect was not seen with cells fixed in 1:1 ethanol/acetone before staining, apparently because of disruption of lysosome membranes.

Fluorescein diacetate

Fluorescein diacetate, which is itself non-fluorescent, is hydrolysed by intracellular esterases to yield the fluorescent product, fluorescein. The fluorescein molecule is polar and extruded from the cell more slowly than the non-polar fluorescein diacetate enters, resulting in intracellular accumulation of fluorescein. Retention of fluorescein is dependent on the integrity of the cell membrane (Rotman & Papermaster, 1966). Thus, the production and intracellular persistence of fluorescein provide an excellent index of cell viability (Persidsky, 1973; Sernetz, 1973; Von Sengbusch & Hugemann, 1974). The rate of escape of fluorescein from the cell is temperature-dependent (Rotman & Papermaster, 1966), and also influenced by the concentration of medium components such as albumin (Bruning, Kardol & Naipal, 1975). Visser, Jongeling & Tanke (1979) reported an interesting correlation between the fluorescence excitation spectrum and intracellular pH.

Measurements of cell fluorescence intensity and polarization have been applied to the detection of stimulation of lymphocytes incubated with fluorescein diacetate. Wilder & Cram (1977) demonstrated differential staining intensities of human T and B lymphocytes by flow cytometry, and Braunstein *et al.* (1979) observed increased fluorescence of mouse spleen cells after 4 hr stimulation with PHA. Following the initial observations of Cercek, Cercek & Garrett (1973), altered polarization of fluorescence after lymphocyte activation has been confirmed in several studies discussed below.

Cercek and colleagues reported that patients with malignant disease can be recognized by polarization changes in their lymphocytes stimulated for 30 min with PHA and with cancer basic protein (Cercek, Cercek & Franklin, 1974; Cercek & Cercek, 1977). After tumour resection, polarization values returned to normal (Cercek & Cercek, 1975a), and tumour specificity of the test has been claimed (Cercek & Cercek, 1975b, 1977). Other workers confirmed the usefulness of this test in the diagnosis of cancer (Takaku, Yamanaka & Hashimoto, 1977; Hashimoto, Yamanaka & Takaku, 1978; Kreutzmann *et al.*, 1978; Pritchard & Sutherland, 1978). Change in fluorescence polarization has also been observed in mixed lymphocyte reactions at 30 min between lymphocytes of two human subjects (Cercek & Cercek, 1976a), and of two inbred strains of mice (Nairn *et al.*, 1978).

The reproducibility and reliability of the fluorescence polarization test is entirely dependent on meticulous attention to technical detail, including the preparation of glassware and of cells (Bagshawe, 1977; Cercek & Cercek, 1977). Systematic polarization changes are found only when carefully defined cellular fractions are tested; mononuclear cells in the upper layer or part of the layer of cells sedimenting above a medium of defined density and osmolality must be used (Cercek & Cercek, 1978; Hashimoto, Yamanaka & Takaku, 1978; Pritchard & Sutherland, 1978). Carbonyl-iron-ingesting cells are removed from the blood before sedimentation. Differential counting of the final cell suspension reveals 80–90% lymphocytes, 5–10% monocytes and 3–7% neutrophils and eosinophils (Kreutzmann *et al.*, 1978). Measurements on single cells, using a microscope fluorimeter, instead of on the whole suspension in a cuvette, indicate that 45–54% of the normal human lymphocyte fraction responds to PHA (Cercek & Cercek, 1976b). Adaptation of the measurements to flow systems should permit more detailed analysis of the cell type responding (Price *et al.*, 1977; Stewart *et al.*, 1979; Udkoff & Norman, 1979).

Interpretation of this phenomenon is complex. The Cerceks suggest that the fluorescence polarization responds to change in local microviscosity or 'structuredness of the cytoplasmic matrix' (SCM). The SCM is a reflection of the forces of interaction between macromolecules and smaller entities such as water molecules and ions (Cercek & Cercek, 1977), and according to Cercek & Cercek (1973) and Cercek, Cercek & Ockey (1973) could conceivably have a regulatory function on aspects of cellular metabolism.

On the other hand, polarization changes have been ascribed in part to altered kinetics of fluorescein accumulation, because polarization and intensity values are inversely related (Epstein *et al.*, 1977). Inferred binding of fluorescein to intracellular proteins is supported by shifts in the absorption and emission maximum wavelength; variations in this binding could also cause the observed polarization changes associated with cell activation (Udkoff & Norman, 1979).

NUCLEAR PROBES

Fluorescence cytochemistry of nucleic acids is the longest established of the fluorescent cell probe techniques. It has provided information on the secondary and tertiary structure and functional activity of nucleic acids as well as practical application to cell discrimination (reviewed by Zelenin, 1977). Reagents for these studies include intercalating dyes (e.g. AO, ethidium bromide, propidium iodide), fluorescent Schiff-type reagents (e.g. acriflavine, flavophosphine N, Auramine-O), and fluorescent natural product antibiotics (e.g. mithramycin, chromomycin A_3), also discussed by Kasten, Burton & Glover (1959), Trujillo & Van Dilla (1972), Crissman, Oka & Steinkamp (1976). Staining procedures have often required fixation of the cell or temporary disruption of membrane permeability. An interesting new group of bisbenzimidazole dyes developed by the Hoechst Company permits staining of DNA in viable cells, localizing at particular base pairs without intercalation (Loewe & Urbanietz, 1974; Latt, 1977).

Acridine orange (AO)

Investigation of nuclear changes during lymphocyte activation has been achieved largely by labelling with AO. This dye fluoresces green in monomer form when in low concentration in solution or intercalated into double-stranded DNA, and fluoresces red when polymerized in concentrated solution or after binding to single-stranded RNA or DNA. Analysis of fluorescence from AO-stained fixed cell smears has permitted recognition of changes in lymphocytes within minutes of mitogen stimulation (Killander & Rigler, 1965, 1969; Rigler, 1966; Barbaruk & Brit, 1976; Mysliwska, Mysliwski & Witkowski, 1976). We ourselves have also observed increased green fluorescence in lymphocytes after 30 min stimulation by the mitogens PHA, PWM, Con A, and by allogeneic cells (Nairn *et al.*, 1978).

Increased fluorescent staining has been interpreted as increased availability of AO binding sites in the deoxyribonucleoprotein (DNP) complex caused by a weakening of ionic bonds between DNA and chromatin proteins. That a release of histones occurs on lymphocyte activation is supported by the rapid decrease in staining observed with alkaline fast green after PHA stimulation of lymphocytes (Burton, 1968). Weakening of the DNP complex has also been correlated with its decreased heat stability 60 min after PHA stimulation (Rigler & Killander, 1969; Zlobina, 1974). Uncritical acceptance that changes in AO staining of fixed cell smears are due to increased availability of nucleic acid binding sites has been challenged by Bolund, Darzynkiewicz & Ringertz (1970), who reported that increased fluorescence could be caused by local cell crowding.

AO staining of cell suspensions has now been achieved by such procedures as treatment with Triton X-100 at low pH (Traganos *et al.*, 1977). The cell populations in the G_1 (increased red fluorescence), S (increased red and green fluorescence), and G_2+M (increased green fluorescence) cell cycle phases, after mixed lymphocyte culture, could be detected and analysed by flow cytometry.

FLOW MICROFLUORIMETRY

The availability of flow microfluorimetry techniques permits individual cell analysis on much larger cell numbers than is possible by manual procedures. This provides greater accuracy and sensitivity, and the possibility of recognizing changes in a small proportion of cells in the sample studied. Application of flow systems to the detection of lymphocyte activation has so far been largely restricted to analysis of nuclear changes. Nucleic acid staining techniques allow determination of the proportion of cells in particular phases of the cell-cycle, and also automated cytopathology screening. Methodology and applications are discussed in a special number of the *Journal of Histochemistry and Cytochemistry*, in which highly relevant papers to the present review are by Braunstein *et al.* (1979) and Lalande & Miller (1979).

In comparison with ^{3}H-thymidine uptake studies, cell flow techniques permit identification of particular responding populations and appear to be more sensitive (e.g. Utsinger *et al.*, 1977). Furthermore, correlation with assays of cellular synthetic events suggests that different reactivities are measured at earlier times after stimulation (Pollack *et al.*, 1979). Combined analysis thus provides a more accurate total picture of cell behaviour.

Use of flow systems to monitor membrane and cytoplasmic events associated with lymphocyte activa-

tion will greatly enhance the usefulness of fluorescent probe assays. Vital staining techniques permit viable reactive cells to be sorted and further characterized functionally (Lalande & Miller, 1979). The rapid analysis by sophisticated automated equipment has an important bonus value in allowing study of adequate samples of test and control living cell populations before material changes occur in viability or other physiological attributes which would otherwise affect the results of the analysis.

In addition to providing new insight into the cellular events and regulation of lymphocyte activation, analysis of fluorescence parameters of probes by flow systems facilitates fast and sensitive routine diagnostic procedures. Testing of patients' lymphocyte response to antigens and mitogens is feasible, and detection of individual antigens, such as cancer cell products, should be possible through their activation of known reactive fluorescent immunocytes.

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