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FLUORESCENCE STUDIES ON THE MECHANISM OF LIPOSOME-CELL INTERACTIONS IN VITRO

FRANCIS SZOKA [a,*], KENNETH JACOBSON, ZENON DERZKO [b] and DEMETRIOS PAPAHADJOPOULOS **

Department of Experimental Pathology, Roswell Park Memorial Institute, Buffalo, NY 14263 and Departments of [a] *Biochemistry and* [b] *Biophysical Sciences, State University of New York at Buffalo, Buffalo, NY 14214 (U.S.A.)*

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Summary

Sonicated unilamellar liposomes containing fluorescent lipid analogs or biotinyl phosphatidylethanolamine as a ligand for fluorescein avidin have been used to study the mechanism of interaction of phospholipid vesicles with eucaryotic cells. Microscopy revealed that after short incubations the fluorescence was associated with the cell surface in a punctate as opposed to a uniform staining pattern. Fluid vesicles, regardless of charge, were found to associate with cells to the same degree. Solid neutral and negatively charged vesicles associated to a 3-fold greater extent, while solid positively charged vesicles associated to a 10-fold greater extent than fluid vesicles. Fluorescence recovery after photobleaching, a technique used to measure the lateral mobility of cell surface components, was used to measure the lateral mobility of the associated fluorescence probes. No recovery was observed, implying that greater than 90% of the fluorescent lipid analogs are not free to diffuse over distances of the order of 1 μm. When these analogs were introduced into the cell membrane by an ethanol-injection technique, rapid and full recovery after photobleaching was observed. This can be accounted for by a lateral diffusion coefficient characteristic of phospholipids in model and biomembranes. The image and

* To whom reprint requests should be addressed at (present address): Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111, U.S.A.

** Present address: Cancer Research Institute and Department of Pharmacology, University of California, San Francisco, CA 94143, U.S.A.

A preliminary account of this work has appeared in abstract form [34].

Abbreviations: w_s, $1/e^2$ radius of the Gaussian profile laser beam used for photobleaching; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DPPC, dipalmitoyl phosphatidylcholine; DSPC, distearoyl phosphatidylcholine.

photobleaching results suggest that the majority of liposomes that become cell-associated under the conditions used here are adsorbed on the surface. The consequences of this blinding for liposome-mediated delivery of molecules into the cytoplasm or plasma membrane of the cell are discussed.

Introduction

The interaction of phospholipid vesicles (liposomes) with cells is a promising area in cell biology since it holds forth the possibility of introducing new material into the cytoplasm and plasma membrane of the cell. Thus far, growth-regulating nucleotides [1,2], drugs [3], proteins [4,5], nucleic acids [6,7] and intact viruses [8] have been introduced into cultured cells after these agents had been encapsulated in vesicles. In addition, new lipid molecules [9] have been introduced into the red cell membrane and water-soluble fluorescent molecules into the cytoplasm of lymphocytes [10,11] after vesicles containing these compounds had presumably fused with the recipient cell membrane. Such studies have helped to generate considerable excitement about the potential of lipid vesicles as a new pharmacological tool. However, the mechanism of vesicle uptake by cells appears to be complex and remains poorly understood, although the subject has been the focus of considerable recent study (for reviews see Refs. 12 and 13). In brief, various investigators have shown that adsorption, phospholipid exchange, endocytosis and fusion are all possible pathways for the interaction of vesicles with cells.

In an attempt to define more clearly the contribution of each pathway to the cell-vesicle interactions, we have used two fluorescence-microscopy techniques. First, we examined the fluorescence-microscopy image of cells after they had been reacted with phospholipid vesicles containing either fluorescent lipid analogs or biotinyl phosphatidylethanolamine which serves as a bilayer-situated ligand for fluorescein avidin. Secondly, we employed fluorescence recovery after photobleaching [14] which allows measurement of lipid diffusion in bilayers [15] and on single cell surfaces [16]. We reasoned that a large amount of fusion of lipid vesicles containing embedded fluorescent lipid analogs with the plasma membrane would be detectable by this technique, since the vesicle lipids should diffuse rapidly within the plasma membrane after fusion resulting in rapid recovery kinetics, characteristic of bilayer lipids. Based on our observations with a number of different tissue culture cells after interaction with phospholipid vesicles of various composition, it appears that the majority of liposomal lipid that becomes cell-associated during a short incubation time is adsorbed onto the cell surface, without extensive fusion into the plasma membrane.

Materials and Methods

Lipid and other chemicals

Palmitic acid (puriss) and stearic acid (puriss) were obtained from Fluka A.G., Switzerland. Stearylamine (cetylamine) was obtained from K and K Laboratories, Plainview, NY, cetyltrimethylammonium bromide (CTAB) was

obtained from Mann Research, New York, NY. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were extracted from egg yolks as described [17]. Dipalmitoyl phosphatidylcholine (DPPC) and distearoyl phosphatidylcholine (DSPC) were synthesized as previously described [17] and contained greater than 99% of the appropriate fatty acid. Phosphatidylserine (PS) was extracted from bovine brain as previously described [18]; [^{3}H]DPPC was prepared as previously described [17], and greater than 99% of the radioactivity comigrated as a single spot with non-labeled DPPC. It was used within 3 months of purification. All phospholipids were purified on silicic acid and found to be pure on thin-layer chromatography [17]. The fluorescent lipid analog, *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine was obtained from Avanti Biochemicals, Birmingham, AL, and yielded a single spot on thin-layer chromatography. 3,3′-Dioctadecyloxacarbocyanine (diO-C_{18}(3)), 3,3′-dioctadecylindocarbocyanine (diI-C_{18}(3)) and 3,3′-dihexadecylindocarbocyanine (diI-C_{16}(3)) were the generous gifts of Dr. A.S. Waggoner. Biotin-labeled egg phosphatidylethanolamine and biotin-labeled bovine brain phosphatidylserine were prepared by using the method of Boyer et al. [19] and purified by acetone precipitation followed by high-pressure liquid chromatography in chloroform/methanol/water (60 : 15 : 1, v/v). These analogs yielded one spot on silicic acid-chloroform/methanol/water (80 : 25 : 2, v/v) thin-layer chromatography which reacted with both biotin and phosphate-specific reagents [19]. They were stored in chloroform under N_2 at −50°C until use. Fluorescent microspheres, 0.25 and 0.88 μm in diameter were purchased from Polysciences, Warrington, PA. Tissue culture media were obtained from Gibco, Grand Island, NY. Avidin and biotin were obtained from Sigma Chemical Company, St. Louis, MO.

Preparation and properties of lipid vesicles

Unilamellar vesicles were prepared by sonication of the phospholipid suspension [17,20] in phosphate-buffered saline without Ca^{2+} and Mg^{2+} [25], pH 7.4, at a concentration of 5 μmol lipid/ml for 1 h at 25°C under N_2. These optically clear preparations were centrifuged at 105 000 × *g* for 60 min to remove any large vesicles. Dynamic light-scattering [21] and negative-stain electron microscopy on selected vesicle preparations confirmed that greater than 95% of the vesicles were between 250 and 350 Å in diameter.

Lipid concentration was determined by phosphate analysis [17]. Fluorescent lipid analogs were included at 0.5—1 mol%, a concentration that does not affect the gel-to-liquid crystalline phase transition temperature as measured by differential scanning calorimetry. In some cases, trace amounts of [^{3}H]-DPPC ($2 \cdot 10^6$ dpm per μmol lipid) were included in order to follow cell-vesicle interactions. Thin-layer chromatographic examination of [^{3}H]DPPC extracted from vesicles following sonication showed no degradation of the label. Vesicles were prepared from the following starting materials: neutral fluid, egg phosphatidylcholine; neutral solid, DPPC or DPPC/DSPC (1 : 1); negatively charged fluid (PS/PC, 1 : 9); negatively charged solid (PS/DPPC/DSPC, 1 : 4.5 : 4.5); positively charged fluid, (stearylamine/PC, 1 : 9); positively charged solid (CTAB/DPPC/DSPC, 1 : 4.5 : 4.5). Fluid vesicles were sonicated at 25°C. Solid vesicles were sonicated at 50°C and held at this temperature for at least 30 min

following sonication. The T_m value for the solid DPPC/DSPC sonicated vesicles has previously been reported at 43°C [22]. Vesicles containing biotin-labeled phosphatidylethanolamine were prepared with phosphatidylcholine at a biotin-labeled phosphatidylethanolamine-to-phosphatidylcholine mol ratio of 1 : 4.

Cells

Mouse L929 cells were grown in RPMI 1640 with 10% fetal calf serum on 35-mm plastic petri dishes (Falcon Plastics, Oxnard, CA) as previously described [2]. L929 suspension cells were grown in RPMI 1640 with 10% fetal calf serum buffered with 25 mM Hepes buffer. The cells were seeded at $4 \cdot 10^4$ cells/ml and subcultured at a cell density of $5 \cdot 10^5$ cells/ml. WI-38, NIL-8 and human fibroblasts were cultured on 35-mm plastic petri dishes [14,23]. Subconfluent cultures were studied and these were usually obtained 24 h after plating. Mouse L1210, ELD and HTC cells were grown in suspension culture [3,6,24]. Suspension cultures were routinely harvested when the cells were at densities between 5 and $9 \cdot 10^5$ cells/ml. The cell density was determined by using a hemocytometer and cell number was adjusted to either $1 \cdot 10^6$ or $2 \cdot 10^6$/ml in the appropriate media prior to the experiment. HTC cells were obtained from Dr. D. Doyle and L929 suspension cells from Dr. H. Weinfeld, both of Roswell Park Memorial Institute, Buffalo, NY.

Interaction of vesicles with cells

Cells grown on petri dishes were washed three times with phosphate-buffered saline without Ca^{2+} and Mg^{2+} and then incubated with vesicles in 1 ml RPMI 1640 without fetal calf serum for the indicated period of time at 37°C. The vesicle lipid concentration was 200 nmol/ml unless otherwise indicated. After the incubation period, the vesicles were aspirated off the cell monolayer and the cells washed an additional three times with phosphate-buffered saline without Ca^{2+} and Mg^{2+} and placed under 1 ml Dulbecco's phosphate-buffered saline with Ca^{2+} and Mg^{2+} unless otherwise indicated, and examined immediately for the various fluorescence-microscopy studies.

Cells grown in suspension were incubated with 200 nmol lipid at $2 \cdot 10^6$ cells per ml of RPMI 1640 without fetal calf serum for 60 min at 37°C while being rotated in a slanted position at 12 rev./h. All cell suspensions were greater than 95% viable as determined by trypan blue exclusion. After the incubation period, 10-ml of ice-cold phosphate-buffered saline without Ca^{2+} and Mg^{2+} were added and the cells centrifuged at $200 \times g$ for 5 min. The cell pellets were resuspended in the same buffer and transferred to a clean centrifuge tube. The cell pellets were washed an additional three times with 10 ml of the same buffer at 40°C and the final pellet was suspended in 1 ml of the same buffer per $2 \cdot 10^6$ cells and kept at 4°C. Fluorescence measurements were made immediately after washing and an aliquot was transferred to a scintillation vial for radioactivity measurements. If the cells were examined for fluorescence recovery after photobleaching, they were warmed to room temperature immediately before any measurements were taken. When the cells were washed using the same protocol but at room temperature, similar fluorescence images and fluorescence recovery results were obtained.

In some experiments, Hepes-saline buffer containing various concentrations

of Ca^{2+} was used in the incubation of the cells with vesicles. The composition of this buffer was 8.0 g NaCl, 0.4 g KCl, 1 g glucose, 2.38 g Hepes, titrated to pH 7.4 with 1 M NaOH with the final volume adjusted to 1000 ml with distilled water. $CaCl_2$ was added to give a final concentration of 10, 50, or 200 mM. In experiments where the cells were incubated with biotin-labeled phosphatidylethanolamine/phosphatidylcholine vesicles, they were washed as indicated above, incubated in 1 ml Dulbecco's phosphate-buffered saline at 4°C with 5 μg fluorescein-conjugated avidin for 1 min and washed an additional three times in Dulbecco's phosphate-buffered saline at 4°C and the fluorescence image was examined or fluorescence recovery after photobleaching measurements were performed immediately.

Fluorescence microscopy and fluorescence recovery after photobleaching measurements

The microscope used for these observations and measurements has been described in detail [14]. It should be noted that with our instrumentation and a 1.5 μm beam diameter ($2w_s$) we could measure diffusion coefficients of approx. $1 \cdot 10^{-8}$ cm^2/s. The half-time for such a recovery will be about 170 ms given by $\tau_{1/2} = (w_s^2/4D)\gamma$, where D is the diffusion constant and γ is taken as 1.2 (see Ref. 14). While the actual observation of such rapid diffusion depends on the nature of the cell, entry of the probe into the plasma membrane and the subsequent capability of the probe to diffuse over the entire cell surface should lead to observable recovery.

Lens designations were those used previously [14]. In this study, the 50 cm focal length lens (FL) previously used to focus the laser beam in front of the objective was changed to a 17.8 cm focal length anti-reflection coated lens (ESCO Optics, Oak Ridge, NJ). To view the specimen, the laser beam was expanded by placing a biconcave lens (L1, f = 6.4 cm) before the focusing lens (FL) such that the spot created by L1 was about one focal length away from FL. The expanded parallel beam emerging from FL was brought to focus near the rear focal plane of the objective by a second converging lens (L2). The result was approximately parallel light emanating from the objective with a reasonably uniform intensity profile. To remove fringes in the image, presumably due to interference phenomena in the coherent excitation beam, an etched-glass wheel was rotated at low frequency (approx. 10 Hz) in the beam before lens L1. Rotation averages out the 'speckle' pattern produced by the etched glass. Most photography was performed with a Leica 35 mm eyepiece camera and GAF 500 or Ektachrome 400 color film using exposure times of 10—60 s, with about 2—5 mW of monochromatic (488 or 496 nm) defocused laser power as an excitation source.

We found the fluorescence images from carbocyanine dyes were photographed more readily than images from nitrobenzoxadiazole and fluorescein dyes, which faded more rapidly.

The fluorescence intensity from selected areas of the cells could be quantitated using the measuring beam of the fluorescence microscope. We term this a microphotometric measurement. This microphotometric technique measures the fluorescence excited by a Gaussian laser beam impinging on the cell having a 1.4 μm $1/e^2$ width; thus, a measure of the average density of vesicles bound to

the cell in small regions is correlated with the fluorescence signal. Such spot measurements were performed on 10 to 15 cells (one measurement per cell) in order to arrive at an estimate of the fluorescence intensity per unit area associated with a given cell in culture after incubation with a given type of vesicle. Cells were located by dark- or bright-field microscopy without knowledge of fluorescence distribution in that region and then the emission measured to provide a random sample of fluorescence intensity per unit area.

Spectrophotofluorometry of fluorescent lipid analog labeled vesicles

The association of vesicles containing *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine or diI-C_{16}(3) with cells was measured in an Aminco-Bowman spectrophotofluorimeter equipped with an averaging digital photometer. The excitation and emission wavelengths for *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine and diI-C_{16}(3) were 460/520 nm and 520/560 nm, respectively. A 2 mm slit was positioned on the excitation side, and a 1 mm slit on the emission side of the cuvette with a 1 mm slit before the photomultiplier. The fluorescence from 1 ml of washed cells ($1—4 \cdot 10^6$) was measured in phosphate-buffered saline without Ca^{2+} and Mg^{2+} containing 1.0% Triton X-100. In each case the same number of cells treated under similar conditions with vesicles lacking the fluorescent probes were used as controls. The fluorescence signal obtained from these control suspensions was subtracted from that obtained from the cells incubated with vesicles containing fluorescent probes. The cell-associated fluorescence was compared to the original fluorescence added to the vesicles and expressed as a percentage of fluorescence that remained cell-associated. Following the fluorescence measurements, the entire aliquot was transferred to a scintillation vial and [^{3}H]DPPC that had become cell-associated was quantitated by liquid scintillation counting as previously described [25].

Results

Fluorescence-microscopy images

As an indication of the cellular location of vesicle membrane or encapsulated fluorescence probes, we have examined the fluorescence-microscopy images of various cell lines incubated with sonicated unilamellar vesicles containing *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine or diO-C_{18}(3), or diI-C_{18}(3). Fig. 1A and B shows fluorescence micrographs of monolayer L929 cells incubated with phosphatidylserine/phosphatidylcholine vesicles labeled with diO-C_{18}(3), where the top and middle of the cell are in focus, respectively. This 'optical sectioning' suggests that the bound vesicles are predominantly located on or close to the cell periphery. Furthermore, the stain is punctate in nature; for reference, the inserts in Fig. 1A and B show that size of 0.25 and 0.88 μm mono-disperse fluorescent microspheres, respectively.

When monolayer L929 cells were incubated with phosphatidylserine/phosphatidylcholine vesicles containing diO-C_{18}(3), washed to remove non-associated vesicles, detached with 0.1% trypsin and immediately examined by fluorescence microscopy, a punctate peripheral image was observed. Similar results were obtained when monolayer L929 cells were first detached with

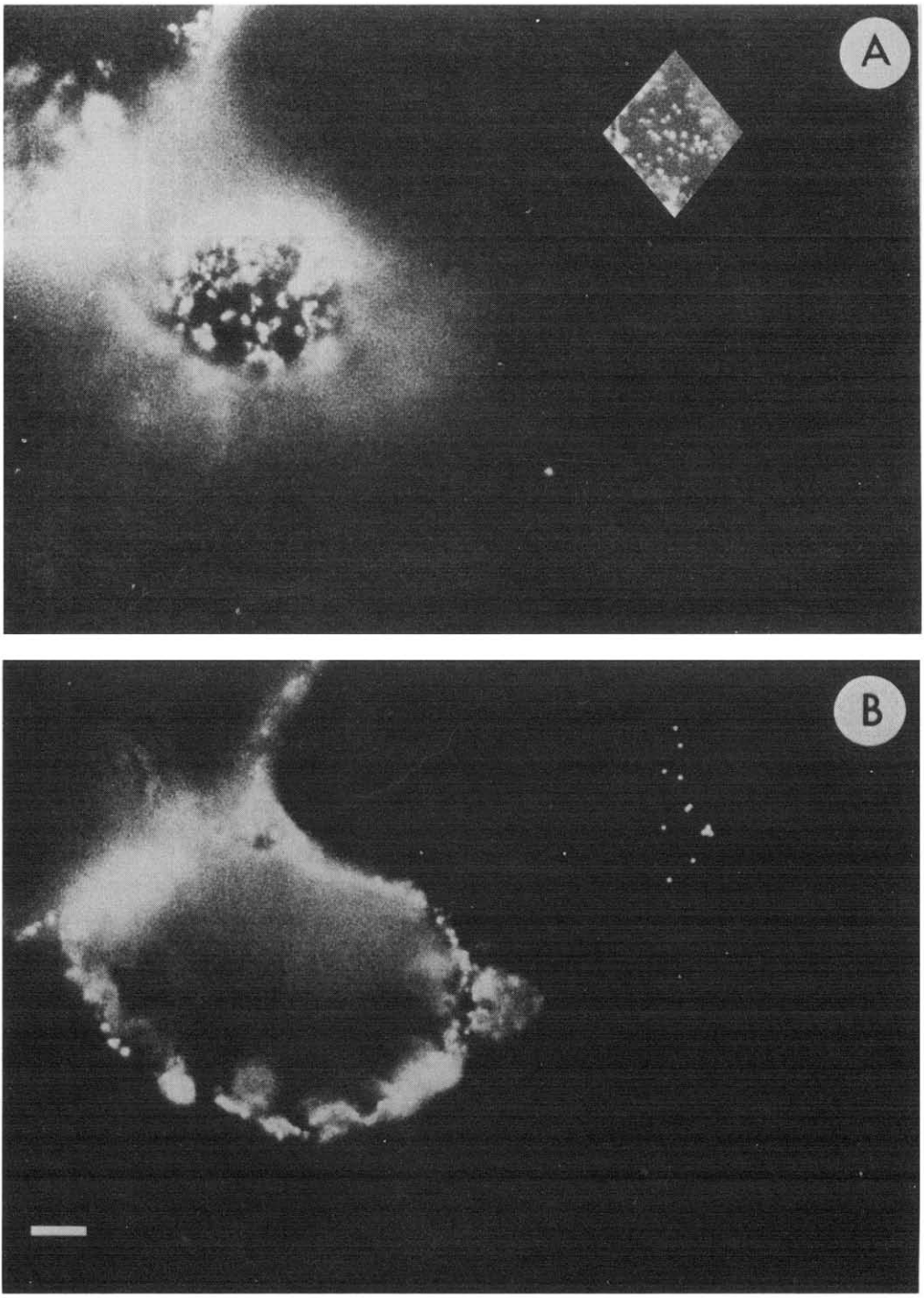

Fig. 1. The distribution of fluorescent phospholipid vesicles on L929 monolayer cells. A and B are fluorescent images of cells incubated with (phosphatidylserine/phosphatidylcholine) vesicles containing diO-$C_{18}(3)$ at 37°C for 1 h. Non-associated phospholipid vesicles were removed as indicated in Materials and Methods. The insert in A is from cell incubated with 0.25 μm fluorescein latex beads. The insert in B is from cells incubated with 0.88 μm fluorescein latex beads. Magnification ×840, bar represents 5 μm.

0.1% trypsin, then incubated with phosphatidylserine/phosphatidylcholine vesicles containing diO-C_{18}(3), washed as indicated to remove non-associated vesicles, and immediately examined by fluorescence microscopy. Similar results were also obtained with L929 cells that had been adapted to suspension culture. These results indicate that the mode of vesicle-cell interaction was not drastically altered by detachment of cells from the substratum or by growth of L929 in suspension cultures.

All other tissue culture cells examined in this project showed a similar punctate fluorescence when incubated with vesicles containing various fluorescence analogs (*N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine, diO-C_{18}(3), diI-C_{18}(3)) in the bilayer; for example, L1210 cells incubated in suspension with vesicles regardless of composition or temperature of incubation (4 or 37° C) yielded a punctate staining pattern similar to that shown in Fig. 2A. This punctate staining pattern was also observed when the biotin-labeled phosphatidylethanolamine (BPE) (BPE/PC, 1 : 4) containing liposomes were incubated with L1210 cells, followed by fluorescein-avidin (Fig. 2B). The similarity of this image with Fig. 2A argues for a predominant localization of the associated lipid at the cell surface since it was accessible to the avidin macromolecule.

Fluorescence images of highly spread NIL-8 cells or human fibroblasts incubated with phosphatidylserine/phosphatidylcholine vesicles labeled with diO-C_{18}(3) (micrographs not shown) were also punctate in nature. However, with such spread cells, the optical sectioning employed on the L929 cell is not possible because the depth of field of the ×50 objective will exceed the cell's thickness, particularly near the periphery. Consequently, though we believe that the punctate fluorescence is localized at the cell surface, the image cannot be used to differentiate between surface and internal fluorescence. With these monolayer cells a similar peripheral, punctate staining pattern was observed with vesicles, phosphatidylcholine, phosphatidylserine, PS/PC (1 : 9), PS/PE (6 : 4) containing diI-C_{18}(3), diI-C_{16}(3) or *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine (NBD-PE).

Estimation of the amount of cell-associated lipid

In order to quantitate the visual impressions of differing amounts of cell-associated lipid obtained by fluorescence microscopy, we measured the intensity of fluorescence from small areas on single cells which had been incubated with vesicles (Fig. 3).

Vesicles (either phosphatidylserine/phosphatidylcholine or DPPC) containing diO-C_{18}(3) as the fluorescence probe yield a similar fluorescence signal when associated with ELD and NIL cells. L929 monolayer cells exhibit a higher level of fluorescence than the other two cell types for both the DPPC and the phosphatidylserine/phosphatidylcholine vesicles. For all three types of cell examined, vesicles that were close to or below their transition temperature (DPPC or DPPC/DSPC mixtures, respectively) exhibited a greater amount of association with cells. Since the entire binding curve was not measured for each cell and vesicle type, the differences observed could represent a true difference in vesicle binding capacity per unit area of differences in the rate of the vesicle cell-association reaction.

The use of fluorescence microphotometry yielded information on the rela-

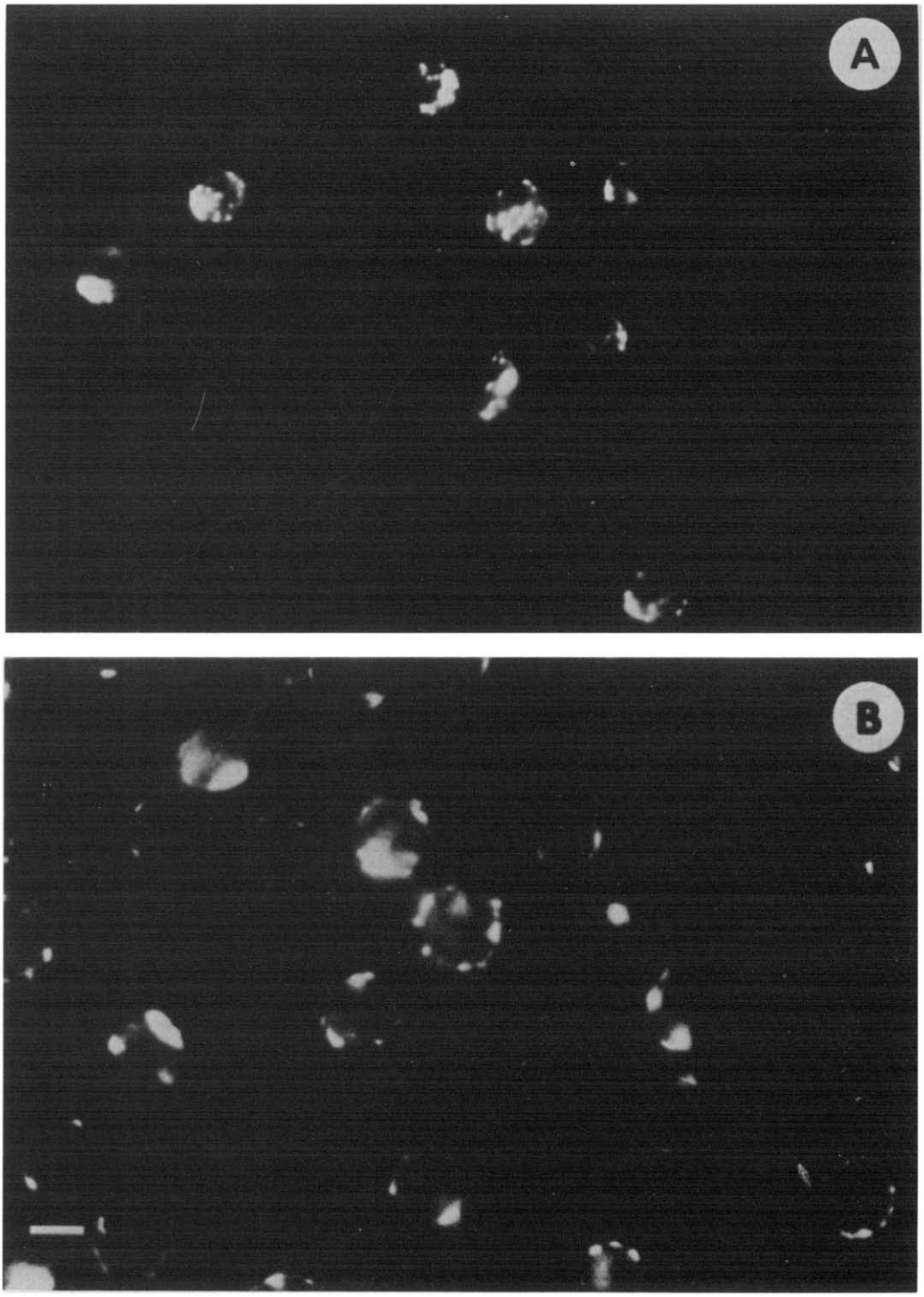

Fig. 2. (A) L1210 cells incubated with phosphatidylserine/phosphatidylcholine vesicles containing diI-$C_{16}(3)$ (magnification X420, bar represents 10 μm). (B) L1210 cells incubated with biotin-labeled phosphatidylethanolamine(BPE)-containing vesicles (BPE/PC, 1 : 4): control cells in the absence of fluorescein-conjugated avidin, or incubated with phosphatidylcholine vesicles followed by fluorescein-conjugated avidin exhibited no fluorescence over the barely detectable autofluorescence associated with untreated cells. The cells were examined immediately after the final wash (magnification X840, bar represents 5 μm).

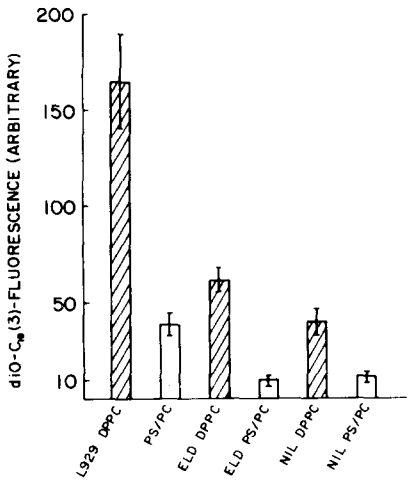

Fig. 3. Microphotometry of diO-C_{18}(3)-labeled phospholipid vesicles associated with various subconfluent monolayer cell cultures as detailed in Materials and Methods. Measurements made using a salt-water immersion X50 objective with a spot size of 1.4 μm. Excitation power: 7.5 μW at 488 nm. All measurements are normalized to the same photometer amplification factor. Fluorescence was measured through an S525 nm interference filter and the values reported are the average of 10—15 measurements on separate cells PC, phosphatidylcholine, PS, phosphatidylserine.

tive level of association of various vesicles to various cells but was not used to obtain an estimate of the absolute amount of vesicle lipid associated with the cell. To quantitate the actual amount of lipid associated with the cells, we have used vesicles containing [^{3}H]DPPC. As seen in Table I, in the cell types examined, fluid vesicles regardless of charge, associate with the cells to a similar degree. Solid neutral and negatively charged vesicles show a 2- to 3-fold greater level of association. Finally, solid positively charged vesicles associate with cells to the greatest extent of any of the vesicle types studied, at levels at least 10-fold greater than that of positively charged fluid vesicles.

Comparison of the uptake of radioactively labeled vesicles with and without

TABLE I

% UPTAKE OF VESICLE-LIPID ([^{3}H]DPPC) BY VARIOUS CELLS

Except where indicated, values given are the mean ± S.E. of three separate experiments with freshly prepared vesicles for each experiment. The values for the HTC cells are the mean of two separate experiments that agreed to within 20%. Where no standard error is indicated, the number in parentheses is the number of observations on which the value is based. F, 'fluid' or liquid crystalline vesicles; S, 'solid' or gel state vesicles; +, vesicles with net positive charge; —, vesicles with net negative charge. PC, phosphatidylcholine; PS, phosphatidylserine; STAM, stearylamine; CTAB, cetyltrimethylammonium bromide.

Vesicle composition	L1210	ELD	HTC	L929
PC (F)	0.42 ± 0.069	0.84 ± 0.31	0.80	0.61 ± 0.075
DPPC/DSPC (S)	1.5 ± 0.35	1.91 ± 0.48	1.62	2.37 ± 0.38
STAM/PC (F+)	0.59 ± 0.052	0.93 ± 0.30	1.14	0.88 ± 0.23
CTAB/DPPC/DSPC (S+)	4.87 ± 0.97	12.36 (2)	11.15	8.67 ± 1.07
PS/PC (F—)	0.55 ± 0.037	0.78 ± 0.25	2.0	0.91 (2)
PS/DPPC/DSPC (S—)	1.37 ± 0.22	1.27 ± 0.49	3.45	1.99 ± 0.30

fluorescent probes shows a close correspondence between the two vesicle types. Vesicles (PC/NBD-PE, 100 : 1) containing both the fluorescent probe *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine and [^{3}H]DPPC, associated with the L1210 cells under the standard conditons at a level of 0.45 ± 0.053 nmol lipid/10^6 cells, while vesicles (phosphatidylcholine) without *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine associated with the L1210 cells to a level of 0.42 ± 0.069 nmol lipid/10^6 cells as determined by scintillation counting (Table I). A simultaneous comparison of lipid uptake (PC/NBD-PE, 100 : 1) measured by *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine fluorescence reveals that 0.32 ± 0.057 nmol lipid is associated with 10^6 cells. In a separate experiment, when phosphatidylcholine vesicles containing both diI-C_{16}(3) and [^{3}H]DPPC were used to follow uptake of vesicle lipid with L1210 cells, 0.54 ± 0.18 nmol lipid/10^6 cells became cell-associated as measured by diI-C_{16}(3) fluorescence compared to 0.55 ± 0.11 nmol lipid/10^6 cells measured by [^{3}H]DPPC association.

These data indicate that relatively small differences exist between different cultured cell types in their interactions with vesicles of similar composition and that solid vesicles associate with cells to a greater degree than fluid vesicles, irrespective of surface charge. Uptake of solid vesicles was significantly enhanced by the inclusion of positively charged cetyltrimethylammonium bromide. Furthermore, the inclusion of the fluorescence probes in the bilayer did not appreciably alter vesicle-cell association. In terms of vesicle lipid associated with the cells, under the conditions in these experiments, a 1% association of vesicles with cells is equivalent to 1 nmol lipid per 10^6 cells, or approx. $2 \cdot 10^5$ vesicles per cell. This assumes that solid and fluid vesicles contain the same number (2000) of phospholipids per vesicle.

Fluorescence recovery after photobleaching

Although the microscopic image suggested, as discussed above, that the majority of vesicles were associated with the cell surface, the question remained as to whether the lipid molecules were still in adsorbed vesicles or embedded within the bilayer of the plasma membrane. To distinguish between these two possibilities we measured the lateral diffusion of molecules in the plane of the plasma membrane by fluorescence recovery after photobleaching.

A typical fluorescence recovery result obtained with vesicles labeled with diI-C_{16}(3) following incubation with L929 monolayer cells is shown in Fig. 4B along with a typical punctate image (Fig. 4A) as discussed above. The recovery kinetics shown in Fig. 4B are indicative of no recovery or complete immobilization of the probe within the time scale of the measurement (5 min).

Similar results were obtained when vesicles (phosphatidylcholine; PS/PC (1 : 9); phosphatidylserine; PS/PE (6 : 4), PC/PE (6 : 4), stearylamine/PC (2 : 8); or DPPC) labeled with diI-C_{18}(3) or *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine were incubated with L929 cells. When these vesicles labeled with diO-C_{18}(3) were incubated with trypsin-detached L929, suspension L929, monolayer NIL-8, human fibroblasts, WI-38, 3T3, ELD, and suspension ELD and L1210 cells, no evidence for rapid recovery kinetics was observed. In addition, when vesicles containing biotin-labeled phosphatidylethanolamine were incubated with either L1210 suspension cells or spread NIL cells and then

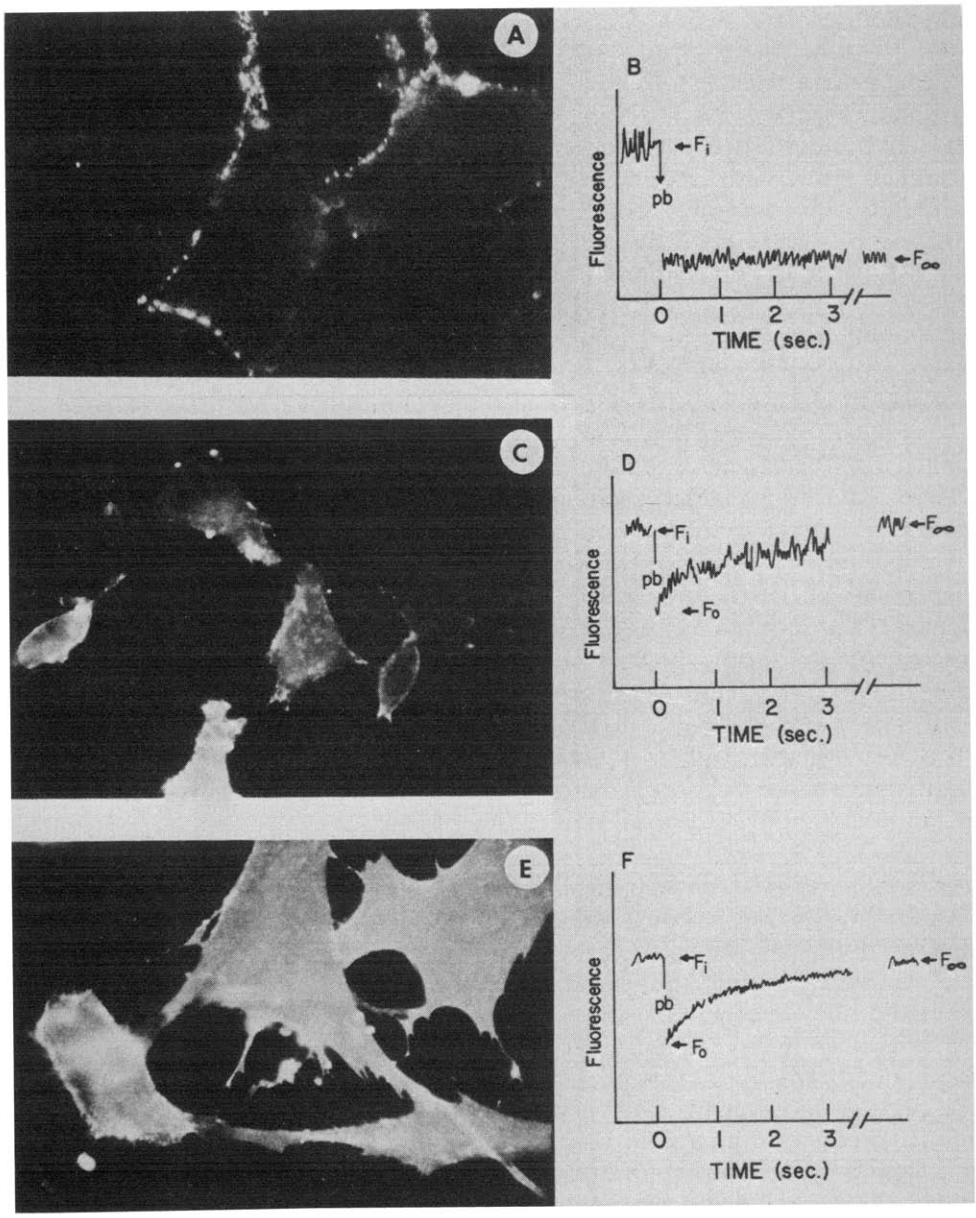

Fig. 4. (A) Subconfluent L929 cells incubated with phosphatidylserine/phosphatidylcholine (1 : 9) phospholipid vesicles containing 1 mol% diI-C_{16}(3) at a lipid concentration of 100 nmol/ml for 30 min at 37°C. (b) Fluorescence recovery kinetics from cells labeled as in A. Bleaching parameters: bleach time, 100 ms; bleach power, 5.5 mW at 488 nm; bleaching beam attenuated by 100 000 for measurement; beam diameter, $2w_s \simeq 1.4$ μm (X53 oil objective employed). (C) L929 cells incubated for 5 min at 25°C with diI-C_{16}(3) at a final probe concentration of about 1.25 μg/ml (5 μl of dye stock solution in ethanol injected into 1 ml of medium overlaying the monolayer culture). (D) Fluorescence recovery kinetics at ambient temperature from cells labeled as in C; apparent $D \simeq 2 \cdot 10^{-9}$ cm^2/s with 95% recovery. Bleaching parameters: bleach time, 20 ms; bleach power, 5 mW at 496 nm; bleaching beam attenuated by 100 000 for measuring; beam diameter, $2w_s \simeq 1.7$ μm (X50 salt-water objective employed). (E) Human fibroblasts incubated for 5 min at 37°C with diI-C_{16}(3) at a final probe concentration of about 1.5 μg/ml (5 μl of dye stock solution in ethanol injected into 1 ml of medium overlaying the monolayer culture). (F) Fluorescence recovery kinetics at ambient temperature from cells labeled as in E; apparent $D \simeq 1.3 \cdot 10^{-8}$ cm^2/s with 95% recovery. Bleaching parameters: bleach time, 50 ms, bleach power, 5 mW at 496 nm; bleaching beam attenuated by 31 600 for measuring; beam diameter, $2w_s \simeq 3.4$ μm (X25 dry objective employed).

stained with fluorescein-conjugated avidin, no fluorescence recovery was observed.

To determine whether the Ca^{2+} concentration of the buffer was important for the cell-vesicle interactions, we incubated L1210 cells with phosphatidylcholine vesicles containing diI-C_{16}(3) in a Hepes-saline buffer for either 5 or 30 min at Ca^{2+} concentrations of 0, 10, 50 and 200 mM at 37° C. Following the removal of non-associated vesicles as described in Materials and Methods, the fluorescence image and recovery kinetics were examined. At all Ca^{2+} concentrations and times of incubations a punctate peripheral image was observed which showed no recovery. It was not possible to incubate phosphatidylserine vesicles with cells in high Ca^{2+}-containing media because the vesicles underwent a Ca^{2+}-induced aggregation [20]. However, when cells were first incubated under the standard conditions with phosphatidylserine vesicles containing diI-C_{16}(3), washed to remove non-associated vesicles, then resuspended in media containing high concentrations of Ca^{2+}, a punctate peripheral image was observed that did not recover when photobleached.

As a control we introduced the same probe into the plasma membrane via another technique and showed that rapid and complete fluorescence recovery occurs following photobleaching. This has been accomplished with diO-C_{18}(3), diI-C_{16}(3) and diI-C_{18}(3) when these probes are introduced into the cell cultures in a small amount of ethanol; however, the diI probes label the cells much more readily as noted earlier [16]. With L929 cells, rapid and nearly complete recovery occurs (Fig. 4C and D). This labeling was most effective when performed at approx. 1 μg/ml diI for less than 5 min at 25 as opposed to 37° C. Inspection of the labeled L929 cells revealed that some probe appeared to be internalized into the cells. This observation is in contrast to NIL or human fibroblast cells which exhibited a more uniform staining pattern. Furthermore, with L929, the surface after labeling shows a fine punctate pattern which may arise because of the microvillae on the surface of L929 cells [23]. Both internalization via relatively immobile endocytotic vesicles and a high density of labeled microvillae could give rise to incomplete or slow recovery. The complication of internalization can be minimized since measurement of recovery kinetics immediately after labeling yields a complete recovery with D in the range of $1—5 \cdot 10^{-9}$ cm^2/s. (Fig. D). This value appears lower than the usually measured value of $D \simeq 1 \cdot 10^{-8}$ cm/s probably because the laser beam intercepts the 'plump' L929 cell with two different diameters. This can result in a slower measured D with the additional possibility of overbleaching the cell surface which intercepts the laser beam at or near its waist [26]. These limitations mean we can use the 100% recovery as an indication of diffusion of the lipid analog probe within the plasma membrane of the L929 cell but we must rely on the highly spread NIL or human fibroblast cell for the quantitative control measurement. An image of diI-C_{16}(3) stained human fibroblast cells and typical fluorescence recovery after photobleaching kinetics are seen in Fig. 4E and F. The diffusion coefficient calculated from Fig. 4F is $1.3 \cdot 10^{-8}$ cm^2/s.

In these experiments the cell-associated fluorescence following incubation with vesicles containing 3 mol% diI-C_{16}(3) was equivalent to that obtained by direct staining with 1 μg/ml diI-C_{16}(3) injected with ethanol. Conditions that result in a punctate fluorescence image with the vesicle-treated cells but a uniform fluorescence image with the ethanol-injected cells.

Discussion

The basic observations of this study are: (1) the fluorescent image of certain cultured cells either in suspension or in monolayers incubated with phospholipid vesicles of varying surface charge and fluidity was punctate and peripheral in nature, and (2) when such cells were photobleached, fluorescence recovery was not observed within the experimental time period. The former observation suggests sites for interaction of the vesicles occur in discrete regions of the cell surface. The latter indicates that the cell-associated lipid is not free to diffuse distances comparable to the beam diameter (approx. 1.4 μm). Furthermore, the similarity of cellular images using fluorescein-conjugated avidin to label biotinyl phosphatidylethanolamine-containing vesicles following their interaction with cells suggests that a considerable amount of the cell-associated lipid resides on the outer surface of the cell where it is accessible to the macromolecule, avidin.

Two hypotheses may be constructed to account for these data. First, the punctate image simply represents collections of adsorbed vesicles which are not mobile enough to contribute to any fluorescence recovery. Second, fusion of most of the vesicles occurs but the fluorescent probe is restricted to discontinuous locations at or near the cell's surface (the punctate areas).

The second of the two hypotheses above is less attractive because the same fluorescent lipid analogs used to label the vesicles can be added to the cells via an ethanol-injection technique and result in a similar amount of fluorescent analog becoming cell-associated. In such experiments, the cells are uniformly labeled and rapid and complete fluorescence recovery was measured with the calculated diffusion coefficients similar to those obtained for the same dye in multibilayer model membranes [15] and biomembranes [16]. This strongly suggests that the same lipid fluorescent probes, when incorporated in the plasma membrane, are free to diffuse over distances comparable to the beam diameter. We would expect the fusion of vesicles containing fluorescent lipid analogs to result in similar behavior. Therefore, the simplest interpretation of our data would be that within the experimental time period, the majority of the vesicles are adsorbed onto the cell surface.

Several points need to be made about these fluorescence recovery data. First, vesicle-bound lipid probes could rapidly diffuse within the plane of an intact vesicle membrane without any recovery of fluorescence because the width of the measurement beam (approx. 1.4 μm) is much larger than the diameter of a sonicated unilamellar vesicle (0.030 μm). A further consideration relates to the possibility that the punctate fluorescence observed on cells after vesicle incubation could result from discrete binding sites for the probe which are localized into domains large enough to be visualized by fluorescence microscopy. This might arise from fusion of the vesicles at specific locations on the cell surface or to a time-dependent fusion with the surface, followed by the rapid sequestration of the probe to discrete locations resulting in a punctate fluorescence image. Such a process might not be distinguishable from intact vesicles that are associated with the cell surface. Thus, the inability to detect fluorescence recovery when these probes are introduced in vesicles may be due to a kinetic phenomenon; the actual residence time of the probe as freely

diffusing molecule in the membrane might be brief before it redistributes into the punctate structures. In a photobleaching experiment, these small regions are bleached but are not connected to any reservoir of unbleached material so that fluorescence recovery cannot occur. If cells labeled via ethanol injection took up considerably more dye than when an equivalent amount of probe is added in vesicles it could be that the putative static probe binding sites are saturated leaving the excess dye to become incorporated into the plasma membrane where it freely diffuses. This possibility is remote since with L1210 cells we obtained as much cell-associated fluorescence through an incubation under standard conditions with vesicles containing 3 mol% diI-C_{16}(3) as by directing staining with 1 μg/ml diI-C_{16}(3) injected with ethanol and the punctate image of the vesicles associated with the cells was unaltered.

In bleaching a punctate cell in which the bright areas were small compared to the beam diameter and not mobile, a priori, one would expect little or no recovery. The question is whether there exists a rather dim diffuse component provided by fusion or exchange diffusion which is obscured when compared to the bright punctate image. Indeed, a small component of probe incorporated in the plasma membrane which recovers rapidly and completely could be overlooked in the noise accompanying the recovery curve. We estimate that this component, if it exists, would be no greater than 10% of the total cell-associated lipid. Furthermore, it does not appear that high external Ca^{2+} concentrations enhance fusion of cell-associated vesicles. When vesicles comprised of phosphatidylcholine/diI-C_{16}(3) were incubated with cells in Hepes-glucose buffer containing 10, 50 and 200 mM Ca^{2+}, the vesicles did not aggregate yet still yielded a punctate staining pattern with no fluorescence recovery. Thus, the presence of divalent cations in the incubation media did not influence the nature of the staining pattern or the fluorescence recovery kinetics.

Although we believe the majority of vesicles are adsorbed to the cell surface some degree of endocytosis is not excluded by our experiments, since endocytotic vesicles could still be located close to the plasma membrane. Furthermore, the staining with fluorescein-conjugated avidin does not preclude that a fraction of the lipid has been endocytosed, leaving only a portion still at the periphery. Experiments with metabolic inhibitors of endocytosis could, however, eliminate this possibility.

The punctate fluorescence image was observed with: (1) vesicles having several different fluorescent lipid probes (diO-C_{18}(3), diI-C_{18}(3), diI-C_{16}(3), and *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine), and (2) with a variety of cultured cells in both monolayer and suspension. For self-luminous green objects, the resolution limit for a X100 objective (N.A. = 1.3) is about 0.25 μm. On this basis alone, one would not know if the fluorescence from an individual punctate particle were generated by a single vesicle or a group of vesicles. However, the large amount of cell-associated lipid coupled with the relatively sparse punctate pattern strongly suggests that aggregates of vesicles and/or restricted vesicle-binding regions occur on the surface of the cell. Scanning electron microscopy studies have shown that the L929 cells display a 'rough' surface morphology with many blebs and microvillae [23]. It is possible that the punctate stain may, in part, be caused by vesicle-binding to these structures; when these features are nearly coincident with the optical

axis, a bright spot of fluorescence would occur due to the longer excitation path for fluorescence.

At this juncture, the origin of the cellular differences in vesicle association (putative adsorption) is not understood nor are the reasons for enhanced association of solid compared to fluid vesicles (Table I, Fig. 3). Since fluid vesicles, regardless of charge, associate with cells to a similar degree, the fluidity of the lipid matrix may be more important in mediating the interaction than surface charge. Alternatively, the solid vesicles may be slightly larger than fluid vesicles so that the increase in cell-associated lipid with the solid vesicles is due to larger vesicles, not more vesicles. In regard to the enhanced adsorption of solid vesicles, Pagano and Takeichi [27] suggested that cell surface proteins provide binding sites for solid vesicles, based on the ability of trypsin to remove a significant fraction of cell-associated solid vesicles, and on the protection against iodination of certain cell surface proteins afforded by solid vesicles. As other investigators have also demonstrated [4,7], solid positively charged vesicles or positively charged vesicles containing cholesterol associate with the cell surface to a much greater degree than negative or neutral vesicles. It is plausible that electrostatic interaction with the negatively charged cell surface mediates this association. However, a more rigid or clustered charge distribution on the vesicle membrane may be required since the fluid charged vesicles do not exhibit such pronounced cell association. One assumption in the evaluation of the effects of vesicle charge on amount of cell-associated lipid following incubation of vesicles with cells is that the charge on the vesicle surface remains unaltered. This may not be the case since under similar conditions, both lipid exchange [28] and modification of vesicles by cell surface proteins have been reported [29].

The conclusion that a large fraction of cell-associated vesicles is adsorbed to the cell surface is not inconsistent with previous studies demonstrating the entry of growth-controlling [1,2], cytotoxic [3], or viral components [8] into cultured cells, since such qualitative bioassays can be extremely sensitive to the entry of small amounts of these agents. Furthermore, the incubation times in many of those reports were considerably longer than those employed in this study. In those cases, a low but constant rate of internalization of the contents of cell-associated vesicles could account for the observed biological effects.

However, our conclusions must be contrasted with reports that a significant fraction of cell-associated fluid vesicles fuse or transfer their contents during short-term experiments [5,10,11,22,27,30,31]. Some of these studies have employed the carboxyfluorescein transfer technique [10,11,27,30], the limitation of which we have previously discussed [25] and which would tend to overestimate fusion. Other studies have used the association of radioactive lipid [22,31] with cells in the presence of metabolic inhibitors. In the latter studies, the fraction of vesicles fused with the cell surface could also be overestimated because the fate of the vesicle lipid or contents was not followed.

When considering the possibility of vesicle-cell fusion, it is instructive to recall that in model systems, it is necessary for the close apposition of opposing bilayers to occur before fusion will result [18,20]. Thus, for cell-vesicle fusion to become an important event, it is probably necessary for the vesicle bilayer to penetrate the 'exoskeleton' and interact with the plasma membrane bilayer

[32]. It is quite possible that with many cell types, the structure of the exoskeleton prevents the frequent apposition of vesicles with the plasma membrane bilayers; thus, the structure of this surface component is most likely an important parameter in determining the relative magnitudes of the pathways involved in vesicle-cell association.

The existence of a large number of adsorbed vesicles on the surfaces of many tissue culture cells should be recognized as a pool of precursors for relatively infrequent vesicle-cell fusing events or for an eventual endocytosis by an energy-dependent process. Moreover, these vesicles would act as a depot of exogenous material that could enter the cell via well known permeability and exhange mechanisms during the course of long-term incubations.

Such adsorption, if general, could frustrate attempts to modify the surface of cells by fusion of the vesicle with the plasma membrane: while some fusion may occur, there could still be a large background of vesicles which are simply adsorbed to the surface. For many physical studies, e.g., spectroscopic measurements, the signal component arising from the adsorbed vesicles would have to be separated from that arising from the fused vesicle. On the other hand, for surface modifications that depend on the correct insertion of a membrane component into the plasmalemma in order to be registered in the appropriate bioassay (e.g., proper transduction of a hormonal signal by a membrane receptor), the adsorbed vesicle component will remain effectively silent.

Attempts to modify the physiology of cells by the introduction of various molecules that are required in relatively high concentrations may be similarly frustrated by a large fraction of surface-adsorbed vesicles. However, as a technique for introducing regulatory substances that can be amplified within the cell, such as nucleic acids, vesicles seem to be a valuable tool for cell biologists.

In this context, methods to enhance vesicle-cell fusion by perturbation of the cell surface are probably worthy of development. Attempts to date using polylysine [33], and poly(ethyleneglycol), while showing some promise have been limited by relatively low cell viability after the treatment (Szoka, F., unpublished data). Finally, considering that fusion may generally be a low probability event, the advantage of larger vesicles in any delivery scheme should be stressed: increasing the internal diameter of the vesicles from 150 Å to 1500 Å would increase the amount of material delivered per fusion event by 1000-fold.

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