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Liposome–lymphocyte interaction: Saturable sites for transfer and intracellular release of liposome contents

(lipid vesicles/fusion)

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ABSTRACT **The water-soluble dye 6-carboxyfluorescein was trapped in the internal aqueous compartments of small sonicated dioleoyl lecithin vesicles and used to assess the kinetics of transfer of vesicle contents to human lymphocytes. By using flow microfluorometry, the initial rate of dye transfer to the cells was measured as a function of the concentration of vesicles in the external medium. The rate of transfer consists of at least two components, one of which saturates at high vesicle concentration and the other of which does not saturate in the range of concentrations explored. The saturable component was competitively inhibited by vesicles not containing dye. Both the saturable and nonsaturable components of transfer were inhibited by fetal calf serum or bovine serum albumin but neither component was affected by bovine IgG, choline chloride, or heparin. Pretreatment of the lymphocytes with trypsin or Pronase had no effect on either component. The saturable component can be interpreted in terms of a two-step process in which vesicles bind reversibly to sites on the cell surface, and dye is then transferred into the cell from the vesicle–site complex.**

Liposome–cell interaction is currently being studied in a number of laboratories as a model for the fusion of biological membranes occurring in such processes as phagocytosis, secretion, cell division, and heterokaryon formation (1). The use of liposomes‡ makes it possible to substitute a synthetic membrane of known and controllable composition for one of the natural ones (2, 3). Liposomes are also under intensive investigation as vehicles for introducing membrane-impermeant substances (e.g., drugs, enzymes, chelating agents) into cells (2–4). Less attention has been devoted to the kinetics of vesicle–cell interaction, although a number of attempts have been made to define the mechanisms involved (5–10).

Liposomes can become associated with cells by stable adsorption to the cell surface, by endocytosis, or by fusion with the cell membrane (5, 6). Studies in our laboratory (11) and that of Huang *et al.* (12) indicate that neither stable adsorption nor metabolically active endocytosis is significant in the interaction of lymphocytes with vesicles whose lipids are in the fluid phase. Fusion and, perhaps, a form of endocytosis insensitive to glycolytic and respiratory inhibitors are therefore left as the major possible mechanisms for transfer of the contents of such vesicles to lymphocytes. The combination of lymphocytes and fluid phase vesicles thus provides a relatively simple system for a detailed study of the kinetics of the transfer process.

In collaboration with Hagins and Yoshikami we recently developed a sensitive technique for monitoring the transfer of a trapped marker from liposomes into cells (11) using the highly water-soluble fluorescent dye 6-carboxyfluorescein (6-CF). In those studies, incubation of human peripheral blood lymphocytes with small unilamellar lipid vesicles containing a high concentration of 6-CF resulted in a widespread distribution of fluorescence throughout the cytoplasm of each cell. "Self-quenching" largely prevented the dye from fluorescing as long as it remained trapped at high concentration in vesicles; relief of self-quenching as the dye was released into the much larger volume of a cell enabled us to monitor transfer of 6-CF from vesicle to lymphocyte. To avoid the mechanistic implications of terms such as "fusion," we use the operational term "transfer" to indicate passage of 6-CF, with accompanying dilution, into the cell interior.

In the present study, we measured the initial rate of 6-CF transfer from fluid phase unilamellar lipid vesicles to lymphocytes as a function of liposome concentration under various conditions. Kinetic analysis revealed both a nonsaturable and a saturable component of the transfer, the latter obeying Michaelis-Menten kinetics (13).

MATERIALS AND METHODS

Preparation of Lymphocytes. Human peripheral blood lymphocytes from healthy donors were purified by using carbonyl iron and Ficoll-Hypaque (14). The cells, which included fewer than 1% monocytes, were cultured overnight in Eagle's medium with 10% fetal calf serum and then resuspended in Hanks' balanced salt solution (pH 7.4) prior to use.

For protease treatment, cells were washed twice by centrifugation with Eagle's Hepes medium containing no protein and then resuspended at 10^7 cells per ml. Digestion, with pancreatic DNase I (20 μg/ml) and either trypsin (1 mg/ml) or *Streptomyces griseus* protease (equivalent to Pronase) (1 mg/ml), was carried out at 37° for 20 min, and then the cells were washed three times in Eagle's Hepes medium containing +10% fetal calf serum.

Preparation of Liposomes. Twenty-five milligrams of L-α-dioleoyl lecithin (DOL) was dried under nitrogen into a thin film on a glass vial, further dried overnight in high vacuum, and then hydrated with 4 ml of 100 mM 6-CF in H_2O (pH 7.4). The suspension was vortex mixed and then sonicated in a glass tube under nitrogen for 1 hr (microtip, Bronson sonifer, model W-350, power setting 3) at about 35°. The sonicate was passed through a 0.22-μm Millipore filter, after which free 6-CF was removed by passage through a short column (26 × 60 mm) of Sephadex G-50 (fine) at 5° with 167 mM NaCl/7 mM KCl/10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethylsulfonic acid, pH 7.4, as eluant. The 6-CF content of each preparation was determined from fluorometric measurements before and after addition of Triton X-100 detergent. Empty vesicles were pre-

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Abbreviations: 6-CF, 6-carboxyfluorescein; DOL, L-α-dioleoyl lecithin.

‡ The terms "liposome" and "vesicle" will be used interchangeably.

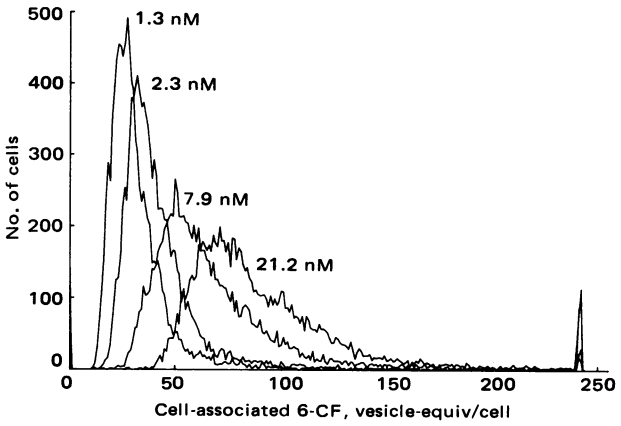

FIG. 1. Frequency distributions of total cell fluorescence obtained by flow microfluorometry. Histograms are shown for 10-min incubations with liposomes at concentrations indicated. The cell fluorescence signals from each incubation fell in a single broad peak.

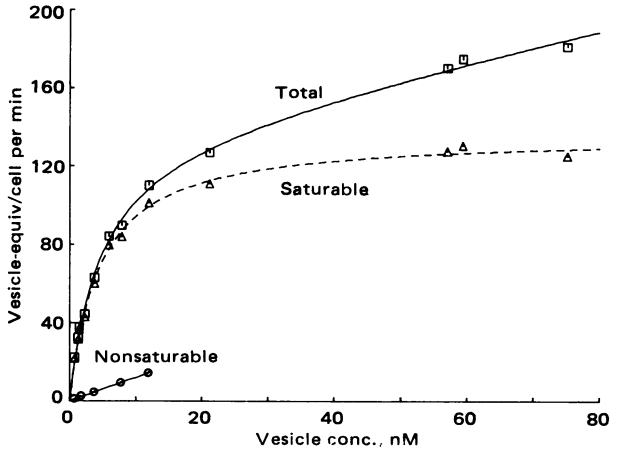

FIG. 2. Initial rate of transfer of 6-CF from vesicles to lymphocytes at 37° as a function of vesicle concentration in the medium. Squares represent observed values for the total rate of transfer. The curve through the squares is the best fit (nonlinear least squares, constant weighting coefficients) by using Eq. 1. The saturable rates of transfer (triangles) were calculated by subtracting the nonsaturable component from the total. The dashed line represents the best fit for the saturable transfer according to Eq. 1. Circles represent the rate of transfer in the presence of excess (99 nM) empty vesicles.

pared by hydrating 25 mg of DOL in 4 ml of eluent and sonicating and filtering as for 6-CF vesicles but omitting column chromatography. Lipid concentrations were determined by phosphate analysis (15). DOL vesicles prepared in this way have previously been characterized by quasi-elastic light-scattering and negative staining electron microscopy (11). For calculation of the concentration of vesicles per mol of lipid or 6-CF, we use the following numbers determined by Hauser *et al.* (16) for 250-Å lecithin vesicles: internal volume, 2.2×10^{-18} cm^3 per vesicle; lecithin molecules per vesicle, 4000. Those numbers agree well with our measured values of lipid/6-CF ratio.

Incubations. The lymphocytes were preincubated in polystyrene tubes at 37° for 30 min with the appropriate incubation medium. Each tube contained approximately 4×10^6 cells in 0.5 ml of incubation medium. Vesicles containing 100 mM 6-CF were then added in small volume to make a final volume of 0.54 ml. The tubes were shaken gently to disperse the vesicles and then were not agitated again during incubation. Incubation was continued for 10 min at 37° and terminated by addition of 2.5 ml of iced Hanks' balanced salt solution containing 10% fetal calf serum. The cells were washed twice by brief centrifugation and then resuspended in 0.8 ml of iced medium for flow microfluorometry. There was little leakage of fluorophore from the cells over a period of hours at 4°. Supernatants of the original incubations were generally checked to assess the total concentration of 6-CF in vesicles and the amount of leakage of dye from the vesicles. Supernatants from final centrifugations were also checked and found to contain negligible amounts of fluorophore.

Flow Microfluorometry. Population distributions of cell fluorescence and the average number of molecules of 6-CF incorporated into each viable cell were determined by flow microfluorometry using the fluorescence-activated cell sorter (Becton–Dickinson Laboratory, Mountain View, CA) as described (11). In all cases the cells were more than 95% viable by a criterion based on light scattering (17). We express the results here in terms of "vesicle-equivalents"—that is, in terms of the number of vesicles whose total 6-CF contents would be required to produce the observed cell fluorescence. Assuming an internal volume of 2.2×10^{-18} cm^3, each vesicle with 100 mM 6-CF contains 130 molecules of the dye. Hence, 1 vesicle-equivalent corresponds to 130 dye molecules.

It should be noted that, if the transfer process were not 100% efficient (i.e., if it were leaky), the number of vesicles interacting would be greater than the observed number of vesicle-equivalents. The conversion factor could be determined only by measuring lipid incorporation as well as 6-CF incorporation. The kinetic description of liposome-mediated transfer would not be different, however. The data in the figures were fitted by using MLAB, an interactive computer (PDP-10) modeling system developed by G. Knott at the Division of Computer Research and Technology, National Institutes of Health.

Materials. DOL was obtained on special order from Applied Science (Philadelphia, PA). It was shipped in dry ice and kept in sealed ampoules at −20° until use; 100-μg samples gave single spots on thin-layer chromatography (visualization with I_2 vapor), and similarly prepared DOL showed less than 1% fatty acid contamination on gas/liquid chromatography. Heat-inactivated fetal calf serum was obtained from GIBCO (Grand Island, NY). 6-CF (Eastman, Rochester, NY) was treated with activated charcoal and recrystallized from ethanol/water (approximately 1:2). Sodium azide, 2-deoxy-D-glucose, heparin, and *S. griseus* protease were obtained from Sigma (St. Louis, MO); bovine serum albumin fraction V containing less than 0.25% (wt/wt) total lipid (manufacturer's data) and bovine IgG were obtained from Miles (Elkhart, IN); choline chloride was from Eastman (Rochester, NY);, trypsin was from Calbiochem (San Diego, CA); and DNase I was from Worthington (Freehold, NJ). Hanks' balanced salt solution, eluant, and Eagle's medium were prepared by the National Institutes of Health media production unit.

RESULTS

Saturable and Nonsaturable Components of Transfer. Fig. 1 shows fluorescence frequency histograms obtained by flow microfluorometry on lymphocytes that had incorporated 6-CF from various concentrations of DOL vesicles during 10-min incubations. The cell signals from each incubation appeared in a single broad fluorescence peak, indicating that all cells in the population had taken up dye. The mean numbers of dye molecules transferred from vesicle to cell were calculated from the histograms.

In Fig. 2, the squares indicate rates of 6-CF transfer during the 10-min incubations as a function of liposome concentration.

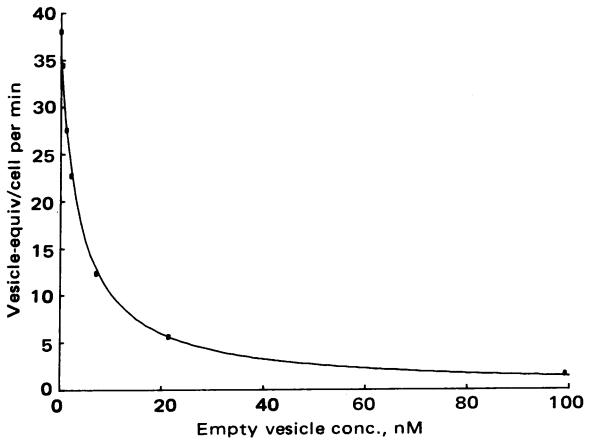

FIG. 3. Initial rate of transfer of 6-CF from vesicles to lymphocytes as a function of empty vesicle concentration. The concentration of 6-CF-containing vesicles was 1.6 nM. Circles represent measured points; the curve represents the best fit to Eq. 1 as a function of $[I]$ varying K_I, but leaving K_m, V_{max}, and k_N unchanged.

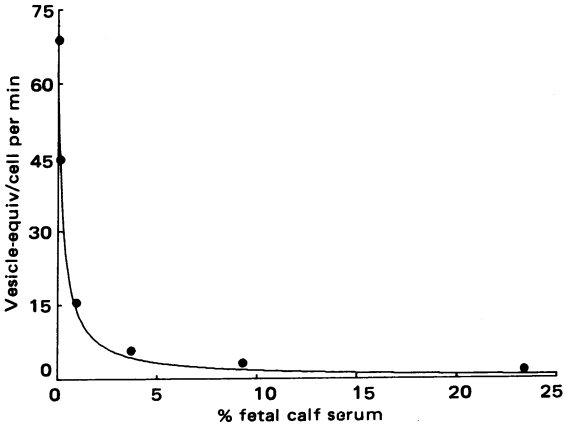

FIG. 4. Initial rate of transfer of 6-CF from vesicles (concentration 3.7 nM) to lymphocytes as a function of fetal calf serum concentration. Conditions of incubation were as for Fig. 1. ●, Measured points; the curve represents the best fit to Eq. 1, varying K_I but leaving V_{max}, K_m, and k_N unchanged.

The time course of increase in fluorescence is approximately linear over the first 20 min of incubation (18). Hence, 10-min incubations give good approximations of the initial rates of transfer. In no case was liposome 6-CF in the incubation decreased by more than 0.3% by transfer into the cells. The data are compatible with the hypothesis that the liposome-mediated transfer consists of two processes, one of which is saturable and the other of which is nonsaturable (i.e., linear) over the concentration range explored. At low liposome concentration, most transfer occurs through the saturable process.

The saturable component of transfer can be treated by a Michaelis–Menten model in which the vesicles bind reversibly to sites and the 6-CF is transferred from the vesicle–site complex into the cell. The nonsaturable transfer is assumed to be proportional to vesicle concentration with a clearance rate constant k_N. The total rate of transfer (v) is then given by:

$$v = \frac{V_{max}[L]}{[L] + K_m(1 + [I]/K_I)} + k_N[L] \qquad [1]$$

in which $[L]$ is the liposome concentration, $[I]$ the concentration of a competitive inhibitor (to be discussed later), K_m the Michaelis constant, K_I the inhibition constant, and V_{max} the maximal rate of saturable transfer.

We fitted the data represented by squares in Fig. 2 to Eq. 1 with $[I] = 0$ and obtained the following values for the kinetic parameters: $V_{max} = 137 \pm 7$ vesicle-equivalent cell^{-1} min^{-1}; $K_m = 4.5 \pm 0.4$ nM vesicles (i.e., 0.0144 mg of lipid per ml); and $k_N = 0.75$ (vesicle-equivalent cell^{-1} min^{-1})/(nmol vesicle liter^{-1}). (The units of k_N indicate that it represents a slope in Fig. 2.) In separate experiments the clearance rate for transfer of free 6-CF from the medium into lymphocytes was found to be 0.1 (molecules cell^{-1} min^{-1}/nmol liter^{-1}). This is about a factor of 8 smaller than the nonsaturable liposome-mediated transfer rate constant k_N, indicating that the nonsaturable transfer is not due to free dye that had leaked from vesicles. The triangles in Fig. 2 were calculated from data for total transfer with the nonsaturable component subtracted. Incubations at higher vesicle concentrations than those shown in Fig. 2 (at which the nonsaturable component becomes more important) also showed single-peak fluorescence histograms, indicating that the two components do not correspond to different cell populations.

Inhibition by Empty Vesicles. Measurements of transfer in the presence of an excess (99 nM) of empty vesicles (i.e., vesicles containing no 6-CF) were also consistent with Eq. 1. The results are indicated by circles in Fig. 2. At that concentration of empty vesicles the saturable component is almost completely abolished and only the nonsaturable component, proportional to liposome concentration, is seen.

Fig. 3 shows the rate of transfer as a function of the concentration of empty vesicles. The experiment was carried out at a low fixed concentration of 6-CF vesicles (1.6 nM), at which the saturable component dominates. Empty vesicles were added to the incubation medium a few seconds before addition of 6-CF-containing vesicles, but the inhibition was the same if the empty vesicles were added 20 min earlier. These results confirm that the saturable transfer was indeed almost completely eliminated by the concentration of empty vesicles used in the experiments of Fig. 2.

Because the empty vesicles and the 6-CF vesicles were made the same way, we expected *a priori* that K_m and K_I would be equal. The inhibition constant obtained by fitting the data to Eq. 1 as a function of $[I]$, leaving K_m, V_{max}, and k_N unchanged ($K_I = 3.0 \pm 0.3$ nM) was 67% of K_m. Considering the usual uncertainties in determining K_m and the possible systematic errors in determining the concentration of vesicles from 6-CF and phosphate analysis, the agreement is quite good. Empty multilamellar DOL liposomes (formed without sonication) also inhibited transfer, indicating that the competitive effect is not dependent on the geometry of the liposome.

Effects of Serum Components. Fig. 4 shows the rate of transfer as a function of fetal calf serum concentration. The experiment was carried out at a concentration of 6-CF vesicles at which the saturable component of transfer dominates (3.7 nM). The inhibition was almost complete at 9% fetal calf serum. At this concentration, serum affects vesicle leakage by at most a few percent. The value obtained for K_I by fitting the data in Fig. 4 to Eq. 1 was 0.15% fetal calf serum.

Fig. 5 is a Lineweaver–Burk plot of the saturable component of liposome-mediated transfer in the absence and presence of 1% fetal calf serum. The data are fitted to Eq. 1 with the constraint that V_{max} remains the same with and without serum but K_m is allowed to vary. Using that constraint, we obtain an approximately 10-fold larger apparent K_m in the presence of serum. The reasonably good fit with an unchanged V_{max} is consistent with competitive inhibition by serum. The K_I calculated from the data is 0.17% serum; this value agrees well with

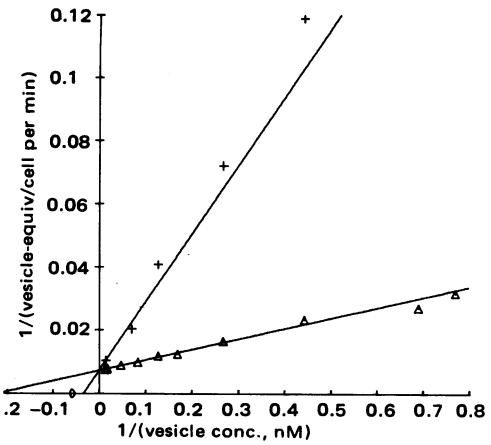

FIG. 5. Lineweaver–Burk plot for the saturable component of 6-CF transfer from vesicles to lymphocytes as a function of liposome concentration in the presence (+) and absence (△) of 1% fetal calf serum. The lines represent simultaneous fits to Eq. 1 for transfer with and without serum, with the constraint corresponding to the hypothesis of competitive inhibition by serum (i.e., unchanging V_{max}). Constant weighting coefficients were used (on the uninverted data).

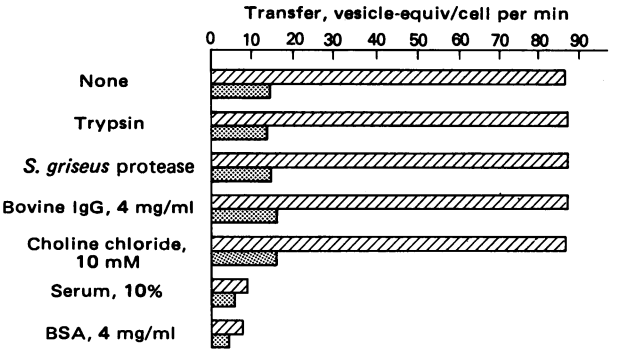

FIG. 6. Effect of various compounds or treatments on saturable (hatched bars) and nonsaturable (stippled bars) components of liposome-mediated transfer of 6-CF from vesicles to lymphocytes. See *text* for conditions of proteolysis and of incubations. The concentration of 6-CF liposomes was 35 nM and that of the empty liposomes was 132 nM. Corrections were made for the residual 21% of the saturable component not blocked by empty vesicles. BSA, bovine serum albumin.

that found when the serum concentration was allowed to vary.

Fig. 6 summarizes the results of experiments in which we used empty vesicles to separate the two components of transfer. In the absence of empty vesicles we obtained the total transfer, whereas in the presence of excess empty vesicles we obtained the nonsaturable component; the difference was the saturable component. The data indicate that the saturable transfer from 6-CF vesicles at a concentration of 35 nM was inhibited by 90% and the nonsaturable transfer by about 50% in the presence of 10% fetal calf serum. In an initial effort to identify the component(s) of fetal calf serum responsible for this inhibition of transfer, we tested bovine serum albumin and found it to mimic the effect of whole fetal calf serum. In contrast, bovine IgG, a protein that does not bind lipids, had no comparable effect on transfer, suggesting that inhibition by bovine serum albumin was not a general effect of proteins. It should be noted that the albumin preparation contained some lipid, so further experiments will be required to determine whether protein or lipid was responsible for the inhibition.

Proteolytic Digestion. Exposing lymphocytes to trypsin (1 mg/ml) for 20 min did not affect either component of transfer (see Fig. 4), although such treatment does cleave a number of lymphocyte surface proteins (19). A more drastic proteolytic digestion with *S. griseus* protease (Pronase) (1 mg/ml) for 20 min, a treatment that results in more extensive release of lymphocyte surface proteins [for example, the Fc receptor (19)], also had no effect.

Other Compounds. To test the possibility of a membrane receptor for the choline portion of the phospholipid head group, we added an excess (10 mM) of choline chloride to the incubation medium. As shown in Fig. 4, there was no effect on transfer. Heparin, a compound that has been found to affect binding of low density lipoprotein to its cell surface receptor (20), had no signficant effect on the transfer or on its susceptibility to fetal calf serum. Addition of 0.1% Na azide and 50 mM 2-deoxyglucose, a combination of inhibitors sufficient to block other active lymphocytes functions such as capping and cytotoxicity, inhibited total transfer by about 25% over the whole concentration range. Cytochalasin B (50 μg/ml) had no effect.

DISCUSSION

In investigating the transfer of liposome contents into lymphocytes, we have identified a saturable and a nonsaturable component of the process and have found that the two components are not associated with different subpopulations of cells. The saturable component can be analyzed by the Michaelis–Menten paradigm for enzyme kinetics. The liposome–site and the inhibitor–site interactions are depicted as simple reversible bimolecular reactions (one liposome treated as one macromolecule):

$$[L] + [S] \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [LS] \xrightarrow{k_{+2}} \text{(6-CF into the cell)}$$

$$[I] + [S] \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} [IS] \qquad [2]$$

$$K_m = \frac{k_{-1} + k_{+2}}{k_1}; K_I = \frac{k_{-3}}{k_{+3}} \qquad [3]$$

$$[LS] + [IS] + [S] = [S_t] \qquad [4]$$

in which $[S]$ is the number of unoccupied sites per cell, $[LS]$ is the number of liposome–site complexes, $[IS]$ is the number of inhibitor–site complexes, $[S_t]$ is the total number of sites, and the ks are rate constants. The actual mechanism of transfer cannot be stated with certainty, but other studies on peripheral blood lymphocytes in our laboratory (11) and on a similar cell type by Huang *et al.* (12) implicate either fusion or a metabolically passive form of adsorptive endocytosis (21). These two possibilities have not yet been distinguished in any study involving interaction of lipid vesicles with mammalian cells.

One possible mechanism for the nonsaturable transfer is nonadsorptive endocytosis, a process in which uptake is found to be directly proportional to the concentration of solute in the medium (21). If we assume that the rate of free dye incorporation into lymphocytes gives an upper limit for the rate of nonadsorptive endocytosis, the nonsaturable component of the vesicle-mediated transfer is 8 times too large to be explained on the basis of that mechanism. The simplest interpretation of the nonsaturable component is that it represents fusion or else an adsorptive endocytosis that does not saturate in the range of concentrations studied.

The most straightforward interpretation of the saturable component is that it represents transfer at a finite number of sites on the lymphocyte membrane. We can not distinguish whether these are discrete sites or simply coverage of a con-

siderable area of the surface. If, in the extreme case, saturation resulted from coverage of the entire cell surface (about 25×10^4 "sites"), the turnover rate for the transfer process (i.e., $V_m/[S_t]$) would have to be very slow (about 5.7×10^{-4}/min). The character of the sites still remains to be determined but our initial observations are that (*i*) there is competitive inhibition by empty vesicles and also by some component of fetal calf serum, (*ii*) the sites are unaffected by proteolytic digestion of the cell surface, and (*iii*) there is inhibition by bovine serum albumin, but not by bovine IgG, choline chloride, or heparin. Using gel filtration, Zborowski *et al.* (22) studied the interaction of serum albumin with phosphatidylcholine liposomes and found no measurable quantities of protein associated with the liposomes. This finding suggests that bovine serum albumin interacts with the sites rather than with the liposomes themselves.

The insensitivity of the saturable transfer to proteolytic enzymes contrasts with findings by Pagano and Takeichi (23) on stable adsorption of solid-phase vesicles to the plasma membranes of Chinese hamster fibroblasts. They found the adsorption to be moderately sensitive to pretreatment with trypsin and associated with proteins of the cell surface. Huang *et al.* (12) showed that mild trypsinization released solid-phase vesicles adsorbed to mouse thymocytes, and we have found that trypsinization decreases stable adsorption of solid-phase vesicles to human lymphocytes (unpublished data). It seems likely, therefore, that the presumed sites for transfer and those for stable adsorption are of different natures. The findings on transfer of vesicle contents also contrast with those on the receptor for low density lipoprotein, which is sensitive to proteolysis and to the effects of heparin (24). It is tempting to speculate that the saturable component of transfer will prove to be associated with "bare lipid" patches (25) on the membrane.

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