*Proc. Natl. Acad. Sci. USA*
Vol. 75, No. 8, pp. 3766–3770, August 1978
Cell Biology

# Retention of susceptibility to mitogens after direct dansylation of viable human lymphocytes

(plasma membrane/fluorescent probe/lectin)

PETER S. COLEMAN*, ANTHONY J. EWELL†, AND ROBERT A. GOOD†

* Laboratory of Biochemistry, Department of Biology, New York University, New York, New York 10003; and † Memorial Sloan-Kettering Cancer Center, New York, New York 10021

*Contributed by Robert A. Good, April 20, 1978*

ABSTRACT **The covalently binding fluorescent probe 5-dimethylamino-1-naphthalenesulfonyl (dansyl) chloride was affixed directly to the plasma membrane of viable human peripheral blood lymphocytes via a solid phase transfer method utilizing Sephadex G-10 as the transfer vehicle. After dansylation, lymphocytes retain maximal short-term viability. Dansyl, as the protein conjugate or as the free acid, does not appear to penetrate the cells to any significant extent. Dansylated mixed lymphocyte cultures respond to lectin mitogen stimulation for at least 72 hr. Furthermore, differential response of dansylated lymphocytes in culture to three plant lectin mitogens provides a clue to the binding loci of concanavalin A with respect to phytohemagglutinin and pokeweed mitogen on the lymphocyte surface receptors for these lectins. The ability to sustain functionally responsive dansylated lymphocytes for several days in culture suggests that such probe-tagged cells may be useful in elucidating aspects of the plasma membrane in the regulation of cell behavior.**

In the study of biological membranes, extrinsic fluorescent probe molecules provide very sensitive measures of their immediate environment by the emission they produce upon excitation. The use of fluorescent probes has made possible the estimation of relative rotational mobility of lectin-binding sites, and fluorescence anisotropy measurements have permitted the determination of relative membrane viscosities of malignant transformed and normal cells (1, 2). With viable intact cells, the fluorophore is usually conjugated first to a surface receptor ligand, such as the sugar-binding lectin concanavalin A (Con A), and then this fluorescent conjugate is reacted with the surface of the plasma membrane of whole cells (3). However, the direct introduction of covalent fluorescent probe molecules onto the surface of whole cells and the subsequent analysis of cellular function have not been extensively studied (4).

We have instituted studies on the effects of fluorescent probes covalently bound to the surface of viable human peripheral blood lymphocytes. Two familar fluorogenic molecules which react with primary amino groups were examined: 5-dimethylamino-1-napthalenesulfonyl chloride (dansyl-Cl) and fluorescein isothiocyanate. We have had more methodological success employing a solid-phase probe transfer technique with the water-insoluble dansyl-Cl, and the data we report here deal exclusively with the effects of this probe.

We have investigated several properties of isolated, cultured human peripheral lymphocytes after direct dansyl labeling via our probe transfer method. Our data provide information about the effect of plasma membrane-bound dansyl on short-term cell viability, the tendency for dansyl to penetrate the intracellular milieu together with the extent of probe binding to lymphocyte plasma membrane, and the responsiveness of such probe-labeled lymphocytes to lectin stimulation.

A most important finding of these studies is that for at least 72 hr the covalently bound probe does not extinguish the broad functional events that characterize the responses of mixed populations of lymphocyte cultures exposed to mitogens.

## METHODS

**Lymphocyte Isolation from Peripheral Blood.** Whole blood (type A-positive) was collected from random, healthy adults (18–65 years old), heparinized, and diluted 1:1 (vol/vol) with Hanks' balanced salt solution (HBSS). The lymphocytes were separated according to Boyum (5) in Ficoll/Hypaque and washed three times by centrifugation with HBSS, then resuspended in the same medium. Dye exclusion (trypan blue, 0.4%) cell counts were routinely performed in a hemocytometer.

**Direct Dansylation of Viable Lymphocytes.** Dried, acetone-washed Sephadex G-10 beads (Pharmacia), devoid of fines, were added to an acetone solution of dansyl-Cl (Sigma), resulting in a system that was 4.2% probe/bead (wt/wt). The acetone was removed by rotary evaporation under reduced pressure, and the dried dansyl-Cl-bead adduct was stored in the dark at −20°. To label the cells, $4.5 \times 10^6$ viable lymphocytes per ml of HBSS, containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer, pH 7.2, were incubated with the dansyl-Cl-bead adduct (2.0 mg of adduct per $10^6$ viable lymphocytes) for 30 min at room temperature, with initial gentle inversion. A Ficoll/Hypaque centrifugation of the cell–bead suspension separated the dansyl-labeled lymphocytes from the probe-bead adduct. The lymphocytes were washed three times in HBSS or, if the preparation was to be cultured, in RPMI 1640 medium (Gibco).

For all studies that involved subsequent culture of the dansylated cells, two control preparations were examined simultaneously (see Fig. 1). Sham-treated lymphocytes were incubated with plain ("nude") acetone-washed Sephadex G-10, according to the dansyl-Cl labeling protocol. Time control lymphocyte suspensions were incubated without beads but otherwise handled by means of the dansyl-Cl labeling procedures.

**Plasma Membrane Isolation: Fluorescence Localization of the Dansyl.** Lymphocyte components were fractionated (6) in RNase-free 25–50% (wt/vol) linear sucrose gradients, made with membrane buffer (5 mM $MgCl_2$/10 mM Tris·HCl buffer, pH 7.4) (see Fig. 2). Aliquots (250 μl) of the isopycnic fractions were collected from the centrifuge tube bottoms, and sucrose densities were calculated from refractive index measurements made on alternate aliquots. Fluorescence measurements were performed on the remaining aliquots, after 1:50 dilution with the membrane buffer. A modified Cary 14 spectrophotometer,

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: dansyl-Cl, 5-dimethylamino-1-napthalenesulfonyl chloride; HBSS, Hanks' balanced salt solution; PHA, phytohemagglutinin; PWM, pokeweed mitogen; Con A, concanavalin A; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

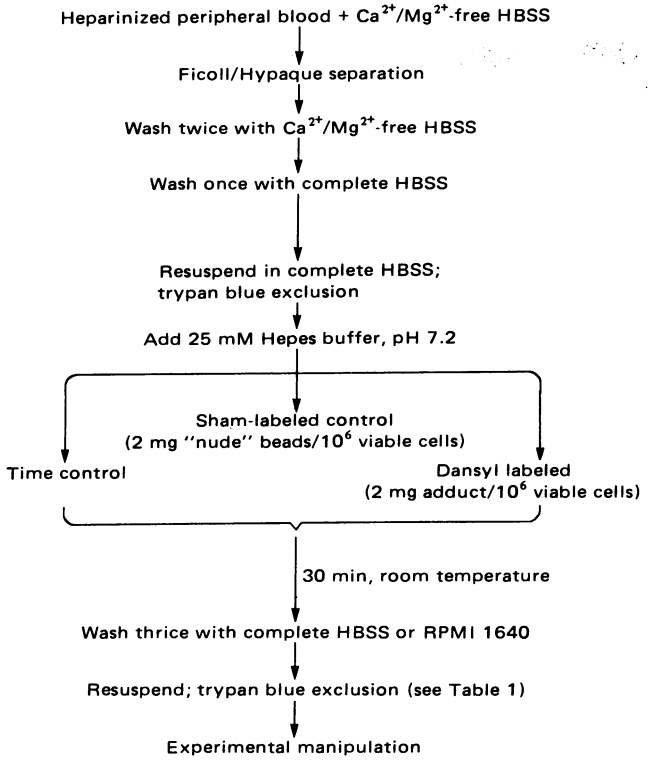


FIG. 1. Sequence of methods used to covalently label viable, isolated, human peripheral circulating lymphocytes in suspension with dansyl-Cl. Three parallel cell aliquots were routinely processed (time control, sham labeled, and dansyl labeled) for subsequent experimental study.

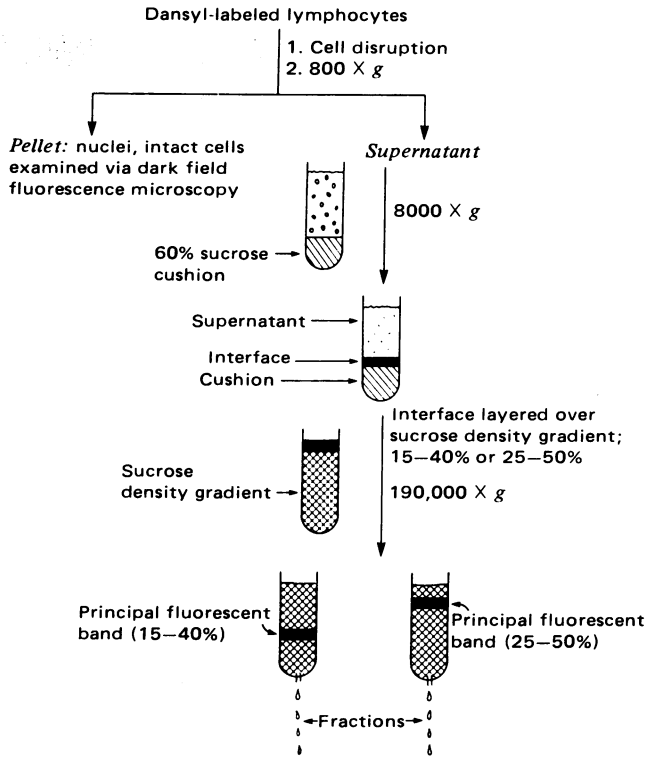


FIG. 2. Isolation of the plasma membrane fraction of danylated lymphocytes. The use of two different linear sucrose gradients yielded differentially sedimenting fluorescent bands corresponding to plasma membrane fractions according to isopycnic density.

equipped with front-face fluorescence data acquisition, was employed. Light scattering corrections were not applied.

**Specificity of Dansyl Localization: $^{125}$I/Dansyl Mixed-Label Analysis.** (Figs. 3 and 4.) The surfaces of viable lymphocytes were radioiodinated. Cells ($2 \times 10^7$) were incubated at room temperature in a total of 285 $\mu$l containing 10 $\mu$l of 0.03% $H_2O_2$, 25 $\mu$l of lactoperoxidase at 5 mg/ml (Sigma), 2 mCi of $Na^{125}I$ (New England Nuclear), and 250 $\mu$l of phosphate-buffered saline, pH 7.4. The incubation was terminated after 10 min by rapid dilution with 12 ml of phosphate-buffered saline.

A simultaneously prepared suspension of dansyl-labeled lymphocytes was combined with the $^{125}$I-labeled cells, and the mixed-probe cell suspension was washed with HBSS. Trypan blue exclusion for this system was greater than 97%. This suspension was then taken through plasma membrane isolation techniques, employing both 15–40% and 25–50% (wt/vol) linear sucrose density gradient centrifugation. Fractions (250 $\mu$l) were collected from the tube bottoms and the radioactivities of 100-$\mu$l aliquots were measured in an LKB model 80,000 gamma counter. After radioactivity assay, these 100-$\mu$l aliquots were diluted 1:5 with membrane buffer and assayed for fluorescence intensity at 490 nm.

**Probe Permeation: [$^3$H]Dansyl Cell Fraction Enrichment.** (Fig. 5.) [*Methyl*-$^3$H]dansyl-Cl (New England Nuclear) was bound to Sephadex G-10 in the presence of carrier dansyl-Cl by the procedures outlined ($5.4 \times 10^5$ cpm/mg of adduct). A suspension of viable lymphocytes, prepared as described above, was divided in half. One aliquot of whole cells was dansylated as described. The second aliquot was first subjected to Dounce homogenization after a 30-min osmotic shock in cold deionized water, and the homogenate was then incubated with the radioactive probe-bead adduct. Both whole cells and homogenate were allowed to react with the same amount of [$^3$H]dansyl-Cl under identical conditions. The dansylated whole cell aliquot was disrupted via osmotic swelling and Dounce treatment. Centrifugal fractions of the homogenates were examined for $^3$H probe enrichment. The covalently bound dansyl was selectively precipitated with ice-cold 10% (wt/vol) trichloroacetic acid, and the precipitate was collected on 0.45-$\mu$m pore diameter cellulose filters in a filter manifold device (Millipore). The filters containing precipitate were washed with cold 10% trichloroacetic acid, removed to scintillation vials containing 15 ml of Instagel (Packard), and assayed for radioactivity in a liquid scintillation spectrometer. Both homogenate systems were assayed for protein content (7) to normalize the radioactivity data.

**Cell Culture of Mitogen-Stimulated, Dansylated Lymphocytes.** Lymphocytes were cultured for 72 hr in the presence of plant lectin mitogens (8) in wells of microtiter culture plates

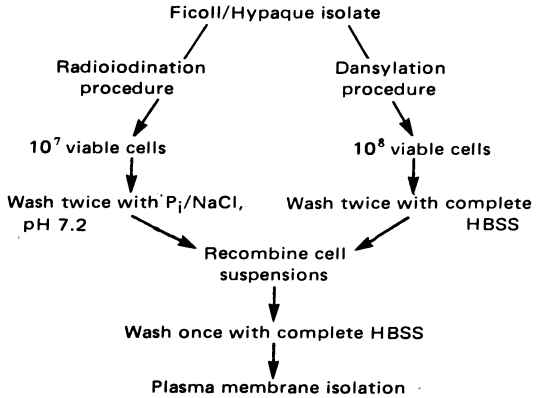


FIG. 3. Preparation of the mixed probe-labeled lymphocyte suspension. The cell suspension contained $^{125}$I-labeled and dansyl-labeled lymphocytes. The plasma membrane fraction was obtained according to Fig. 2. $P_i$/NaCl, phosphate-buffered saline.

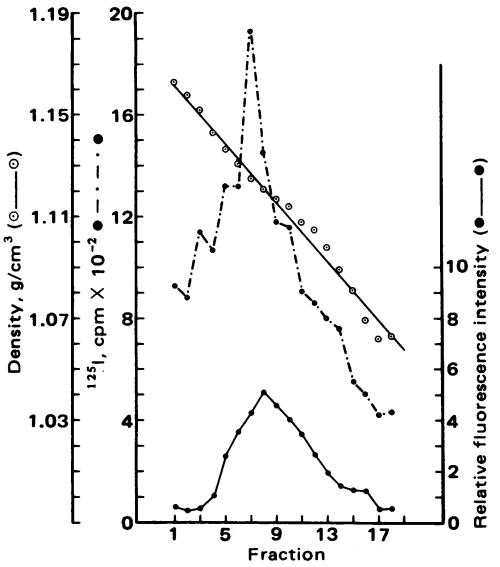


FIG. 4. Mixed probe-labeled plasma membranes in suspension. Aliquots of the combined plasma membrane fraction were collected from isopycnic 15–40% continuous sucrose density gradients and assayed for both $^{125}I$ and dansyl fluorescence at 490 nm. Fractions were ~0.28 ml.

(Cooke Engineering, London). [2-$^{14}$C]Thymidine (0.025 μCi/ml; New England Nuclear, 54.6 Ci/mol) was added to each culture well at 68 hr, and after a 4-hr pulse the cultures were terminated. The contents of each microtiter plate well were collected onto AH glass fiber filters (Reeve Angel), using an automated cell harvesting device (Otto Hiller, Madison, WI). The filters were washed, air dried overnight, cut, and assayed with a liquid scintillation spectrometer in a cocktail containing 3.0 g of 2,5-diphenyloxazole (PPO) plus 0.1 g of 1,4-bis[2(5-phenyloxazolyl)]benzene (POPOP) per liter of toluene.

Three plant lectin mitogens were assayed for their ability to promote DNA synthesis in dansylated and control lymphocyte cultures. Dose–response data for solutions of phytohemagglutinin (PHA) P and Con A (Difco) and pokeweed mitogen (PWM) (Gibco), prepared aseptically in RPMI 1640 medium, were generated from 25-μl additions of the lectin to the culture wells. Control cultures received 25 μl of RPMI 1640 medium.

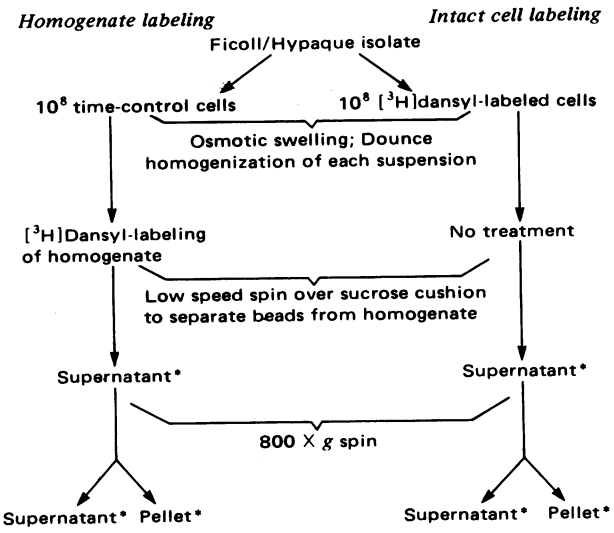


FIG. 5. Probe penetration studies via dansylation of intact and disrupted lymphocytes. The procedures outlined were performed with aliquots from the same initial Ficoll/Hypaque lymphocyte suspension (see Table 2).

* Fractions normalized for protein content before and after trichloroacetic acid precipitation.

## RESULTS

**Direct Dansylation and Short-Term Viability.** Table 1 demonstrates that neither dansylation nor the solid phase labeling had deleterious effect on the short-term permselective functions of the labeled lymphocytes. Moreover, routine phase contrast microscopy of labeled and time control suspensions revealed no discernible alterations in cellular morphology over a period of several hours. Significantly, dansyl labeling was performed under physiological conditions.

**Dansyl Enrichment of the Plasma Membrane.** Fig. 2 diagrams the process employed for the isolation of lymphocyte plasma membranes. Cells that escaped fragmentation via Dounce homogenization fluoresced clearly, whereas nuclei from disrupted cells did not fluoresce. This was the first indication of the apparent restriction of the dansyl probe to the cell periphery. The nucleus of the mitotically quiescent lymphocyte occupies more than 80% of the internal cell volume, and would be readily accessible to any penetrant probe. Fig. 6*D* is a photograph of dansyl-labeled lymphocytes obtained via dark field fluorescence microscopy. The large nucleus is clearly discerned as a dark, intracellular, light-absorbing filter, surrounded by highly fluorescent regions of the cell periphery. Were the dansyl bound to both the nuclear envelope and the plasma membrane, the nucleus would appear at least as bright as the cell periphery.

Further processing of the homogenate supernatant resulted in a strongly fluorescing, minimally diffuse band in the centrifuge tubes, observed via UV lamp, as depicted in Fig. 2. Gradient fractions were examined in a spectrofluorometer and relative fluorescence intensity was correlated with isopycnic sucrose density. Peak emission intensity appeared in plasma membrane fractions of sucrose densities between 1.11 and 1.13 g/cm$^3$ (9–11).

Confirmation of the plasma membrane localization of the fluorescent probe was obtained by means of a "mixed probe" experiment. Separate portions of a viable lymphocyte suspension were tagged with either dansyl-Cl or $^{125}I$ and then recombined, and the plasma membrane fraction was isolated. Fig. 3 presents our general procedural sequence, and Fig. 4 displays the results obtained from this heterogeneously labeled plasma membrane fraction derived from the mixed probe lymphocyte population. The isopycnic sucrose density gradient fractions examined by fluorometry and radioisotope counting show coincident peaks of fluorescence and radioactivity at gradient densities that are characteristic of lymphocyte plasma membrane preparations.

**Studies on Intracellular Penetration of Dansyl.** Our data suggest that, when dansyl is transferred to the lymphocyte

Table 1. Plasma membrane integrity after dansyl labeling

| Condition | Mean dye exclusion, % | Median and CI, % |
|---|---|---|
| Time control | 95.68 ± 0.50 | 96.80, 94.31–97.77 |
| Sham labeled | 95.98 ± 0.72 | 96.99, 95.70–98.31 |
| Dansyl labeled | 95.20 ± 0.62 | 96.24, 93.91–97.24 |

Trypan blue dye exclusion by peripheral blood lymphocytes. The median and 95% confidence interval (CI) agree with the mean values for dye exclusion. Cells from 38 volunteer blood donors were used to compile these data.

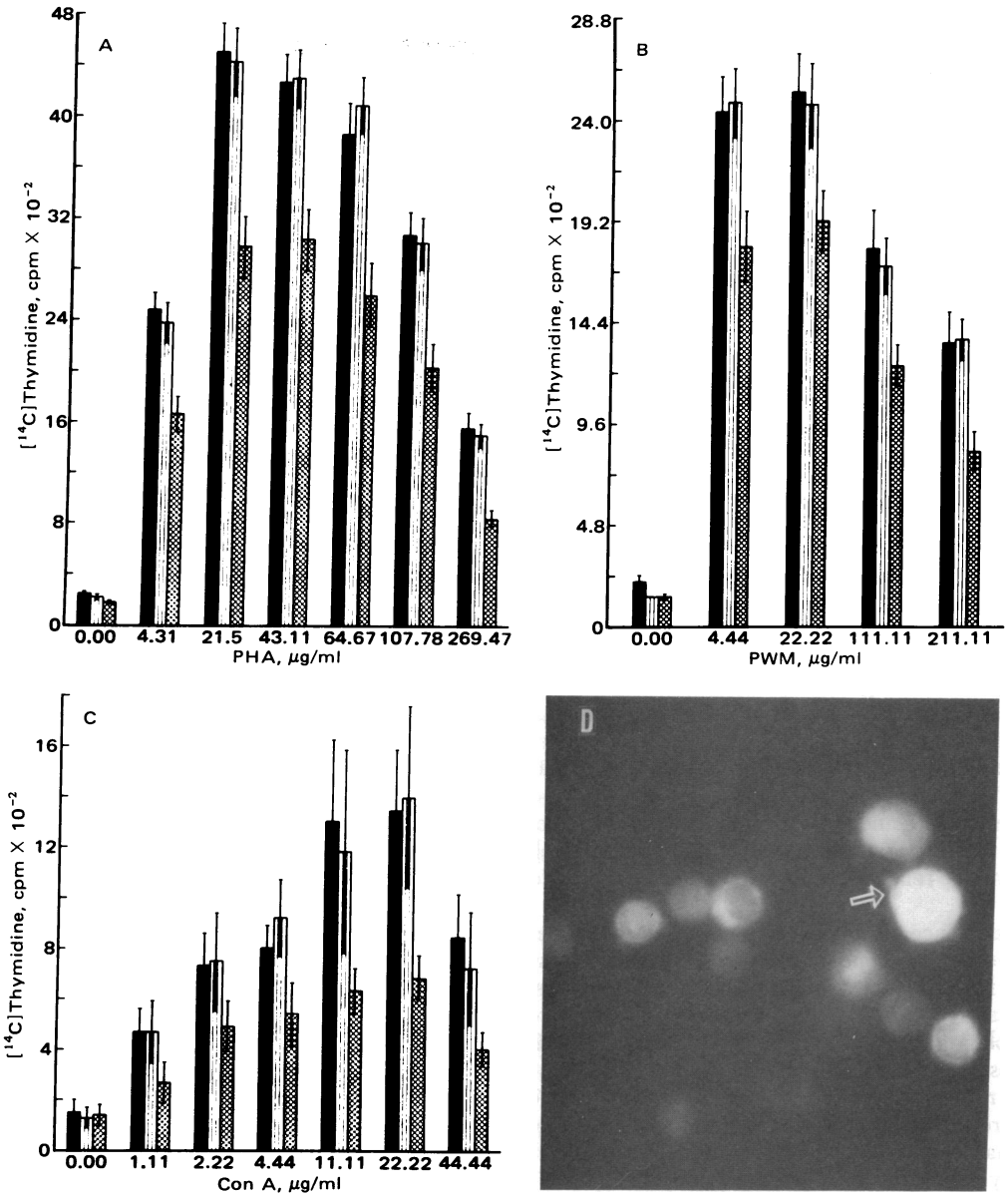


FIG. 6. Mitogen-induced blastogenesis of dansylated lymphocytes in 72-hr culture. Dose–response pattern with (*A*) PHA; (*B*) PWM; (*C*) Con A. For each mitogen level tested, time-control (black bars), sham-labeled (clear bars), and dansyl-labeled (cross-hatched bars) cells were assayed. Vertical lines within each cell group indicate SEM. (*D*) Photomicrograph of viable, unfixed dansylated lymphocytes as seen under dark field fluorescence conditions. (×1200.) A transparency was made with high-speed Ektachrome (daylight) film and printed via the Cibachrome process (Ilford, Paramus, NJ). Dansyl has penetrated the cell indicated by the arrow, and its nucleus is highly labeled. The remaining cells have excluded the probe such that the nuclei appear as dark intracellular shadows with roughly spherical outlines.

surface by the methods described above, its localization is rather stringently restricted to the plasma membrane. The experiment outlined in Fig. 5 provides a direct demonstration.

Dansyl-Cl is known to be readily hydrolyzed to the free acid (dansic acid) in aqueous medium at physiological pH (12). Both dansyl moieties display some distinguishing characteristics. The dansic acid is incapable of covalent interaction with $\alpha$-$NH_2$ groups, yet is rather lipophilic and quite fluorescent, with peak emission in the blue-green region of the spectrum (near 500 nm). On the other hand, covalently bound dansyl, as the protein conjugate amide, displays a green to yellow-green fluorescence (near 530 nm). Furthermore, the free acid is soluble in cold trichloracetic acid, whereas protein-conjugated dansyl is not, the thioamide bond being stable to such treatment. Thus, the two types of dansyl moiety can be separated by precipitation of the protein-bound probe with trichloroacetic acid (13).

In Table 2 we show that in fact both forms of the probe, the free acid and the protein-conjugated dansyl, did not appear to significantly permeate the intracellular space of intact viable cells, because the cells were shown to exclude approximately 95% of the radioactivity (total or covalent) otherwise retrieved when broken cells were dansylated.

**Retention of Response to Mitogens after Dansylation.** Dansylated lymphocytes were treated with lectins for three days in culture. The dose–response profiles, compared to sham-treated and true-time control cultures, are given in Fig. 6 *A*, *B*, and *C*. The general pattern of lectin dose–response was similar in probe-labeled, sham-treated, and time-control cultures. However, the dansyl labeling of lymphocytes differentially reduced the amount of thymidine incorporated at maximal doses of lectin. Peak Con A response was diminished about 50%, while that for PHA and PWM was lowered by approximately 30%. Bound dansyl itself is not mitogenic.

Table 2. Intracellular [$^3$H]dansyl penetration

| Fraction | cpm/μg protein: Intact cells | Homogenate | % intracellular dansyl exclusion |
|---|---|---|---|
| | Total dansyl | | |
| Total homogenate | 571.1 | 10,019.0 | 94.3 |
| 800 × *g* supernatant | 1101.2 | 13,235.3 | 91.7 |
| 800 × *g* pellet | 154.6 | 7,564.1 | 98.0 |
| | Covalent dansyl | | |
| Total homogenate | 33.3 | 689.1 | 95.2 |
| 800 × *g* supernatant | 32.4 | 406.1 | 92.0 |
| 800 × *g* pellet | 11.4 | 292.3 | 96.0 |

Cellular location of [$^3$H]dansyl. Total dansyl is measured prior to protein precipitation with 10% trichloroacetic acid, while covalent dansyl is assumed to be that measured in the precipitate subsequent to such treatment. Analysis was performed according to the procedures given in Fig. 5.

## DISCUSSION

The use of fluorescent organic molecules to investigate membrane structure and function is now widespread (see ref. 14 for a comprehensive review). According to a recent review on cell surface labeling (15), a nonpermeant reagent should react exclusively with chemical groups at the cell periphery and manifest no internal labeling. Because the overwhelming majority of membrane probe studies have utilized the mammalian erythrocyte, the extent of hemoglobin labeling after cell lysis is a sensitive measure by which to assess probe penetration with such cells (16). With nucleated cells, however, such probe localization studies are few, and there exist no criteria based upon corroboration from different laboratories that can be safely employed to indicate relative probe permeation into the cytoplasmic milieu.

Despite the presence of covalently bound dansyl on their surface, lymphocytes respond to mitogenic stimuli. Dansyl appears to have little effect on viability, mitogen sensitivity, and cell proliferation even through the fluorophore is highly restricted to the plasma membrane of the lymphocyte (Table 2). Differences observed in the response to several plant lectin mitogens may reflect an effect of dansyl on some aspect of lectin–surface receptor interaction. A given surface receptor glycoprotein may share the ability to bind different lectins independently by possessing mixed affinity for several lectins.

Studies on the inhibition of PHA and Con-A-induced mitogenesis by a porcine thyroglobulin-derived glycopeptide (17) suggest that these two lectins share an oligomeric carbohydrate moiety as a binding locus. PHA appears to prefer D-mannose residues that are more distal (from the cell surface) than the residues preferred by Con A, evidenced by shorter binding-activation times required by the PHA (18). Furthermore, whereas *N*-acetylneuraminic acid does not display specific affinity for PHA, Con A, or PWM, its removal should nevertheless allow for a less steric and charge-dependent hindrance of Con A diffusion to its receptor site close to the plasma membrane surface. If these considerations have merit, then Con A binding should be enhanced by neuraminidase treatment of cells, while that for PHA should be minimally affected, and this is what is found (19). T-lymphocyte blastogenesis is also known to be promoted by $NaIO_4$ (20). The periodate reaction effects aldehyde formation of vicinal alcoholic groups on the oligosaccharide side chains, and agents that block such alcohol-to-aldehyde oxidations (e.g., $KBH_4$) inhibit $NaIO_4$-induced blast transformation. Significantly, $KBH_4$ does not affect T-cell transformation by Con A (21). These arguments fortify the proposition that PHA, and by extension PWM, binds to the cell surface at a somewhat greater distance from the plasma membrane bilayer than does Con A.

The covalent attachment of dansyl-Cl to the lymphocyte surface principally involves reaction with primary amines (ε-amino groups of lysine residues). Dansic acid presumably associates hydrophobically with the plasma membrane lipid bilayer. Thus, this fluorophore may be considered to reside in close apposition with the lipoprotein milieu of the membrane itself. This would place the dansyl moiety (approximate dimensions 70 × 120 Å) in the neighborhood of the Con A receptor locus, but farther from the more distal PHA (and PWM) site. This may account for the ability of Con A to stimulate mitogenesis of dansylated lymphocytes only 50% as well as it does with control cells, while PHA and PWM stimulated DNA synthesis in our dansylated cells more than 70% as effectively as controls.

These studies can be extended to address the problem of identifying populations of peripheral circulating lymphocyte subclasses. Preliminary indications are that T cells yield different dansyl fluorescence characteristics than do B cells.

We thank Dr. M. Sonnenberg for the use of the modified Cary 14 spectrofluorometer. This work was supported in part by a grant from the American Cancer Society, IN-14-0 (to P.S.C.); and by grants from the U.S. Public Health Service, CA-08748, CA-17404, and AI-11843; the Zelda R. Weintraub Cancer Fund, and the Fund for the Advanced Study of Cancer (to R.A.G.).

1. Inbar, M., Shinitzky, M. & Sachs, L. (1973) *J. Mol. Biol.* **81**, 245–253.
2. Shinitzky, M., Inbar, M. & Sachs, L. (1973) *FEBS Lett.* **34**, 247–250.
3. Mallucci, L. (1976) in *Concanavalin A as a Tool*, eds. Bittinger, H. & Schnebli, H. P. (Wiley, New York), pp. 69–78.
4. Lalezari, P., Nehlsen, S. L., Novodoff, J. & Lalezari, I. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 697–700.
5. Boyum, A. (1968) *Scand. J. Clin. Lab. Invest.* **21**, suppl. 97, 51–76.
6. Melera, P. W. & Cronin-Sheridan, A. P. (1976) *Biochim. Biophys. Acta* **432**, 300–311.
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
8. Waithe, W. I. & Hirschhorn, K. (1970) in In Vitro *Methods in Cell Mediated Immunity*, eds. Bloom, B. R. & Glade, P. (Academic, New York), pp. 455–461.
9. Allan, D. & Crumpton, M. J. (1970) *Biochem. J.* **120**, 133–143.
10. Ferber, E., Resch, K., Wallach, D. F. H. & Imm, W. (1972) *Biochim. Biophys. Acta* **266**, 494–504.
11. Demus, H. (1973) *Biochim. Biophys. Acta* **291**, 93–106.
12. Gray, W. R. (1967) *Methods Enzymol.* **11**, 139–151.
13. Gray, W. R. (1972) *Methods Enzymol.* **25**, 121–138.
14. Azzi, A. (1975) *Quart. Rev. Biophys.* **3**, 237–316.
15. Hubbard, A. L. & Cohn, Z. A. (1976) in *Biochemical Analysis of Membranes*, ed. Maddy, A. H. (Chapman and Hall, London), pp. 427–501.
16. Schmidt-Ullrich, R., Knufermann, H. & Wallach, D. F. H. (1973) *Biochim. Biophys. Acta* **307**, 353–365.
17. Toyoshima, S., Fukuda, M. & Osawa, T. (1972) *Biochemistry* **11**, 4000–4005.
18. Lindahl-Kiessling, K. (1972) *Exp. Cell. Res.* **70**, 17–26.
19. Han, T. (1973) *Clin. Exp. Immunol.* **13**, 165–170.
20. Novogrodsky, A. & Katchalski, E. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 3207–3210.
21. Novogrodsky, A. (1976) in *Mitogens in Immunobiology*, eds. Oppenheim, J. & Rosenstreich, D. (Academic, New York), pp. 43–56.