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CHOLESTEROL-FREE PHOSPHOLIPID DOMAINS MAY BE THE MEMBRANE FEATURE SELECTED BY Nϵ-DANSYL-L-LYSINE AND MEROCYANINE 540

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SUMMARY: We have used Nϵ-dansyl-L-lysine as a fluorescent membrane probe, to study cells taken from tissues concerned with immune function. There is a striking similarity between the staining selectivity of this compound and that reported by others for merocyanine 540. Both compounds stain leukemic, human, peripheral leukocytes, an erythroleukemia line, and some mouse bone marrow cells, suggesting common selectivity for a membrane feature of hemopoietic cells. Both compounds fail to stain red blood cells, normal human leukocytes, mouse spleen and thymus cells. We have recently reported that dansyl-lysine apparently selects for cholesterol-free phospholipid domains in liposomes and now report similar selectivity for merocyanine 540 staining of liposomes.

INTRODUCTION: There has been considerable interest in the use of merocyanine 540 (MC540) as a fluorescent membrane probe selective for an unknown feature characteristic of immature hemopoietic cells, and absent from mature blood, spleen, marrow and thymus cells. Demonstration of this selectivity, through microscopy, typically requires the presence of serum during staining. In its absence, the probe may act as a general, impermeant membrane stain (1-3). Dead mature, normal, nucleated cells stain with MC540 but, unlike that of hemopoietic or human leukemic cells, such staining is quenched by inclusion of trypan blue in the staining solution (1).

The present paper reports that Nϵ-dansyl-L-lysine (DL) is a fluorescent membrane probe which appears to have the same cell-staining selectivity as reported by others for MC540, and that MC540 has liposome-staining selectivity similar to that we have previously reported for DL (4).

Abbreviations: MC540, merocyanine 540; DL, Nϵ-dansyl-L-lysine; EDTA, ethylenediaminetetra-acetic acid; PBS, phosphate buffered saline; PC, phosphatidylcholine; DMPC and DPPC, dimyristoyl- and dipalmitoylphosphatidylcholine; FACS, Fluorescence Activated Cell Sorter.

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MATERIALS AND METHODS: Blood from leukemic patients was excess to that required for routine clinical tests, and was donated by a local hematology/oncology practice. It was examined within about 30 hr of withdrawal, as was that taken from normal healthy human volunteers. All bloods were drawn into EDTA vacuum tubes. Counts of DL-staining cells were performed on mixtures of 10 microliters blood and 0.2 ml DL solution, 10^{-3} M DL in phosphate-buffered saline (PBS, 0.145 M NaCl with 0.01 M phosphate at pH 7.2). Background fluorescence is negligible. There is no need to wash cells after (or before) staining. Red cells do not interfere, so were not removed. A Zeiss Universal microscope equipped for epi-illumination by a UV lamp, with band pass filtering at 365 nm, was used. Emission was filtered by a long pass barrier filter, $>$ 395 nm.

Single cell suspensions (approximately 10^7 cells/ml) were obtained by disaggregation of tissues from 8-20 week-old Balb/c mice, killed by cervical dislocation. Cells were routinely suspended in RPMI medium with 5% fetal calf serum (FCS), and other additives as used previously for tissue culture work (5). Staining solutions were 10^{-3} M DL in PBS, with or without 0.1% trypan blue. Equal volumes of staining solution and cells were mixed and examined immediately, without further preparation. Details of the method used to prepare the bone marrow specimen shown in Fig. 1 are given in the figure legend. The microscope was as used for examination of human blood.

Friend virus-induced murine erythroleukemia cells, GM86 clone 745, were obtained from Dr. Douglas Lyles, Bowman Gray School of Medicine, Winston-Salem, North Carolina. They were grown in suspension, without agitation, in Dulbecco's modified Eagle's medium (GIBCO) with 10% fetal calf serum. Staining procedures were as for the single cell suspensions of normal mouse tissues, except as otherwise noted in the next section.

Lipids and liposomes were as previously described (4,5). MC540 was purchased from Eastman Kodak, Rochester, N.Y., and was purified by two sequential separations on 100 micron silica gel preparative TLC plates, using $CHCl_3$:CH_3OH (7:3) as solvent. R_f was 0.72. The purified MC540 was dissolved in C_2H_5OH:H_2O (1:1) at 1 mg/ml to give a stock solution which was stored at -20°C in the dark. This was diluted into PBS, as required, immediately prior to use. Stained liposomes were examined using a FACS analyzer, as described in the Fig. 2 legend, or using a Zeiss Universal microscope equipped for the epifluorescence technique with excitation filtered at 546 nm and emission at $>$ 590 nm. A heated stage permitted temperature variation.

RESULTS AND DISCUSSION: DL fails to stain erythrocytes and $>$ 99% live, freshly-prepared, mouse spleen and thymus cells, whether serum is present or not. MC540 requires the presence of serum for this result (1-3). Dead cells stain with both DL and MC540, and in both cases this is quenched by trypan blue (1). Disaggregated mouse bone marrow shows about 10-20% live cells which stain with DL. A similar figure is reported for MC540 (1-3). DL can be used to study the distribution of staining cells in intact pieces of marrow, as shown in Fig. 1. Such cells typically occur as multicellular balls, often apparently surrounding a large particularly strongly staining cell. These multicellular balls are distributed throughout a matrix of cells which fail to stain appreciably with DL, by eye, although their outlines are visible, in some cases, in

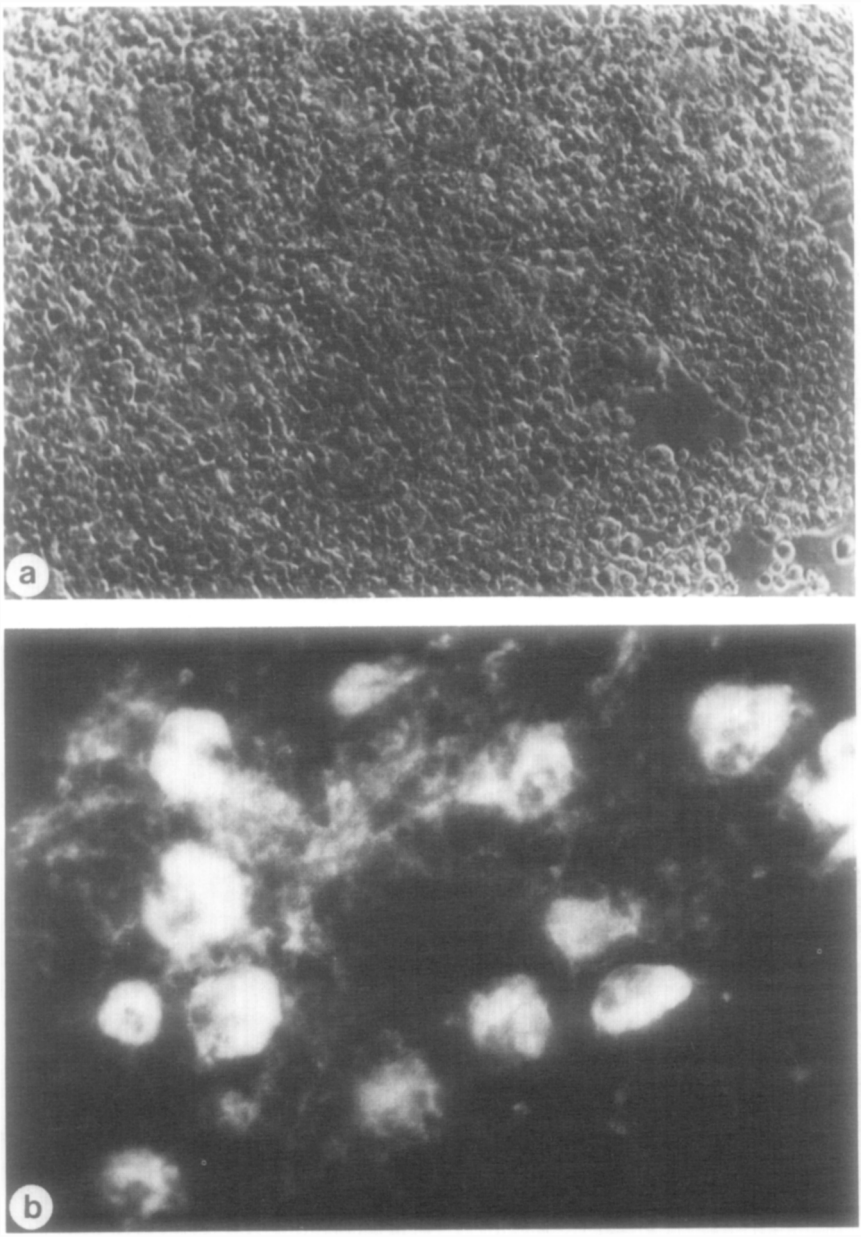

Figure 1. An intact piece of mouse bone marrow, taken from the femur, was soaked for 5 min in 10^{-3} M DL, with 0.1% trypan blue, in PBS. It was then transferred to a slide glass, covered gently with a cover slip and examined at a magnification x 112. It is shown (a) using phase contrast optics and (b) using the UV epi-illumination technique.

the Fig. 1 photograph. Wright stained smears reveal large cells, about the same size as the largest DL-staining cells, which have pale blue cytoplasm and pink nuclei typical of primitive hemopoietic cells. Thus, although we have no functional evidence for the identity of the staining cells, morphological evidence

suggests that they may be hemopoietic. The groups of staining cells may include erythroblastic islands (6).

A small population (~1%) of erythroleukemia cells stains rapidly and intensely with DL. Such cells appear particularly grainy by phase contrast microscopy. The rest of the cells stain after incubation with DL for 30 min at 37°C, the procedure reported for MC540 (2). Such staining is relatively weak and is observed more clearly after centrifugation and resuspension in medium lacking DL. Inclusion of DL, at 0.5 - 1.0 x 10^{-3}M, does not affect the growth of erythroleukemia cells or the viability of normal spleen cells in vitro (5). However, essentially all of the former, but only a small population (~5%) of the latter, are stained after 3-4 days culture. A similar proportion of spleen cells stains with DL when it is withheld during culture and added just prior to examination.

Blood taken from five apparently normal, healthy volunteers failed to reveal DL-staining leukocytes. In contrast, we have found high concentrations of DL-staining leukocytes in blood from patients suffering from the following diseases: lymphoma, chronic myelogenous leukemia, polycythemia vera, acute lymphoblastic leukemia, chronic lymphocytic leukemia. Staining of few of these cells was quenched by trypan blue.

When cells stain with DL it is apparent that the periphery and some internal structures are involved. Marked perinuclear staining can often be seen, as has been described for MC540 (1-3).

Like MC540, DL at concentrations routinely used for staining has no effect on an in vitro spleen cell immune function. For the case of DL, this was determined using lipopolysaccharide, as before, to stimulate a B cell response (7).

DL does not sensitize stained cells to photolysis by visible light. Even the UV lamp, as used for routine microscopic examination, has negligible effect for practical purposes. Stained cells exposed to this UV light for short periods are not killed, as monitored by trypan blue uptake. DL is far more stable than MC540 under most conditions. These characteristics, in addition to the low background provided by aqueous DL (which makes removal of unbound dye

unnecessary in most cases), and the lack of sensitivity to serum content, suggest that DL is far easier to use than MC540 for most purposes.

The MC540 staining profile of liposomes, as a function of cholesterol content, closely resembles that shown in response to DL and reported previously (4). In brief, liposomes with a mole fraction of cholesterol (X_{ch}) ≤ 0.2 are significantly more fluorescent than those with $0.2 < X_{ch} \leq 0.5$. As discussed in Ref. 4, there is a high probability that DL selects for cholesterol-free phosphatidylcholine (PC) domains in synthetic membranes: domains whose existence at low cholesterol content was originally proposed by McConnell and colleagues (8-10). Our results suggest that cholesterol-free phospholipid domains are also preferentially stained by MC540, and that cells which stain with MC540 and DL may have plasma membranes containing such features. Fig. 2 shows that resolution of fluorescence intensity from liposomes with $X_{ch}=0$ and $X_{ch}=0.5$, as detected by a FACS analyzer, is improved by the presence of FCS. On microscopic examination, suppression of staining by cholesterol content is obvious in the presence (but not the absence) of FCS.

Williamson et al (11) have recently described staining of dipalmitoylphosphatidylcholine (DPPC) liposomes in the presence and absence of FCS, and conclude that, in the presence of FCS, MC540 selectively stains "fluid" rather than "solid" DPPC. We have repeated their procedures and agree that there is a very pronounced increase in staining intensity when DPPC liposomes, in the presence of FCS, are heated through the principal thermotropic phase transition temperature (T_c), 41°C (although we find significant staining, which tends to be masked by high background, even below T_c). We see suppression of MC540 staining by high cholesterol content using DPPC or dimyristoylphosphatidylcholine (DMPC) at temperatures below, and above, the T_c of the phospholipid (as with DL staining). As cholesterol at $X_{ch} > 0.2$ increases the "fluidity" of PC membranes below the T_c of the PC, and decreases it above (12), we conclude that staining by MC540 and DL is not a function of membrane "fluidity" per se, i.e. where "fluidity" describes rate of lateral diffusion. In membranes which contain phospholipid and cholesterol, lateral phase separation generates domains which

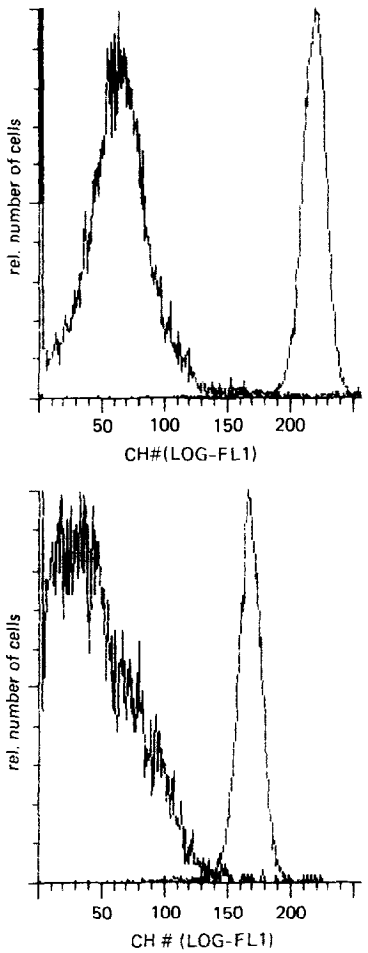

Figure 2. Suppression of MC540 staining of DMPC liposomes at high cholesterol content in the presence (upper histogram) and absence (lower histogram) of 10% FCS. Samples were 10^{-4} M in total lipid and contained MC540 at 1 μg/ml, added after the FCS where applicable. Temperature was 21°C. The data shown were gathered using a Becton-Dickinson FACS Analyzer, Coulter volume-gated for liposomes (called "cells" in the plot) in a restricted size range centered at about 5 microns diameter. Peak occupancy plotted at equal amplitude for each sample. In each histogram, liposomes with $X_{ch}=0$ are shown to have high fluorescence intensity (right hand peak) and those with $X_{ch}=0.5$ have low fluorescence intensity (left hand peak). To optimize resolution of the data, the instrument gain setting was higher for the upper histogram than for the lower. Taking this into account, 10% FCS reduces fluorescence from liposomes with $X_{ch}=0$ to 40% of that generated in its absence. The relative fluorescence intensities of liposomes with $X_{ch}=0$ and $X_{ch}=0.5$, $F_0/F_{0.5}$, is 84 in the presence of serum, and 30 in its absence. Excitation and emission were filtered by BP 545/20 and LP > 580 filters respectively.

differ in composition and in solvation properties towards MC540 and DL, independent of their relative "fluidity."

The existence of "specific, possibly phospholipid-rich membrane domains that mediate complex formation with MC540 in excitable cells" was previously

suggested by Easton et al (13) based on partitioning of MC540 and phospholipids into benzene from an aqueous buffer.

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REFERENCES:

1. Valinsky, J.E., Easton, T.G., and Reich, E. (1978) Cell 13, 487-499.
2. Schlegel, R.A., Phelps, B.M., Waggoner, A., Terada, L. and Williamson, P. (1980) Cell 20, 321-328.
3. Phelps, B.M., Williamson, P., and Schlegel, R.A. (1982) J. Cell Physiol. 110, 245-248.
4. Humphries, G.M.K. and Lovejoy, J.P. (1983) Biophys. J. In press.
5. Humphries, G.M.K. and McConnell, H.M. (1979) J. Immunol. 122, 121-126.
6. Yoffey, J.M. (1973) Ciba Foundation Symposium 13:Haemopoietic Stem Cells. pp 5-45. Associated Scientific Publishers, Amsterdam.
7. Humphries, G.M.K. (1979) J. Immunol. 123, 2126-2132.
8. Recktenwald, D.J. and McConnell, H.M. (1981) Biochemistry 20, 4505-4510.
9. Copeland, B.R. and McConnell, H.M. (1980) Biochim. Biophys. Acta 599, 95-109.
10. Owicki, J.C. and McConnell, H.M. (1980) Biophys. J. 30, 383-398.
11. Williamson, P.L., Massey, W.A., Phelps, B.M. and Schlegel, R.A. (1981) Mol. Cell, Biol. 1, 128-135.
12. Rubenstein, J.L.R., Smith, B.A. and McConnell, H.M. (1978) Proc. Natl. Acad. Sci. USA 76, 15-18.
13. Easton, T.G., Valinsky, J.E. and Reich, E. (1978) Cell 13, 475-486.