J. Membrane Biol. (1984) 77:115–122

The Journal of
Membrane Biology
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Membrane Structure and the Tenuously Maintained Resistance to Staining with Nε-Dansyl-L-Lysine Shown by Many Cells

Gillian M.K. Humphries and John P. Lovejoy
Institute for Medical Research, San Jose, California 95128

Summary. The ability to resist staining by Nε-dansyl-L-lysine is tenuously maintained in the majority of live nucleated cells taken from tissues concerned with immune function. Resistance is lost under a variety of nonphysiological conditions known to, or likely to, cause protein denaturation or aggregation. In contrast to that of dansyl-γ-aminobutyrate, the fluorescence intensity of Nε-dansyl-L-lysine is only weakly enhanced by native proteins. This is further reduced on denaturation or aggregation of the proteins. It is unlikely, therefore, that cellular uptake of, and staining by, Nε-dansyl-L-lysine is a direct consequence of membrane protein denaturation/aggregation but may result from a decrease in protein-phospholipid interactions leading to formation of phospholipid domains. Previous work has indicated that such features are stained by Nε-dansyl-L-lysine (Humphries, G.M.K., Lovejoy, J.P., 1983, *Biophys. J.* **42**:307–310; Humphries, G.M.K., Lovejoy, J.R., 1983, *Biochem. Biophys. Res. Commun.* **111**:768–774).

Although it appears likely that passage through a dansyl-lysine-staining state is a common, if not universal, prelude to cell death (as monitored by uptake of trypan blue), not all cells that lose resistance to dansyl-lysine staining are moribund. Resistance to staining is also lost by macrophages on binding to solid substrates and multivalent ligands. The possible physiological significance of this is discussed.

Key Words Nε-dansyl-L-lysine · phospholipid domains · membrane protein aggregation

Introduction

We have recently reported that Nε-dansyl-L-lysine (DL) has the same cell-staining selectivity as exhibited by merocyanine 540 (MC540) (Humphries & Lovejoy, 1983*a*) and that phospholipid (PL) membrane domains are the likely target for both these fluorescent probes (Humphries & Lovejoy, 1983*a*, *b*). On examination of single cell suspensions freshly prepared from tissues concerned with immune function, such as blood, spleen, marrow, thymus and peritoneal exudate, we find few live cells which stain with DL. Those which do are likely to be hemopoietic. In the present paper we describe conditions under which other cells from these tissues become susceptible to staining with DL, and discuss the possible structural and functional significance.

Abbreviations

DL, Nε-dansyl-L-lysine; PL, phospholipid; FCS, fetal calf serum; GA, glutaraldehyde-treated; PBS, phosphate-buffered saline; DB, dansyl-γ-aminobutyrate; PEG, polyethyleneglycol; PC, phosphatidylcholine.

Materials and Methods

Cells

Spleen cells and peritoneal exudate cells were obtained from 8 to 20-week-old Balb/c mice and prepared as single cell suspensions in RPMI 1640 with 5% fetal calf serum (FCS) and other additives as previously described (Humphries & McConnell, 1979). For production of glutaraldehyde-treated cells (GA cells), Ficoll-Paque-separated cells were suspended in phosphate-buffered saline (PBS, 0.145 M NaCl with 0.01 M phosphate, pH 7.2) at 10^7/ml with 0.05% glutaraldehyde and shaken gently for 15 min at room temperature. At the end of that period, FCS was added to a final concentration of 10% and the cells were centrifuged and washed 2 to 3 times in PBS or complete medium. This procedure generates ~95% cells which stain with DL but not trypan blue, and ~5% cells which stain with trypan blue. For trypsinization of cells, they were exposed to the enzyme at 5 mg/ml, with EDTA·4 Na at 2 mg/ml, in Hanks buffered saline without Ca^{++} and Mg^{++} (GIBCO). After incubation at 37 °C for the times reported, the reaction towards the cells was blocked by addition of FCS, to a final concentration of 20%. Cells were washed in fresh medium prior to examination. Other procedures were as described in the Figure legends.

Fluorescent Probes and Staining Procedures

DL and dansyl-γ-aminobutyrate (DB) were purchased from Sigma. For cell staining, the compounds were prepared as stock 10^{-3} M solutions in PBS, with or without 0.1% trypan blue. Cell suspensions were mixed with these stock solutions at equal volume and examined immediately, without washing or further preparation, under the microscope or using the FACS analyzer.

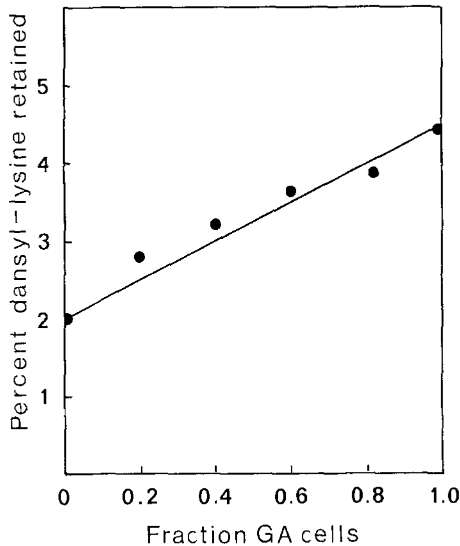

Fig. 1. DL uptake by cells is increased by treatment with glutaraldehyde. Mixtures of GA cells and untreated cells were prepared in the proportions indicated, pelleted and resuspended at 8×10^7 cells in 0.05 ml 10^{-3} M DL solution. After incubation at 21 °C for 10 min, 1 ml PBS was added and the cells were filtered on small Millipore (5 micron) filters. After washing with a further 1 ml PBS, the filters and cells were extracted with methanol for 30 min and the methanol extracts assayed with reference to standard methanol solutions of DL

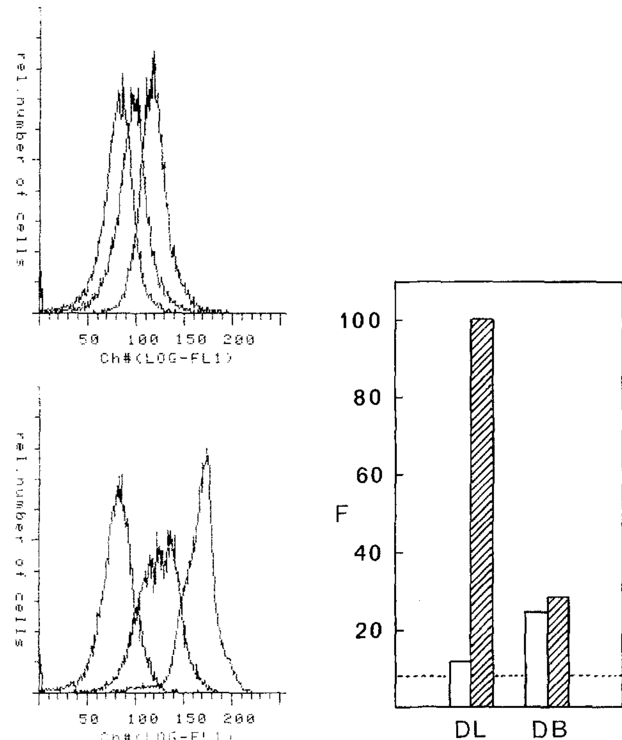

Fig. 2. DL and DB staining of untreated and GA cells, as demonstrated using a FACS analyzer. The upper histogram shows untreated cells; the lower shows GA cells. All plots are for 10^4 cells. Samples were all 10^7 cells/ml with 0.05% trypan blue. In both histograms, the left-hand (lowest fluorescence) peak shows cells with no further addition. The other peaks show cells exposed to DL or DB at 5×10^{-4} M. In the upper histogram (untreated cells), the right-hand (greatest fluorescence) peak is generated by cells exposed to DB. In the lower histogram (GA cells) the right-hand peak is generated by cells exposed to DL. On the right-hand-side of the Figure is a bar chart showing the same FACS data, with the mean peak fluorescence values transformed to an arbitrary linear scale of fluorescence (*F*). The dotted line shows the identical fluorescence value generated by untreated and GA cells in the absence of DB or DL. The open bars show untreated cells; the cross-hatched bars show GA cells. *F* is in response to DL or DB, as indicated at the bottom of the chart

MC540 was as reported previously (Humphries & Lovejoy, 1983*a*) and was used at a final concentration of 10 µg/ml with 10% FCS. After 30 min at 37 °C, cells were washed to remove unbound MC540.

Instruments

A Zeiss Universal Microscope photometer equipped for the epifluorescence technique was used. For DL and DB, excitation was filtered at 360 nm and emission at >395 nm. For MC540, excitation was filtered at 546 nm and emission at >590 nm. A Becton-Dickinson FACS analyzer, fitted with a UG1 excitation filter, a short-pass 375 nm dichroic mirror and two long-pass 400 nm emission filters was also used for DL and DB studies. A Turner fluorometer with excitation filtered at 360 nm and emission at >510 nm was used to assay DL/DB fluorescence in PBS: FCS mixtures. A Brinkman RM3 water bath was used for controlled heating of cell suspensions and FCS.

Results

Effect of Glutaraldehyde Treatment of Spleen Cells

Figure 1 illustrates an experiment which indicates that selective staining by DL parallels selective uptake of DL. Due to fluorescence enhancement of membrane-associated DL, the difference between treated and untreated cells is far more striking when the probe is used to study staining rather than uptake. This is illustrated by Fig. 2. This Figure also shows that DB makes little distinction between GA and untreated cells. Previous trypsinization of spleen cells, for periods up to 30 min, fails to alter these responses to glutaraldehyde treatment. With longer periods of trypsinization, a decrease in viability and increase in DL staining of cells, is noted, prior to any subsequent treatment.

Red cells fail to stain with DL either before or after glutaraldehyde treatment. MC540 distinguished between normal spleen and GA or heated cells in a manner analogous to that of DL.

Effect of Heating Spleen Cells

Nucleated spleen cells, but not red cells, are stained by DL after short periods of heating at temperatures a few degrees above 37 °C. This is illustrated by Fig. 3. In this experiment, DL and trypan blue were used separately. If used in combination, it is apparent that trypan blue reduces the quantity

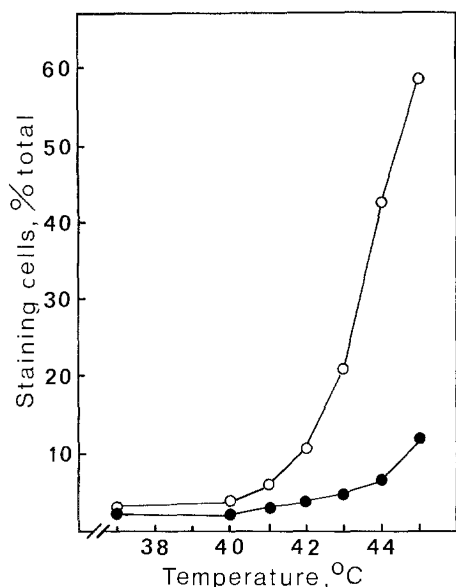

Fig. 3. The staining profile of Ficoll-Paque-separated mouse spleen cells after heating for 20 min at the temperatures shown. Equal volumes of cells at 10^7 per ml. and either DL at 10^{-3} M in PBS (—o—), or trypan blue at 0.1% in PBS (—•—), were mixed and examined immediately. About 250 cells were counted for each point

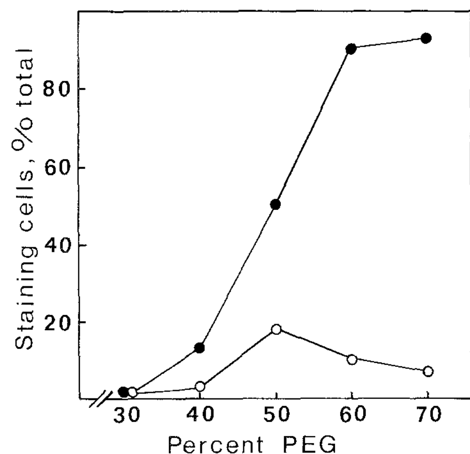

Fig. 4. Staining profile of mouse spleen cells after treatment with PEG. Spleen cells were treated with PEG as recommended for hybridoma production (Oi & Herzenberg, 1980) except that the PEG was prepared at the range of concentrations (wt/wt) shown. After resuspension in fresh medium, cells were mixed with an equal volume of 10^{-3} M DL in PBS with 0.1% trypan blue and counted for DL-staining (—o—) and trypan blue-staining (—•—) cells as a percent of total. It should be noted that this protocol is different from that used for the experiment shown in Fig. 3. In the PEG experiment, trypan blue was used to quench DL fluorescence which would otherwise be emitted from dead cells. About 250 cells were counted for each point

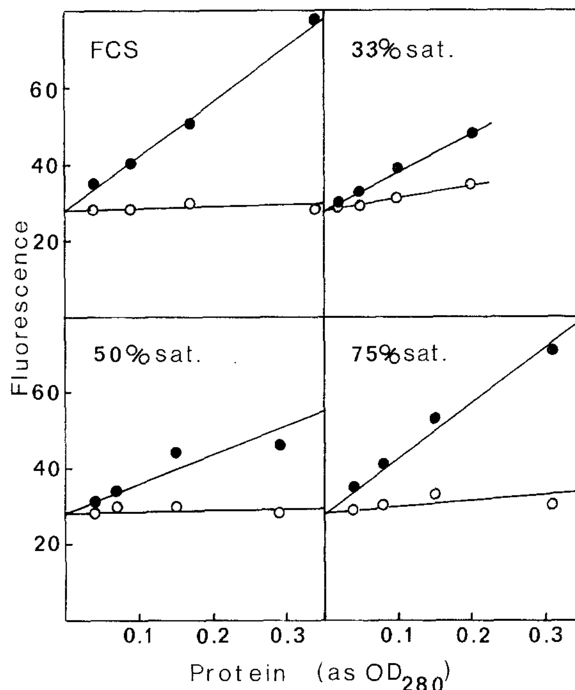

Fig. 5. Proteins in FCS enhance the fluorescence intensity of DB significantly, but have little effect on that of DL. PBS was used as solvent. A 1% FCS solution has an $OD_{280}=0.27$. The top left-hand panel shows the effect of untreated FCS on fluorescence emitted by DB (—•—) and DL (—o—) at 10^{-6} M. The other three panels show the effect of three groups of proteins, prepared by sequential saturated $(NH_4)_2SO_4$ precipitation of the same FCS, reconstituted with PBS, and used with 10^{-6} M DL or DB as for the untreated FCS. The data were measured on an arbitrary scale using a simple fluorometer and corrected for slight background fluorescence from proteins in the absence of DL/DB

of DL-staining cells observed, by a number the same as that which it stains. As reported previously (Humphries & Lovejoy, 1983*a*) we conclude that DL stains dead cells but that such staining is quenched by trypan blue. In the temperature range, 37 to 40 °C, uptake of DL and trypan blue is typically not a function of temperature over a period of 2 hr. With temperatures at or greater than about 41 to 42 °C, DL and trypan blue uptake increase with time. Some heat-treated cells maintain a DL-staining, trypan blue-excluding profile for long periods of time. For example, in a typical experiment using spleen cells heated at 45 °C for 20 min, we found that, after 18 hr *in vitro* culture (*see* Humphries & McConnell, 1979), 96% of the cells stained with DL and 27% with trypan blue. At 0 hr, 61% had stained with DL and 6% with trypan blue. Unheated control cells showed DL-staining cells to increase from 4% to 34% in the same period, and trypan blue-staining cells from 1% to 6% over the same period. Total cell number over the 18-hr period remained essentially constant.

Using methanol extraction in a procedure similar to that described for GA cells, we found that heated-treated, DL-staining cells take up significantly more DL than do control unheated cells (*data not shown*). Also, as is the case for GA cells, DB makes little distinction between heated and control unheated cells (*data not shown*).

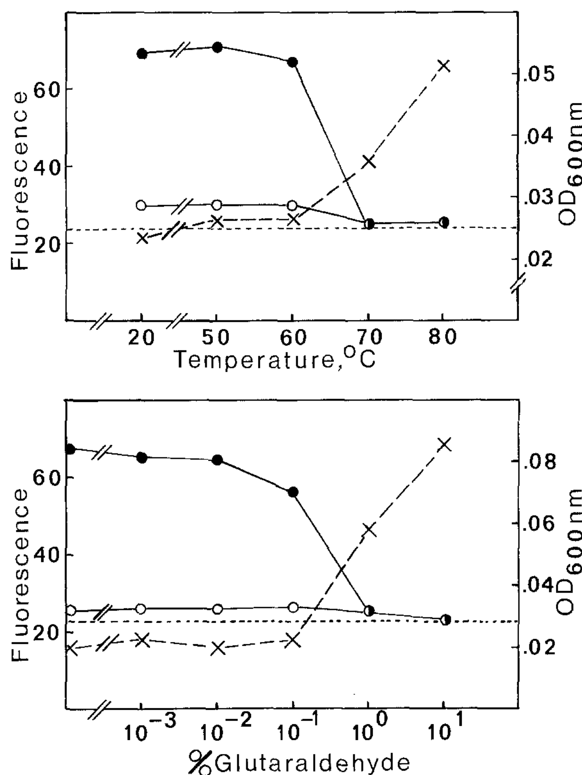

Fig. 6. Heat or glutaraldehyde treatment destroys the ability of FCS to enhance the fluorescence of DB. As for the experiment shown in Fig. 5, PBS was the solvent and DL and DB were used at 10^{-6} M throughout. The dotted line shows fluorescence from DL/DB in the absence of FCS. FCS was pretreated by heating for 20 min at the various temperatures indicated, or by mixing with an equal volume of PBS and the various proportions of glutaraldehyde indicated and leaving for >10 min. Pretreated or untreated FCS was then mixed, at a final concentration of 1%, with the probes. Fluorescence of DB (—●—) and DL (—○—) is shown as a function of FCS pretreatment. Also shown is the turbidity, as $OD_{600\ nm}$ (--×--), of FCS/PBS (50:50) mixtures as a function of the same pretreatment conditions

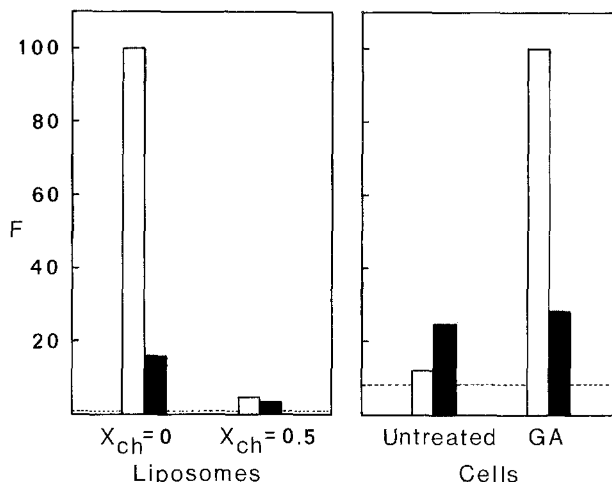

Fig. 7. A comparison of the relative susceptibilities of liposomes, untreated cells and GA cells to staining with DL versus DB. The left-hand chart shows values of F (mean peak fluorescence as detected by the FACS analyzer and transformed to an arbitrary linear scale) for DMPC liposomes having mole fractions of cholesterol, $X_{ch}=0$ and 0.5. Liposomal lipids, DL and DB were all at 10^{-4} M. Liposomes were Coulter volume-gated to a narrow size range centered at about 5 microns diameter. The right-hand chart shows the same data as Fig. 2 rearranged for better comparison with the liposome data. In all cases the filled bars show values of F generated by DB and the open bars show those generated by DL. The linear scales for the left- and right-hand charts are similar but *not* identical. Dotted lines show F for unstained particles

The Effect of Polyethyleneglycol (PEG) on Spleen Cells

Figure 4 illustrates the effect of PEG on spleen cells. The protocol used is as recommended for hybridoma production (Oi & Herzenberg, 1980), except that we have varied the PEG concentration. The recommended concentration is 50%, a condition under which we find that the maximum number of DL-staining, trypan blue-excluding cells are produced.

DL, DB, Proteins and Liposomes

Figure 5 shows that the fluorescence intensity of DB is significantly enhanced by low concentrations of FCS. That of DL is altered very little. Increasing the density of FCS to 1.25 g/ml with KBr and centrifuging at 100,000 × *g* for 24 hr produces an infranate with fluorescence-enhancing properties similar to that of the untreated serum. We conclude that the properties are not confined to lipoproteins. As shown in Fig. 5, they appear to be a common feature of many, but not necessarily all, serum proteins.

We have attached FCS proteins, prepared by $(NH_4)_2SO_4$ precipitation, to activated CH Sepharose 4B beads (Pharmacia) and also prepared control beads in which the active groups are blocked by Tris. Using these beads, we find that uptake of DB and DL is protein-dependent, and that 3 to 4 times as much DB as DL is taken up under conditions which are not saturating. Microscopic examination shows that FCS beads are more intensely stained by DB than by DL under these conditions.

As shown in Fig. 6, treatment with heat or glutaraldehyde destroys the ability of serum proteins to enhance the fluorescence of DB and DL. As is also shown in Fig. 6, loss of fluorescence-enhancing properties is accompanied by a slight increase in turbidity, as measured by $OD_{600\ nm}$. Analogous results are obtained when treated and untreated FCS is examined, in the presence of DL or DB, using the microscope photometer with front surface illumination of the sample. We conclude that the decrease in fluorescence is not merely a consequence of interference due to the increase

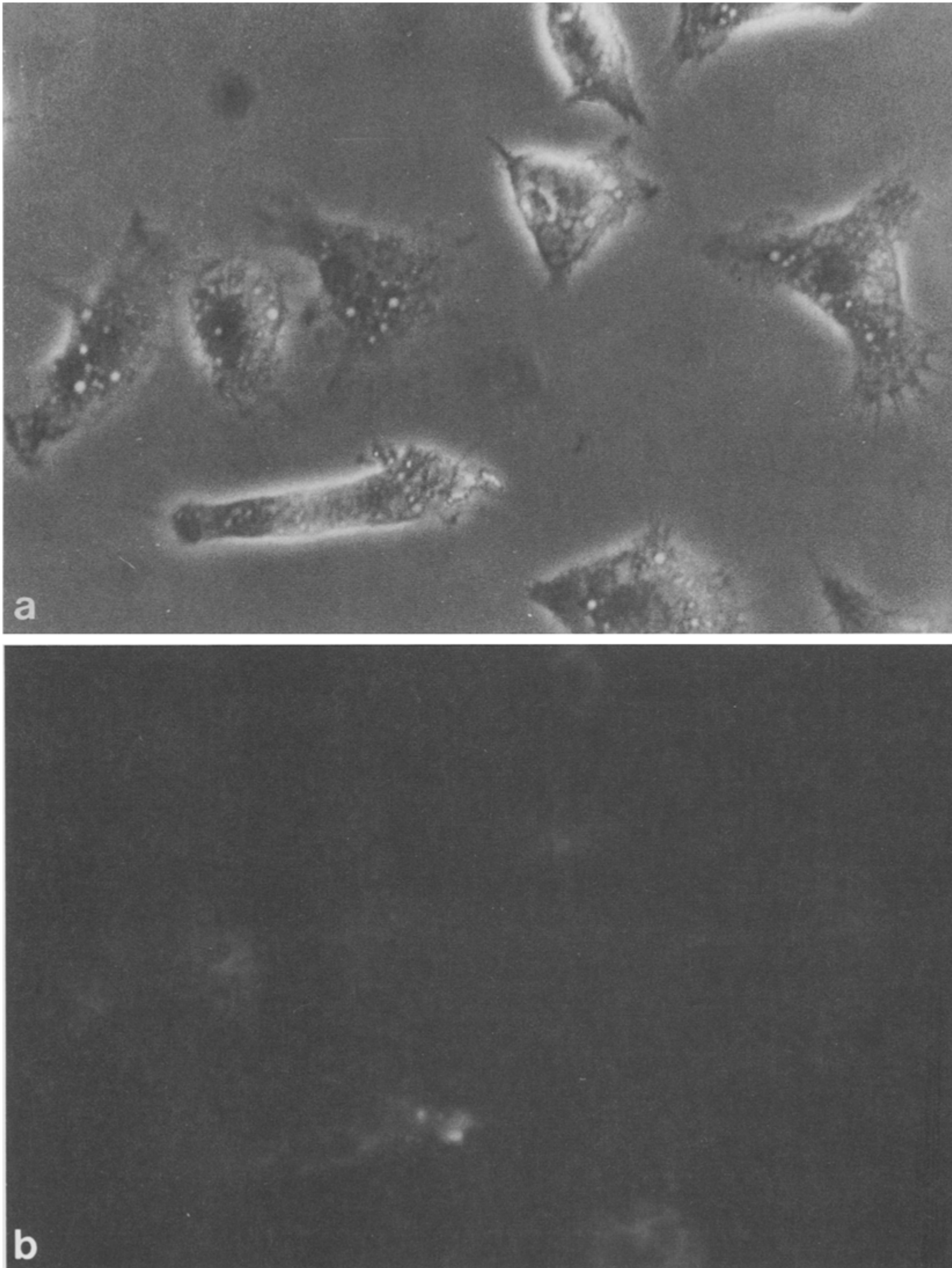

Fig. 8. Mouse peritoneal macrophages after 24 hr adherence to glass cover slips at 37 °C with 5% CO_2. Cells were seeded at 2.8×10^6 in 2 ml medium per 35 mm culture dish. After an initial 90 min incubation period at 37 °C, nonadherent cells and medium were removed, and replaced by 3 ml fresh medium, before resuming incubation. After 24-hr total incubation, the medium was removed and replaced by 1 ml 10^{-3} M DL solution. The cover slip was taken from the dish after 2 to 3 min and placed cell-side down on two pieces of double-sided tape on a microscope slide. The cavity was filled with DL solution and the edges taped off. In (*a*), cells are shown by phase-contrast optics. In (*b*), cells are shown by the epifluorescence technique, with filtering as described in Materials and Methods. Cells such as those shown invariably exclude trypan blue but stain with DL. Initial magnification was 400 ×

in turbidity. We do not know which protein site(s) DB and (with far less affinity) DL select, but assume it is some secondary or higher order structural feature common to proteins which is altered on denaturation/aggregation.

Many cells which fail to stain with DL, such as normal spleen cells, stain with DB when used under similar conditions (*see* Figs. 2 and 7). Such staining is relatively faint and is readily reversed by washing. In contrast, DL staining of cells such

as hemopoietic or GA cells requires extensive washing for reversal. We have been unable to reduce susceptibility to staining with DB by trypsinization of spleen cells. Red blood cells do not stain with DB, but apparently all others taken from tissues concerned with immune function do.

DB interacts with protein-free phosphatidylcholine (PC) liposomes in a manner very similar to that of DL (Humphries & Lovejoy, 1983*b*) except that staining is less intense, as shown in Fig. 7. Uptake of DB is also less than that of DL. The emission maximum for protein-bound probe is at ~515 nm, as is also the case for PC-bound probe. The value for DL and DB in aqueous solution is ~545 nm. As with DL (Humphries & Lovejoy, 1983*b*), DB staining and uptake is markedly suppressed by a cholesterol content greater than 20 mol/100 mol total lipid.

Peritoneal Macrophages Stain with DL after Binding to Solid Substrates

Figure 8 illustrates this phenomenon. Susceptibility to staining develops in all bound cells within about an hour. In some cells it may be within minutes. Mouse peritoneal macrophages also develop susceptibility to staining with DL on binding to dinitrophenylated Sepharose beads coated with anti-DNP antibodies.

Glass-bound cells provide convincing evidence that DL stains and passes through the plasma membrane, and then stains internal membranes as well. Leukemic human leukocytes also demonstrate this unequivocally. Heat-treated or GA cells typically stain intensely, and so diffusely that it is difficult to make out different structures. When cells stain with DL or DB the periphery is invariably stained. Peripheral staining is faint for the case of glass-bound cells.

Discussion

Glutaraldehyde treatment is the most satisfactory procedure we have so far devised for producing a high proportion of cells capable of staining intensely with DL but not with trypan blue. Heat treatment, under controlled conditions, is also quite successful and is of interest because the appearance of cells which stain with DL but exclude trypan blue occurs under conditions similar to those used to study heat treatment of tumors and heat-facilitated drug uptake (Hahn, 1982). PEG treatment yields a low concentration of cells which stain with DL but not trypan blue, but is of interest, with reference to the latter part of this discussion, because it is widely believed that cell fusion may require protein-free domains (*see* Zimmerman, 1982 and references therein).

In addition to the methods described, it appears that any condition or procedure known to decrease viability of cells *in vitro*, over an extended period of time, also leads to a relative increase in DL-staining, trypan blue-excluding cells. These include (a) use of glass rather than plastic tubes, (b) use of medium without, rather than with, FCS, (c) use of PBS rather than medium, (d) use of 0.3 M sucrose rather than PBS (e) use of hypertonic rather than isotonic PBS, (f) variation of pH from 7.2. A microscope slide preparation of cells mixed with DL and trypan blue and left for about 10 min, so that it is drying around the edges of the specimen, will show nonstaining cells in the center, trypan blue-staining cells at the edge of the cover slip, and a band of DL-staining cells between them. It should be noted that such cells cannot be distinguished from the nonstaining cells by using regular or phase-contrast optics and visible light. Azide, at 10^{-2} M, and ouabain, at 10^{-7} M, have no significant effect on DL or trypan blue uptake for the first 4-hr exposure. It appears likely that cells invariably pass through a DL-staining state prior to cell death, as monitored by trypan blue uptake, and that they can maintain this state for long periods of time in some cases.

However, susceptibility to staining with DL does *not* invariably indicate that a cell is moribund. We have previously described DL uptake by hemopoietic cells which are clearly in a very active state, and whose behavior is apparently unaffected by staining with DL (Humphries & Lovejoy, 1983*a*). In the present paper, we also describe DL uptake by peritoneal macrophages on binding to glass or to antibody-coated beads. These are preliminary observations for which we offer no mechanistic explanation at this time but include them to support the thesis that a change from the DL-excluding to the DL-staining state may, in some cases, have a functional significance distinct from heralding cell death. For example, selective uptake of DL may parallel that of molecules of biological significance required for cell function. Uptake of such molecules would be triggered by cellular binding to glass, etc.

Also of interest is the fact that macrophages bound to glass are known to fuse with each other to produce "giant cells" (Chambers, 1978). Conceivably, fusion may require some change in the state of the membrane which is recognized by DL and induced by binding to glass (for the case of

macrophages) or treatment with PEG (for the case of spleen cells).

In view of the strong possibility that DL is capable of recognizing some structural feature of membranes which has great biological significance, we will discuss what this feature might be, based on our experimental observations.

As previously noted, DL-staining is reversible with difficulty using aqueous solvents. It is readily reversible using methanol. As has been reported for MC540 (Easton, Valinsky & Reich, 1978), we conclude that no covalent interaction between DL and membrane components is required for staining to occur.

At pH 7.2, DL carries a net positive charge and DB is neutral. This is predictable from their structures and has been confirmed by us, using paper electrophoresis. MC540 is negatively charged but exhibits the same cell-staining selectivity as does DL (Humphries & Lovejoy, 1983*a*). A positively charged analog of MC540 also shows the same selectivity as the usual form, but a neutral analog stains normal spleen cells (Schlegel et al., 1980) as does the neutral molecule, DB. We conclude that, for the selectivity exhibited by MC540 and DL, a net charge may be important, but its sign is not. This suggests either (a) that DL and MC540 do not stain by virtue of their interactions with special sites on the membrane having a net charge opposite to their own, or (b) that negatively and positively charged sites co-exist on membranes which they stain, giving both probes a similar chance of staining. (For the sake of this discussion, "sites" are of a size at least as big as one whole molecule.) However, we observe that PC liposomes which are negatively charged (5 mol/100 mol dicetyl phosphate) or positively charged (5% stearylamine) are stained with the same intensity as are neutral liposomes when either DL or DB is used. Also, suppression by cholesterol content is maintained, to the same extent, for both probes when charged lipids are included in liposomes. It seems more likely, therefore, that DL is not selectively taken up by virtue of its interaction with membrane sites having net negative charge; that selection is governed by some other feature. (This does not preclude the possibility that an electrostatic interaction between DL and phospholipid headgroups is a factor in its uptake.)

Presumably MC540 and DL select for an inter- or intramolecular structural feature of membrane components. Whatever this feature is, it is certainly very readily formed in cells (other than red blood cells) when they are exposed to agents which cause protein aggregation and/or denaturation. We, therefore, examined the interactions between DL and serum proteins, before and after protein aggregation/denaturation. In doing so, we were fortunate to discover that the similar compound, DB, differs quantitatively from DL in its interactions with native, but not denatured, proteins. DB "stains" native proteins much more intensely than does DL, and neither probe "stains" denatured proteins significantly. On the other hand, DL stains PC appreciably more intensely than does DB. We conclude that the weak, readily reversible DB staining of normal spleen and other nonhemopoietic, nonred cells is due to interactions with surface proteins, and that DL has insufficient affinity for such proteins to exhibit staining. When membrane proteins are denatured and/or aggregated by heat, glutaraldehyde, PEG, etc., they will fail to "stain" with either DB or DL if their behavior is similar to that of serum proteins. It is unlikely, then, that the membrane structural feature selected by DL on exposure of cells to protein denaturing/aggregating agents is protein or protein-protein in nature. Presumably, trypsinization fails to reverse susceptibility to staining by DB because protein is not totally removed.

One of the earliest observations made in regard to synthetic membranes constructed from a single PL, was that they undergo a sharp cooperative "melting" phenomenon at a characteristic temperature, the principal thermotropic phase transition temperature (T_c). This is altered by addition of cholesterol or protein (Owicki & McConnell, 1979; Pink & Chapman, 1979). In either case, cooperative melting at T_c is suppressed, suggesting that PL-PL interactions are replaced by PL-cholesterol or PL-protein interactions. We have recently described staining of PC-cholesterol liposomes by DL (Humphries & Lovejoy, 1983*b*) and proposed that DL is selectively taken up by PC domains occurring at low cholesterol content. The co-existence of such domains with PC-cholesterol complex, as a long-range-ordered structure, was originally proposed by McConnell and colleagues (Copeland & McConnell, 1980; Owicki & McConnell, 1980; Recktenwald & McConnell, 1981). The DL-staining profile of sphingomyelin-cholesterol liposomes is similar to that of PC-cholesterol liposomes, suggesting that selection is for some common feature of PL domains; or, at least, for PL domains with choline headgroups.

If PL domain structure *is* perturbed by protein in much the same way as by cholesterol (Owicki & McConnell, 1979; Pink & Chapman, 1979), and if DL selects for PL domains, then membrane protein should be capable of suppressing DL uptake

through its interaction with lipids. Indirect evidence that this is the case is provided by the experiments reported in the present paper. In brief, if proteins are aggregated (through denaturation or otherwise) it is predictable that their interactions with PL will be diminished. It follows that this may result in the formation of PL domains capable of selective staining by DL. Glutaraldehyde and PEG are well-known protein-aggregating agents. So of course is heat. However, the heating conditions under which resistance to DL-staining is lost are surprisingly mild. However, very recent work examining recovery of fluorescence after photobleaching of FITC-antibody-labeled membranes, indicates that protein lateral mobility is greatly diminished under these heating conditions (Mehdi et al., 1983).

As induced by exposure to a variety of nonphysiological conditions, loss of resistance to staining with DL may reflect the fragile nature of native protein structure and protein-PL interactions. The high cholesterol content of red cell membranes may be responsible for their failure to stain with DL even after treatment with protein-aggregating/denaturing agents: protein may not be required to suppress DL uptake by red cells. (Another paper regarding red cells and DL is in preparation.) As macrophages usually require a significant length of time (during which they change their morphology) to assume a DL-staining state on binding to glass, factors other than rearrangement of membrane components may be involved. A net increase in PL, or decrease in cholesterol or protein, might result in PL domain formation leading to susceptibility to staining with DL.

We do not claim that the experiments reported here provide complete proof for this structural model, but our results are consistent with it. If the model is correct and protein aggregation can lead to generation of a new membrane phase with permeability properties different from those which existed previously, one is presented with a possible mechanism for transduction of the signal generated by aggregation of membrane receptors by polyvalent ligands. Treatment of cells with polyvalent ligands, such as mitogens, *does* lead to susceptibility to staining with DL under certain conditions. We are currently investigating the possibility that this has functional significance.

We thank Dr. Diether Recktenwald and Becton-Dickinson Company for assistance with the FACS analyzer, Dr. Leon Yengoyan and San Jose State University for access to the electrophoresis machine, Dr. Qasim Mehdi for communication of an abstract in press and Ms. Carole Guthrie for preparation of the manuscript. This work was supported by NIH grant AI17525 and ACS grant RD-140.

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Received 15 March 1983; revised 30 June 1983