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Lymphoma–Vesicle Interactions: Vesicle Adsorption, Membrane Fragmentation, and Intermembrane Protein Transfer†

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Appendix: Plasma Membrane Composition of the Cultured Murine Lymphoma Line BL/VL3

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ABSTRACT: Sonicated dimyristoylphosphatidylcholine vesicles interact with cultured murine lymphoma (BL/VL3) to generate complexes of vesicle and cell membrane components. Cell-free supernatants harvested after cell–vesicle incubations contain three distinct lipid species that can be separated by density gradient centrifugation. Analysis of protein and lipid composition and assays for cell and vesicle lumen contents reveal that the densest of the three lipid species comprises sealed plasma membrane fragments complexed with vesicles, while the least dense species is indistinguishable from pure phospholipid vesicles. The third, intermediate density species consists of topologically intact vesicles with associated plasma membrane proteins but without detectable cell lipids or cytoplasmic components. The membrane fragmentation and cell-to-vesicle protein transfer observed during lymphoma–vesicle incubations are examined as functions of cell and vesicle concentrations and incubation time.

Liposomes interact with cells in a variety of ways. Lipid may transfer between the two membranes, liposomes may adsorb to the cell surface or be incorporated by endocytosis, or the two species may fuse, pooling cytosolic and encapsulated vesicle contents [for reviews, see Pagano and Weinstein (1978), Huang (1983), and Margolis (1984)]. In a fifth mode of interaction, protein may transfer between cell and liposome membranes (Newton et al., 1983; Huestis & Newton, 1986). The nature and extent of such interaction(s) are dependent on the cell type, the liposome composition, and experimental parameters such as cell and liposome concentrations and length of incubation. For example, the major mode of interaction between murine P388 cells and dioleoylphosphatidylcholine (DOPC)[1] vesicles is stable adsorption of intact vesicles to the cell surface (Blumenthal et al., 1982). In contrast, when human erythrocytes are incubated with vesicles composed of the more hydrophilic lipid dimyristoylphosphatidylcholine (DMPC), vesicle lipid transfer, vesiculation of the cell membrane (Ott et al., 1981; Ferrell et al., 1985), and spontaneous cell-to-vesicle protein transfer (Newton et al., 1983; Huestis & Newton, 1986) are observed.

Erythrocyte-to-vesicle protein transfer has been characterized by analysis of the resulting protein–vesicle complexes. The composition of these complexes and the function and proteolytic sensitivity of their bound proteins (in particular, the anion transporter band 3) showed that transferred protein inserts into the vesicle bilayer in native orientation; exofacial inhibitor sites are exposed to the suspending medium and the cytofacial protein segment contacts the vesicle lumen (Newton et al., 1983; Huestis & Newton, 1986; Newton & Huestis, 1986). Protein transfer in the reverse direction, from vesicles to cells, has been demonstrated at a low but detectable level. Erythrocyte band 3 transfers in native orientation from protein–vesicle complexes into the membranes of erythrocytes and human and murine lymphoma, conferring increased anion transport capacity on the cells (Newton et al., 1983; Newton & Huestis, 1988).

This paper examines the generality of intermembrane protein transfer in a study of interactions between DMPC vesicles and cultured murine lymphoma (Lieberman et al., 1979). Incubation of BL/VL3 lymphoma with sonicated DMPC vesicles results in the generation of two types of protein–lipid complexes that differ in density and lipid and protein composition. Separation and compositional analysis of these species

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[1] Abbreviations: BTEE, *N*-benzoyl-L-tyrosine ethyl ester; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; FDA, fluorescein diacetate; FITC, fluorescein isothiocyanate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography.

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are described, and their generation is examined as a function of cell and vesicle concentration and duration of incubation.

Materials and Methods

Materials. DMPC was purchased from Sigma Chemical Co. [^{14}C]DPPC was obtained from Amersham Radiochemicals. [^{14}C]DMPC was synthesized from DMPE (Sigma) and [^{14}C]methyl iodide (ICN) according to the method of Stockton et al. (1974). All other chemicals were of reagent grade. The murine lymphoma line BL/VL3 (Lieberman et al., 1979) was maintained at 37 °C in RPMI 1640 supplemented with 15% fetal calf serum and antibiotics (Gibco). Cells were harvested by centrifugation (700*g* for 5 min) and washed once at room temperature in phosphate-buffered saline [138 mM NaCl, 6.1 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 5 mM KCl, 1 mM $MgSO_4$, and 5 mM glucose, pH 7.4 (NaCl/P_i buffer)]. Cell viability was assessed by Trypan blue exclusion and routinely found to exceed 95%. Unless otherwise stated, experiments were conducted in NaCl/P_i buffer.

Preparation of Vesicles. Small unilamellar vesicles were prepared by sonicating a suspension of DMPC (24 mM in NaCl/P_i buffer) to clarity (Bouma et al., 1977). To introduce a radioactive marker for either intact vesicles or vesicle lipid monomers, chloroform solutions containing 1 μCi of [^{14}C]DPPC or [^{14}C]DMPC, respectively, were evaporated to dryness under a stream of nitrogen. Sonicated vesicles were added, and sonication was continued for 20 min. In some experiments, chymotrypsin was introduced into the vesicle lumen by addition of 150 μL of chymotrypsin solution (12.5 mM in NaCl/P_i) to 1.5 mL of sonicated vesicles. The protein solution was added dropwise to the vesicle suspension under continuous sonication, and sonication was continued for 60 s. Vesicles were separated from excluded enzyme by passage through a Sephacryl S-200 column (0.9 × 10 cm) (Huestis & Newton, 1986).

Lymphoma–Vesicle Incubations. Vesicles were incubated with lymphoma at 37 °C for 5–60 min, as noted in the figure legends. Cell concentrations in samples ranged from cytocrits 5–60, corresponding to approximately (0.2–5) × 10^8 cells mL^{-1}. Cytocrit, defined as the percent cell volume in the total suspension, was measured by drawing a volume of the suspension into a 74 × 0.4 mm capillary tube, centrifuging the sample at 13400*g* for 3 min, and measuring the fraction of the capillary occupied by the cell pellet. Final DMPC concentrations in incubation samples ranged from 0 to 12 mM, as noted in the figure legends. After incubations, samples were centrifuged at 400*g* for 5 min, and supernatants were removed from cell pellets. Recovered cells were found to be >90% viable.

Supernatants from cell–vesicle incubations were applied to sucrose density gradients and centrifuged at 2000*g* for 2 h at 4 °C. Unless otherwise indicated, 5–30% (w/v) sucrose gradients were employed, generated by freezing and then thawing solutions of 18% (w/v) sucrose in NaCl/P_i (Baxter-Gabbard, 1972; Huestis & Newton, 1986). Fractions (200 μL) were collected and analyzed for lipid, vesicle contents, and cell contents.

Lipid Analysis. Sucrose density gradient fractions were analyzed for the nontransferrable vesicle marker [^{14}C]DPPC or for the lipid marker [^{14}C]DMPC by liquid scintillation counting of 10–25-μL aliquots. Lipids were extracted (Bligh & Dyer, 1959) from cells and from gradient fractions and analyzed by thin-layer chromatography. Silica gel HL plates were developed with $CHCl_3$/CH_3OH/formic acid (Daleke & Huestis, 1985).

Vesicle Contents. Lymphoma (cytocrit 30) were incubated with vesicles (9 mM DMPC) that contained encapsulated chymotrypsin (approximately one enzyme molecule per vesicle). Cells were pelleted (400*g* for 5 min) and supernatants removed. To effect clean separation between unaltered sonicated vesicles and protein–vesicle complexes, supernatants were subjected to density gradient centrifugation on shallow gradients (5–25%) formed by freeze–thawing 12% (w/v) sucrose. After centrifugation (2000*g* for 2 h) through such gradients, cell fragments formed a pellet at the bottom of the tube, sonicated vesicles banded at the sucrose/aqueous interface (fraction 21), and protein–vesicle complexes banded around fraction 9 (Figure 5). Chymotrypsin in gradient fractions was assayed by monitoring the hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester (BTEE) catalyzed by 100-μL aliquots of gradient fractions (Walsh & Crumpton, 1977). The assay buffer contained 0.01% Triton X-100 to solubilize vesicles.

Cell Contents. The distribution of cytoplasmic esterases in sucrose gradient fractions was monitored by measuring the hydrolysis of the nonfluorescent substrate fluorescein diacetate (FDA) to yield fluorescein (Rotman & Papermaster, 1966). Aliquots (100 μL) of gradient fractions were added to a solution of 5 μg mL^{-1} FDA in NaCl/P_i (diluted from a stock of 5 mg mL^{-1} in acetone), and the appearance of fluorescein was detected with a Perkin-Elmer 650-10S fluorometer.

Chemical Assays. Gradient fractions containing either cell membrane fragments, protein–vesicle complexes, or low-density vesicles were pooled, diluted to 5 mL in NaCl/P_i or a buffer containing 140 mM KCl, 10 mM HEPES, and 0.25 mM $MgCl_2$, pH 7.0 (HEPES/KCl), and pelleted by centrifugation at 200000*g* for 15 min at 4 °C. Membrane samples were resuspended in 200 μL of NaCl/P_i or HEPES/KCl buffer. Samples were assayed for protein (Lowry et al., 1951), phospholipid (Bartlett, 1959), 5′-nucleotidase activity (Michell & Hawthorne, 1965), and ^{14}C (liquid scintillation counting). Membrane fragment and protein–vesicle complex fractions were subjected to SDS gel electrophoresis on 10% acrylamide gels, followed by Coomassie blue (Ames, 1974) or silver staining (Merril et al., 1980).

Results

Analysis of Lipid Species Generated during Lymphoma–Vesicle Incubations

BL/VL3 lymphoma were incubated with DMPC vesicles for the times specified in the figure legends, and cells were removed from aliquots of the suspension by centrifugation. Density gradient fractionation of cell-free supernatants revealed the presence of three lipid species: residual unaltered vesicles and two protein–lipid complexes of differing density. The relative concentrations of these species were a sensitive function of the cell and vesicle concentrations during incubation (Figure 1). At low cytocrit (5) and relatively low DMPC concentration (6 mM), the major lipid component appeared at the sucrose/aqueous interface, comigratory with pure phospholipid vesicles (Figure 1A, fraction 21). A second, denser species banded at 1.045 g mL^{-1} (fraction 19) and accounted for 15% of the total vesicle lipid. An additional 10% of the vesicle lipid was associated with a broad, cloudy band centered around a density of 1.085 g mL^{-1} (fraction 12). The position of the highest density band varied substantially depending on the cell-to-vesicle ratio employed in the incubation. A higher cell-to-vesicle ratio (cytocrit 40; 12 mM DMPC) generated a high-density band migrating at 1.12 g mL^{-1} (Figure 1B, fraction 1). The densities of residual sonicated vesicles and the intermediate-density species (1.045 g mL^{-1}; fraction 19) were not affected by changing cell and vesicle concentrations; however, the relative amount of the interme-

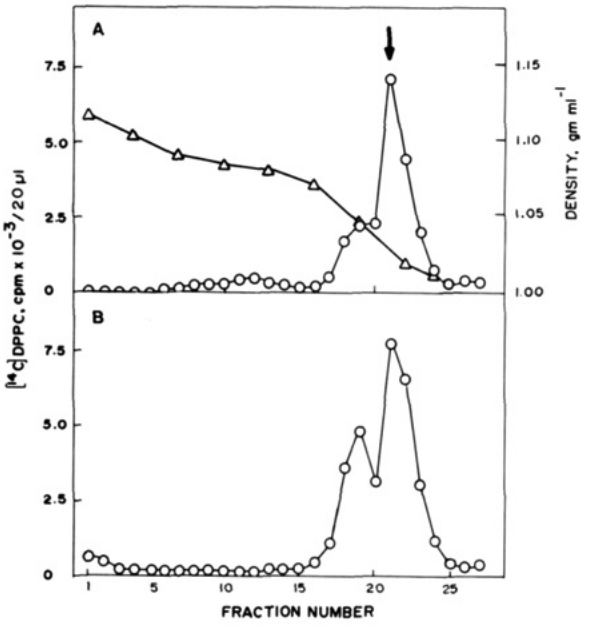

FIGURE 1: Lipid complexes generated by interaction of DMPC vesicles with BL/VL3 lymphoma: (A) 6 mM DMPC plus lymphoma at cytocrit 5; (B) 12 mM DMPC plus lymphoma at cytocrit 40. After a 30-min incubation at 37 °C, cell-free supernatants were fractionated by centrifugation through 18% freeze–thaw sucrose gradients [fraction densities (△)], and the distribution of the nonexchangeable vesicle marker [^{14}C]DPPC (○) was determined. Pure sonicated vesicles band at the arrow.

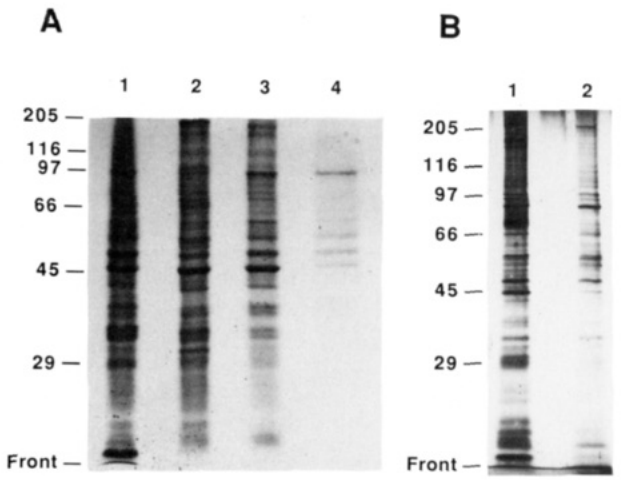

FIGURE 2: Polyacrylamide gel (10%) of protein–lipid complexes generated during lymphoma–vesicle incubations. (A) Coomassie blue stain: whole cell lysate (lane 1); isolated plasma membrane (lane 2); membrane fragments (lane 3); protein–vesicle complexes (lane 4). (B) Silver stain: membrane fragments (lane 1); protein–vesicle complexes (lane 2).

diate-density species increased with increasing cell-to-vesicle ratio. For the experiment described in Figure 1B, this species contained 30–40% of vesicle lipid.

(*a*) *The High-Density Component: Sealed Membrane Fragments.* (*i*) *Protein Composition.* The protein composition of the highest density protein–lipid species is shown on the SDS–polyacrylamide gel in Figure 2A, lane 3 (Coomassie blue stain), and Figure 2B, lane 1 (silver stain). The proteins present in this fraction were qualitatively and quantitatively representative of the plasma membrane protein composition (Figure 2A, lane 2). Particularly notable were high molecular weight proteins (170 and 180 kDa) characteristic of plasma membrane preparations (see Appendix). Additionally, a protein comigratory with erythrocyte actin was prominent in

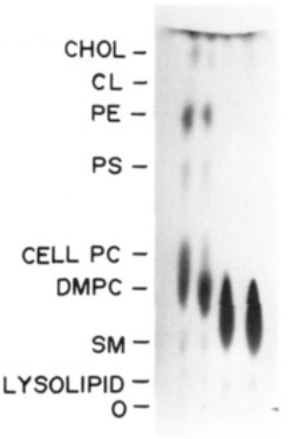

FIGURE 3: TLC separation of lipids from lymphoma and supernatant protein–lipid complexes. Lymphoma (cytocrit 35) were incubated with DMPC vesicles (14 mM) for 30 min at 37 °C. Cell-free supernatants were fractionated as in Figure 1, and lipids were extracted from isolated fractions. Whole lymphoma extract (lane 1); membrane fragment–vesicle complexes (lane 2); protein–vesicle complexes (lane 3); unaltered sonicated vesicles (lane 4). Lane 3 contains 110 nmol of DMPC; under the conditions employed, 0.2 nmol of phospholipid is readily detected by iodine staining. Lipids: CHOL, cholesterol; CL, cardiolipin; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin. In this solvent system, DMPC migrates below cell-derived PC.

both plasma membrane preparations and in the supernatant high-density lipid species.

(*ii*) *Lipid Composition.* Thin-layer chromatographic analysis of the high-density complexes showed the major lipid component to be DMPC, which in this solvent system migrates below cell phosphatidylcholine (Figure 3, lane 2). In incubations at high cytocrit, where formation of the high-density complex is favored (Figure 1B), 70–80% of its component lipid was found to be DMPC. Vesicle–cell incubations at low cytocrit yielded high-density complexes that were >95% DMPC. Cell-derived phosphatidylcholine, cholesterol, phosphatidylserine, and traces of cardiolipin and sphingomyelin were also present, in proportions corresponding to their representation in the plasma membrane (Appendix).

(*iii*) *Cytoplasmic and Vesicle Lumen Markers.* The origin and membrane integrity of the high-density complexes were investigated by assay for cytoplasmic esterase activity (Rotman & Papermaster, 1966) and vesicle lumen components. Cells were exposed to [^{14}C]DPPC-labeled DMPC vesicles at high cytocrit (52) and vesicle concentration (12 mM), conditions promoting formation of the high-density species. Density gradient fractionation of cell-free supernatants yielded the vesicle and esterase distributions shown in Figure 4. Cellular esterase activity comigrated with both high- and low-density lipid species, banding at a density of 1.10–1.12 g mL^{-1} (fractions 1–4) and at the top of the gradient (fractions 16–26). When these fractions were isolated and washed by repeated resuspension and centrifugation, no esterase activity was retained by the lipid complexes in fractions 15–20, while the high-density species retained its esterase activity quantitatively. This observation suggests that the esterase activity at the top of the gradient represents cytoplasmic contents released by lysed cells, while esterase in the high-density lipid complexes is sealed in cell membrane fragments. High-density complexes generated from incubations conducted at low cytocrit migrated at lower sucrose density (Figure 1A, fraction 12). Under these conditions, cellular esterase activity was observed associated with the species in fraction 12 (data not shown). In a separate experiment, the aqueous contents of the vesicles were labeled

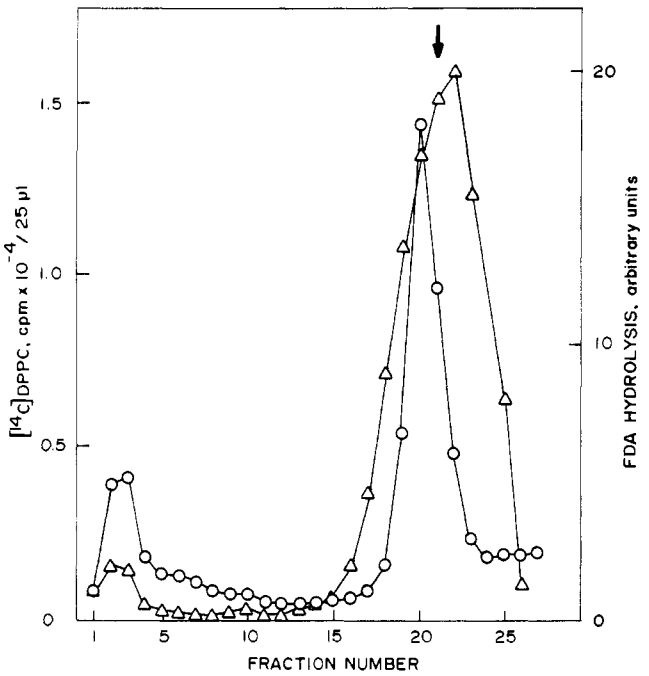

FIGURE 4: Distribution of vesicles (○) and cellular esterase activity (△) on sucrose gradients. Lymphoma (cytocrit 52) were incubated with DMPC vesicles (12 mM) labeled with [^{14}C]DPPC (○) for 30 min at 37 °C. Cell-free supernatants were fractionated as in Figure 1, and fractions were assayed for esterase activity. Activity in fractions 1–5 was stably bound in lipid complexes, while activity in fractions 19–21 was lost completely upon washing (see Discussion). Pure sonicated vesicles band at the arrow. Under conditions in which protein–vesicle complexes account for a large fraction of the total vesicle population (high cytocrit and high DMPC concentration; see Figure 9), resolution between the protein–vesicle band and the unaltered sonicated vesicle band is obscured during fractionation.

Table I: Composition of Supernatant Lipid Species

	sonicated vesicles	protein–vesicle complexes	membrane fragments[a]
chymotrypsin:[^{14}C]DPPC (fmol:cpm)	12	8.6	nd[c]
protein:phospholipid (mg:µmol)	>0.005	0.025	nd
5′-nucleotidase:phospholipid (nmol of P_i/h:µmol)		0.004	nd
5′-nucleotidase:esterase[b] (arbitrary units)		>55	4

[a] As the amount of vesicle lipid associated with membrane fragments varied considerably with incubation conditions, assays involving phospholipid determinations are not listed for this lipid species. [b] The 5′-nucleotidase:cytoplasmic esterase ratio for intact cells was 1.8. [c] nd, not determined.

with the cytoplasmic marker chymotrypsin. Lymphoma incubated with such vesicles generated high-density lipid complexes containing trace activity of the vesicle lumen marker (Figure 5).

The high-density lipid complexes also exhibited activity of the lymphoma ectoenzyme 5′-nucleotidase. The ratio of this plasma membrane marker to esterase activity was 2.2-fold higher in the membrane fragments than in intact lymphoma (Table I), suggesting that the high-density complexes are enriched in plasma membrane over cytosol.

Thus, the lipid, protein, and lumen compositions of the highest density supernatant complexes are consistent with sealed plasma membrane fragments that have DMPC vesicles adsorbed to their surfaces.

(*b*) *The Intermediate-Density Component: Protein–Vesicle Complexes.* (*i*) *Protein Composition.* The species migrating at a density of 1.045 g mL^{-1} (fractions 17–19, Figure 1) was

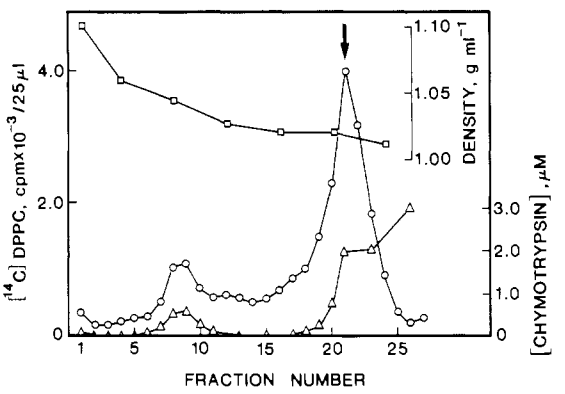

FIGURE 5: Distribution of vesicle membrane marker (○) and aqueous lumen marker (△) on 12% (w/v) sucrose freeze–thaw gradients. Lymphoma (cytocrit 30) were incubated for 30 min at 37 °C with DMPC vesicles (9 mM) labeled with [^{14}C]DPPC and containing entrapped chymotrypsin. Cell-free supernatant was fractionated on a shallow sucrose gradient [densities (□)] to optimize separation of protein–vesicle complexes and pure lipid vesicles. On this gradient, sealed membrane fragments appear in fraction 1 and protein–vesicle complexes band around fraction 9. Pure sonicated vesicles band at the arrow.

found to contain major protein components ranging in molecular mass from 30 to 200 kDa (Figure 2A, lane 4, and Figure 2B, lane 2). Proteins of similar molecular weights appeared in isolated plasma membranes, but the relative quantities of vesicle-bound proteins differed from their representation in either plasma membrane or in membrane fragments. An 85-kDa protein was highly enriched in the intermediate-density complexes, while several species of molecular mass <50 kDa were depleted. Comparison of staining intensities of plasma membrane (lane 2) and protein–vesicle complex (lane 4) bands reveals that on the order of 1% of the cells' 85-kDa plasma membrane protein associated with the vesicles.

The protein:phospholipid ratio of the intermediate-density complexes was found to be 0.025 mg: 1 µmol (Table I), 17-fold lower than that of the lymphoma plasma membrane (Appendix). This ratio, as well as the density of the complexes, was independent of experimental parameters such as cell and vesicle concentrations.

(*ii*) *Lipid Composition.* When the intermediate density complexes were subjected to thin-layer chromatography, the only lipid detectable was DMPC (Figure 3, lane 3). Plates greatly overloaded with lipid extracted from these complexes (100–200 nmol of DMPC) also revealed no detectable cell phospholipids, under conditions where 0.2 nmol of phospholipid could be detected readily by iodine staining.

(*iii*) *Vesicle Contents.* The derivation of the intermediate-density complexes was investigated by labeling the aqueous lumen of the sonicated vesicles with a macromolecular marker, the proteolytic enzyme chymotrypsin. Retention of this marker in intermediate-density complexes would provide evidence that this fraction derives from topologically intact sonicated vesicles. To achieve clean separation between protein–vesicle complexes and the sucrose/aqueous interface, 5–25% (w/v) sucrose gradients [shallower than the customary 5–30% (w/v)] were employed. Figure 5 shows the distribution of vesicle lipid (traced by [^{14}C]DPPC) and vesicle lumen contents (chymotrypsin) on such a gradient after vesicles (9 mM DMPC) had been incubated with lymphoma at cytocrit 30. Under these conditions, membrane fragments pelleted at the bottom of the gradient, and the intermediate density species banded around fraction 9. Trace chymotrypsin activity was associated with the membrane fragment pellet, indicating that some intact

vesicles bound to the fragments. Chymotrypsin activity was also found in the intermediate density complexes, in the unaltered sonicated vesicles (fractions 19–22), and in the soluble fraction above the gradient (fractions 20–26). The last represents enzyme released from vesicles during the cell–vesicle incubation; vesicles not exposed to cells retained >90% of encapsulated enzyme. Incidental entrapment of released enzyme by vesicles interacting with cells could account for no more than 0.3% of the activity found in fractions 8–10.

The lymphoma membrane ectoenzyme 5′-nucleotidase was present in trace amounts in intermediate-density complexes that had been washed by resuspension in buffer and centrifugation. In contrast, no cytosolic esterase activity could be detected in such washed samples. On the basis of the detection limits of the assays, 5′-nucleotidase activity in this fraction was enriched with respect to cytosolic esterase >31-fold compared with intact cells and 14-fold compared with the high-density membrane fragments (fractions 2–3, Figure 4).

(*c*) *The Low-Density Fraction: Unaltered Sonicated Vesicles.* The least dense lipid species in supernatants of lymphoma–vesicle incubations comigrated on sucrose gradients with pure DMPC vesicles. After being washed to remove soluble proteins released from lysed cells, this fraction contained no detectable protein, and the only lipid component detectable by thin-layer chromatography and iodine staining was DMPC (Figure 3, lane 4). On greatly overloaded TLC plates, cholesterol could be detected as approximately 0.1% of total lipid in both the low- and intermediate-density species, likely as a result of cholesterol transfer between membranes (Lange & Steck, 1985). On the time scale of these cell–vesicle incubations (30 min) such transfer is minimal; thus, the lowest density lipid species comprises essentially unaltered sonicated vesicles.

Experimental Parameters Affecting Lymphoma–Vesicle Interactions

(*a*) *Duration of Incubation.* At the cytocrits and DMPC concentrations employed in these experiments, interaction between cells and vesicles was rapid. The composition of cell-free supernatants is shown as a function of incubation time in Figure 6. Protein–vesicle complexes (fractions 17–18, density 1.045 g mL^{-1}) appeared within 5 min after mixture of cells and vesicles. At such early times, membrane fragment–vesicle complexes formed a diffuse band (Figure 6A, fractions 3–12), which migrated at progressively higher density as the incubation proceeded. After 15 min, approximately 10% of the vesicle lipid was found associated with the membrane fragment fraction, and neither its density nor the quantity of bound vesicle lipid changed further with increasing incubation time.

With increasing length of incubation, the amount of vesicle lipid recovered in the supernatant decreased. At the apparent steady state achieved after 30 min, about 40% of vesicle lipid was not recovered (Figure 7), with similar losses found in both protein–vesicle complexes and the pure sonicated vesicle fraction. This loss of lipid was not the result of endocytosis of intact vesicles; when vesicles encapsulating FITC–dextran were incubated with lymphoma, no apparent fluorescence associated with the cells. In contrast, peripheral human blood lymphoma incubated with fluorescently labeled vesicles exhibited readily detectable punctate fluorescence in their cytosol (A. C. Newton, unpublished data). Thus, endocytosis does not appear to be a significant mode of interaction between sonicated vesicles and BL/VL3 cells.

The quantity of vesicle lipid that copelleted with BL/VL3 cells (9 μmol) was 3-fold greater than the native phospholipid

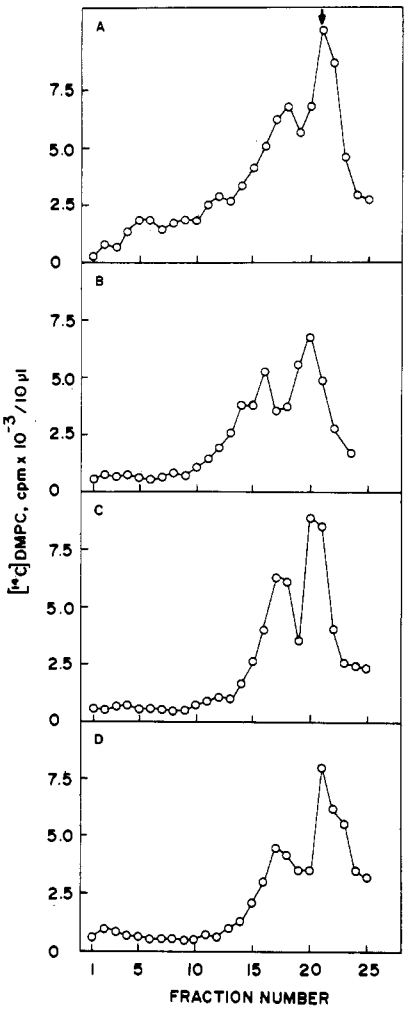

FIGURE 6: Time course of appearance of protein–lipid complexes, shown by distribution of the vesicle lipid marker [^{14}C]DMPC (○). Lymphoma (cytocrit 35) were incubated with DMPC vesicles (12 mM) at 37 °C for (A) 5 min, (B) 15 min, (C) 30 min, and (D) 45 min. Cell-free supernatants were fractionated as in Figure 1. Pure sonicated vesicles band at the arrow.

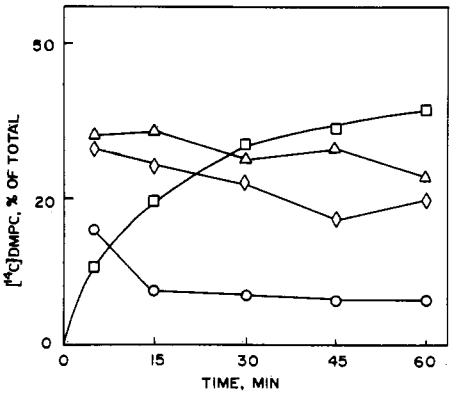

FIGURE 7: Distribution of vesicle lipid as a function of incubation time. Lymphoma (cytocrit 35) were incubated with DMPC (12 mM) at 37 °C for 0–60 min. Cell-free supernatants were fractionated as in Figure 1, and the amount of [^{14}C]DMPC associated with the cells (□), membrane fragments (○), protein–vesicle complexes (◇), and unaltered sonicated vesicles (△) was determined.

content of the cell plasma membrane (approximately 3 μmol/4 × 10^9 cells (Appendix)). A similar amount of vesicle association was found when cells were simply pelleted and when they were centrifuged through a 20% sucrose cushion. However, repeated washing of the cell pellet by resuspension in

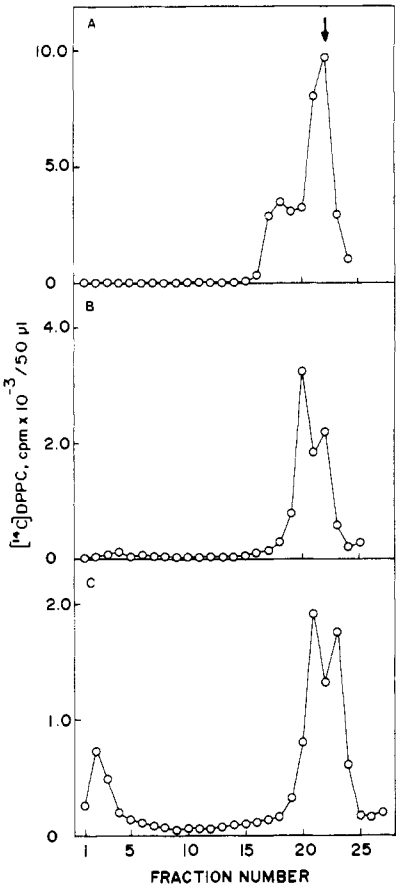

FIGURE 8: Effect of lymphoma concentration on generation of protein–lipid complexes. DMPC vesicles (10 mM lipid) labeled with trace [^{14}C]DPPC (O) were incubated for 30 min at 37 °C with lymphoma at cytocrit (A) 7, (B) 34, and (C) 58. Cell-free supernatants were fractionated as in Figure 1. Pure sonicated vesicles band at the arrow.

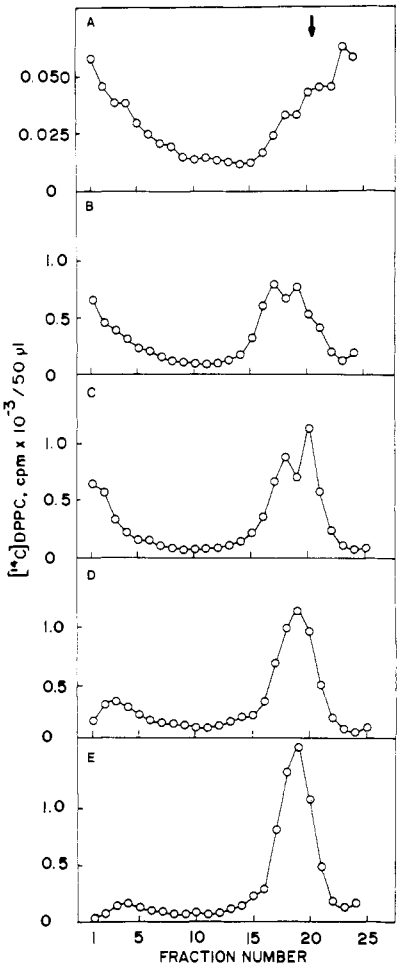

FIGURE 9: Effect of vesicle concentration on generation of protein–vesicle complexes. Lymphoma (cytocrit 33) were incubated for 30 min at 37 °C with DMPC vesicles labeled with [^{14}C]DPPC, at DMPC concentrations of (A) 0 (trace DPPC only), (B) 0.13 mM, (C) 1.3 mM, (D) 6.0 mM, and (E) 12.5 mM. Cell-free supernatants were fractionated as in Figure 1, and the distribution of [^{14}C]DPPC (O) was determined. The total amount of [^{14}C]DPPC was constant for incubations B–E. Unaltered sonicated vesicles band at the arrow. Under conditions promoting significant protein transfer (high cell and vesicle concentrations), the fractionation method employed does not resolve unaltered sonicated vesicles and protein–vesicle complexes, although the species migrate as two distinct bands on sucrose gradients.

buffer resulted in release of >95% of the vesicle lipid. Thus, low-affinity trapping of vesicles or vesicle aggregates appears likely to account for the observed association.

(*b*) *Cell Concentration.* Figure 8 shows the effect of cell concentration on formation of protein–vesicle complexes (the intermediate-density species) and cell fragments (the high-density complexes) at constant vesicle concentration (12 mM DMPC). At cytocrit 7, protein–vesicle complexes accounted for 28% of vesicle lipid. As the cytocrit was raised to 34, approximately 50% of vesicle lipid appeared in the protein–vesicle fraction. This proportion was unchanged upon further increase to cytocrit 58. The amount of vesicle lipid associated with the membrane fragment fraction increased exponentially with increasing cytocrit (fractions 1–5, Figure 8); 21% of vesicle lipid bound to cell fragments in incubations conducted at cytocrit 58. Additionally, the fraction of vesicles pelleting with cells increased with increasing cell concentration, consistent with nonspecific trapping.

(*c*) *Vesicle Concentration.* The effect of changing vesicle concentration at constant cytocrit is shown in Figure 9. Lymphoma membranes were labeled with trace (<60 nM) [^{14}C]DPPC, which in the absence of cells would have migrated as micelles at the top of sucrose gradients. Even in the absence of added DMPC vesicles, the labeled cells released membrane fragments to the supernatant (Figure 9A, fractions 1–5). Micellar DPPC was also present in fractions 15–25. Increasing concentrations of [^{14}C]DPPC-labeled DMPC vesicles were incubated with the labeled cells, generating the supernatant lipid distributions shown in Figure 9B–E. As the vesicle lipid concentration was increased from 0.13 to 12.5 mM, increasing amounts of DMPC bound to the membrane fragment fraction, with a concomitant decrease in the density at which the complexes migrated. For the four incubations shown in Figure 9B–E, the net amounts of DMPC bound to the membrane fragments were 0.008, 0.086, 0.30, and 0.39 µmol, respectively. On the basis of the protein content of the membrane fragments, their cell lipid content was estimated to be 0.1–0.2 µmol. Thus, at the highest vesicle concentration examined, the ratio of vesicle lipid to cell lipid in membrane fragments was between 2 and 4. Chromatographic analysis of the lipid composition of such membrane fragment–vesicle complexes verified that DMPC accounted for approximately 70–80% of the lipid in this species.

The polyacrylamide gel in Figure 10 shows the protein composition of membrane fragments generated from lymphoma (cytocrit 33) incubated in the presence of trace (<60 nM) DPPC, plus DMPC in concentrations ranging from 0 to 12.5 mM. Coomassie blue staining intensities indicate that lymphoma incubated in the absence of exogenous lipid (not shown) or in the presence of trace DPPC released the equivalent of 2–3% of total plasma membrane protein as membrane

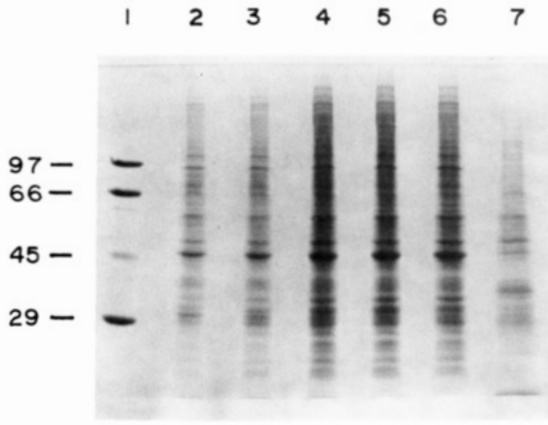

FIGURE 10: Effect of vesicle concentration on generation of cell membrane fragments. Polyacrylamide gel (10%) of the membrane fragment fraction produced by incubation of lymphoma (cytocrit 33; 37 °C, 30 min) with 0 mM DMPC (lane 2), 0.13 mM DMPC (lane 3), 1.3 mM DMPC (lane 4), 6.0 mM DMPC (lane 5), and 12.5 mM DMPC (lane 6). All samples contained trace (<60 nM) [^{14}C]DPPC. Membrane fragments were isolated from sonicated vesicles and protein–vesicle complexes as in Figure 1. Lane 7 contains whole cells; molecular weight markers are shown in lane 1.

fragments. Incubation with DMPC vesicles at concentrations below 0.13 mM did not affect this release significantly. As the vesicle concentration was increased to 1.3 mM DMPC, Coomassie blue staining intensity indicated that membrane fragment release increased 2–3-fold. No further increase was evident as the vesicle concentration was raised to 12.5 mM DMPC.

Discussion

When murine lymphoma are incubated with DMPC vesicles, three types of lipid aggregates appear in cell-free supernatants. Density gradient fractionation of the supernatants separates topologically intact plasma membrane fragments, unaltered sonicated vesicles, and protein–vesicle complexes selectively enriched in plasma membrane proteins. The relative abundance of these species is dependent on experimental variables such as cell and vesicle concentrations. Incubation at low cytocrit produces cell-free supernatants in which unaltered sonicated vesicles are the primary lipid species and cell membrane fragments account for most of the membrane-bound protein. At high cell and vesicle concentrations, a third protein–lipid complex is generated that consists of cell membrane protein associated with vesicle lipid and vesicle lumen contents.

Membrane Fragments. Cell membrane fragments are released spontaneously when BL/VL3 lymphoma are incubated in phosphate-buffered saline at 37 °C. The protein and lipid compositions of these fragments are representative of the plasma membrane, and they contain cytoplasmic esterase activity. The proteolytic susceptibility of proteins in these membrane fragments is indistinguishable from that of proteins in intact cells (A. C. Newton and W. H. Huestis, unpublished results). Thus, these complexes represent topologically intact plasma membrane fragments. The quantity of generated cell fragments increases 2–3-fold in the presence of sonicated vesicles (at [DMPC] > 1.3 mM), possibly as a result of DMPC uptake. The density of membrane fragments produced in the presence of DMPC vesicles is influenced by the cell-to-vesicle ratio. At low vesicle concentrations, these fragments migrate at densities around 1.12 g mL^{-1}, similar to fragments generated in the absence of vesicles. At constant cytocrit, higher vesicle concentrations produce membrane fragments that bind significant quantities of vesicle lipid and lumen contents and migrate at proportionately lower densities on sucrose gradients.

The fraction of vesicle lipid associated with membrane fragments increases exponentially with increasing cytocrit but is relatively insensitive to vesicle lipid concentration in the range examined (Figure 4). Thus, stable adsorption of intact vesicles to the membrane fragment surface appears to be a major mode of association.

Protein–Vesicle Complexes. At high cytocrit and DMPC concentrations, an intermediate-density protein–lipid complex is generated. Analysis of protein, lipid, and lumen contents indicates that this species consists of cell plasma membrane proteins bound to topologically intact sonicated vesicles. The complexes retain macromolecular contents entrapped in the vesicles during sonication but contain no detectable cytoplasmic markers. Their protein composition is distinct from that of the sealed plasma membrane fragments discussed above; notably, they are enriched in an 85-kDa plasma membrane protein and depleted in low molecular weight proteins. These disparities in protein composition indicate that protein–vesicle complexes and membrane fragments are distinct species. Immunochemical characterization also provides evidence for the selective transfer of certain plasma membrane proteins (A. C. Newton and W. H. Huestis, unpublished results).

By TLC analysis, DMPC is the only detectable phospholipid in the intermediate protein–vesicle complexes. On the basis of the sensitivity of the lipid detection method and the protein:phospholipid ratio in protein–vesicle complexes, we can estimate the amount of cell phospholipid that would be expected to appear in these species if the associated protein derived solely from contaminating plasma membrane fragments. The protein:phospholipid ratio of the protein–vesicle complexes (0.025 mg: 1 μmol) is 17-fold lower than that of the lymphoma plasma membrane (0.42 mg: 1 μmol; Appendix). If the vesicle-associated protein is part of a plasma membrane fragment, 0.06 μmol of cell phospholipid should be present for every micromole of total phospholipid. Since 60% of the plasma membrane phospholipid is phosphatidylcholine (Appendix), protein–vesicle complexes thus contaminated with membrane fragments would be expected to contain 0.036 μmol of cell PC/μmol of total phospholipid. Similarly, phosphatidylethanolamine, which accounts for 30% of cell plasma membrane phospholipid, should be present in the protein–vesicle complexes. When TLC plates are greatly overloaded with lipid extracted from the protein–vesicle complexes (110 nmol; Figure 3, lane 3), neither cell PC nor cell PE can be detected by iodine staining. For the particular TLC plate shown, representative membrane fragments sufficient to account for the observed vesicle-bound protein would contain 4 nmol of cell PC and 2 nmol of PE. These quantities of phospholipid are 10–20-fold higher than the detection limit of the system employed. Thus, contaminating membrane fragments can account for at most 10% of the vesicle-associated protein. The most reasonable explanation for the presence of protein in these species is lymphoma-to-vesicle transfer of accessible proteins, perhaps with amounts of associated cell lipid (<25 lipids per protein) that are beneath the detection limit of iodine staining or phosphate assays. For comparison, band 3–vesicle complexes formed during erythrocyte-to-vesicle protein transfer contain on the order of 40 cell phospholipids per band 3 dimer (Huestis & Newton, 1986).

Intermembrane protein transfer is apparent primarily at high cytocrit and DMPC concentrations, where cell–vesicle collisions are relatively high. At low cytocrits, the amount of protein–vesicle complexes formed increases with increasing cell concentration (Figure 1A,B). At and above a threshold cytocrit (approximately 10 for 12 mM DMPC), half of the vesicle lipid appears in protein–vesicle complexes. Generation of the complexes is rapid, with steady-state amounts of protein–vesicle complexes formed within 5 min of cell–vesicle mixing. The distribution of vesicle lipid on sucrose gradients appears similar when vesicles are labeled with the nontransferrable lipid marker [^{14}C]DPPC, or with [^{14}C]DMPC, monomers of which transfer rapidly between membranes on the time scale of these experiments (Ferrell et al., 1985) (compare Figures 1B and 5C). Thus, in this system, transfer of monomeric vesicle lipid to cells or membrane fragments is less extensive than adsorption of intact vesicles.

Intermembrane Protein Transfer in Erythrocytes and Lymphoma. Interactions between murine lymphoma and sonicated vesicles resemble vesicle–erythrocyte interactions in several respects. In both cases, vesicles promote the release of cell membrane fragments that enclose cytoplasmic components and contain membrane protein and lipid. At high cytocrits and vesicle concentrations, both cell types also generate protein–vesicle complexes whose lipid and lumen compositions are inconsistent with the composition of cell membrane fragments. The most likely mechanism for generation of these complexes is selective transfer of cell surface proteins to the bilayer of intact vesicles. In both erythrocyte and lymphoma systems, the generation of protein–vesicle complexes is a sensitive function of cell and vesicle concentrations. In neither system is there evidence for vesicle–cell fusion (with attendant pooling of aqueous contents) or vesicle uptake by endocytosis.

Lymphoma–vesicle interactions also differ from erythrocyte–vesicle interactions in a number of ways. While vesicle lipid is found associated with cell and cell membrane fragments in both systems, lymphoma membranes apparently bind intact vesicles, exhibiting similar binding of the nontransferrable marker DPPC and the relatively hydrophilic DMPC. Erythrocytes and their membrane fragments, in contrast, bind significant amounts of DMPC but no detectable DPPC. This is consistent with uptake of lipid monomers through the aqueous phase, but not with binding of intact vesicles.

The protein:phospholipid ratio of lymphoma protein–vesicle complexes (0.025 mg:1 μmol) is slightly lower than that of erythrocyte protein–vesicle complexes [0.08 mg:1 μmol (Newton et al., 1983)]. This may reflect differences in the molecular weights of the vesicle-bound proteins in the two systems. Erythrocyte band 3 transfers as an oligomer, possibly in association with glycophorin. The proteins associated with the lymphoma protein–vesicle complexes may comprise smaller molecular weight units. Consistent with this suggestion, lymphoma protein–vesicle complexes are slightly less dense than erythrocyte protein–vesicle complexes: the former band at 1.045 g mL^{-1} and the latter at 1.055 g mL^{-1} (Huestis & Newton, 1986).

Generation of protein–vesicle complexes is rapid in both systems, but in contrast to lymphoma protein–vesicle complexes (which are produced within a few minutes of vesicle–cell mixing), erythrocyte protein–vesicle complexes appear only after a time lag of approximately 15 min (Huestis & Newton, 1986). This interval corresponds to the half-time of DMPC transfer from vesicles to erythrocytes and the resultant morphological transformation of the cells from discocytes to spiculate echinocytes (Ferrell et al., 1985). These events, and the consequent release of cytoskeleton-free membrane fragments (Ott et al., 1981), are consistent with DMPC-induced destabilization of the membrane by disruption of its bilayer balance (Sheetz & Singer, 1974). The resultant changes in lipid packing and bilayer–cytoskeleton interactions may predispose proteins to transfer to vesicles. Lymphoma, in contrast, show no evident morphological transformations upon incubation with DMPC vesicles. Their normal morphology features numerous microvilli, whose high surface curvature may, like echinocyte spikes, provide unstable microenvironments that facilitate protein transfer. Thus, the incorporation of DMPC into the cell membrane and/or the presence of highly curved microenvironments may be critical factors in enabling protein transfer to proceed.

Mechanistic analysis of intermembrane protein transfer requires demonstration that vesicle-bound proteins (and proteins delivered from vesicle complexes into intact cell membranes) insert in the recipient bilayer in native orientation and exhibit normal functional properties. This has been accomplished in the case of the transferred erythrocyte anion transporter (Huestis & Newton, 1986; Newton & Huestis, 1986). Ongoing studies of lymphoma protein–vesicle complexes reveal that certain of these proteins also insert in the vesicle bilayer, with their cytoplasmic domain contacting proteases entrapped in the vesicle lumen (A. C. Newton and W. H. Huestis, unpublished results). This suggests that interbilayer protein exchange is a general phenomenon, occurring when other cell types are exposed to the relatively hydrophilic lipid DMPC. Further work is required to identify cellular properties and other experimental conditions that may facilitate or inhibit such events.

Table AI: Purity of BL/VL3 Plasma Membrane Preparation

marker	% of whole cell lysate
DNA (nuclei)	<0.1
esterases (cytosol)	0.09
succinic dehydrogenase (mitochondria)	2
glucose-6-phosphatase (endoplasmic reticulum)	2
cholesterol (plasma membrane)	20
membrane-bound 5′-nucleotidase (plasma membrane)	20

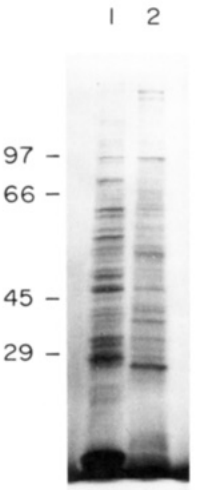

FIGURE A1: SDS–polyacrylamide gel (10%) of whole cell lysate (lane 1) and purified lymphoma plasma membrane (lane 2), after Coomassie blue staining. Indicated molecular weight standards are phosphorylase *b* (97 000), bovine serum albumin (66 000), egg albumin (45 000), and carbonic anhydrase (29 000).

Table AII: Composition of Lymphoma Lysate and Plasma Membrane[a]

	whole cell lysate	plasma membrane preparation[b]
protein (mg/10^{10} cells)	393 ± 14	5.0 ± 0.4
5′-nucleotidase (μmol P_i/h per 10^{10} cells)	212 ± 10	16.8 ± 0.2
phospholipid (μmol/10^{10} cells)	192 ± 8	12.0 ± 0.2
cholesterol (μmol/10^{10} cells)	21 ± 1	4.7 ± 0.2
5′-nucleotidase/protein (μmol P_i/h per mg)	0.63 ± 0.06	3.3 ± 0.3
protein/phospholipid (mg/μmol)	2.1 ± 0.2	0.42 ± 0.04
cholesterol/phospholipid (μmol/μmol)	0.044 ± 0.004	0.36 ± 0.02
cholesterol/protein (μmol/mg)	0.050 ± 0.006	0.84 ± 0.09

[a] Values represent the weighted average, with standard deviation, of three determinations performed in triplicate. [b] Plasma membrane yield is approximately 20% on the basis of recovery of cholesterol and membrane-bound 5′-nucleotidase activity.

Table AIII: Phospholipid Composition of BL/VL3 Plasma Membrane

	total phospholipid[a] (mol %)
phosphatidylcholine	58.9 ± 0.5
phosphatidylethanolamine	29.9 ± 1.3
phosphatidylserine[b] (and phosphatidylinositol)	5.1 ± 1.4
sphingomyelin[b] (and phosphatidylinositol monophosphate)	2.9 ± 0.9
cardiolipin	2.17 ± 0.06
other (including phosphatidic acid)	0.9 ± 0.8

[a] Values represent the weighted average, with standard deviation, of three determinations. [b] In this chromatographic system, phosphatidylinositol migrates with phosphatidylserine and phosphatidylinositol monophosphate with sphingomyelin and phosphatidylinositol bisphosphate remains at the origin.

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Appendix: Plasma Membrane Composition of the Cultured Murine Lymphoma Line BL/VL3

Abstract

The plasma membrane of the murine lymphoma line BL/VL3 has been isolated by a one-step centrifugation and its biochemical composition analyzed. The isolated material represents a 20% yield of plasma membrane, based on recovery of cholesterol and membrane-bound 5′-nucleotidase. In comparison with whole cell lysate, the plasma membrane preparation is enriched (with respect to protein) in cholesterol (17-fold), 5′-nucleotidase (5.2-fold), and phospholipid (5-fold). The molar ratio of cholesterol to phospholipid is 0.36 ± 0.02. Phosphatidylcholine and phosphatidylethanolamine are the major phospholipid classes, accounting for approximately 60% and 30%, respectively, of total phospholipid. Phosphatidylserine, sphingomyelin, and cardiolipin are minor components, each constituting less than 6% of total phospholipid.

Introduction

Lymphoma plasma membranes have highly complex compositions; in contrast to the relatively simple makeup of the erythrocyte membrane, they exhibit a multiplicity of protein components. Although lymphoma membranes have been subjects of extensive investigation, thorough biochemical characterization has been attempted for very few cell lines. Several protocols have been reported for isolation of lymphocyte plasma membranes (Maeda et al., 1983; Misra et al., 1975; Resch et al., 1981; Snary et al., 1976), plasma membrane marker enzymes have been identified, and a variety of monoclonal antibodies have been obtained for antigenic determinants on the cell surface (Ceredig et al., 1984). With the exception of proteins such as Ly 5 (or T200) and the Ia antigens, which have been shown to span the plasma membrane bilayer (Ewald & Refling, 1985; Omary & Trowbridge, 1980; Walsh & Crumpton, 1977), little is known about the orientation and transmembrane distribution of lymphoma plasma membrane proteins.

In studies of lymphoma-to-liposome membrane protein transfer, it was necessary to characterize the composition of the lymphoma plasma membrane. This paper describes the lipid and protein composition of isolated BL/VL3 plasma membranes.

Materials and Methods

Lymphoma. The established T-cell line BL/VL3 (Lieberman et al., 1979) was maintained at 37 °C in RPMI 1640 supplemented with 15% fetal calf serum and antibiotics. Cells were harvested by centrifugation at room temperature (700*g* for 10 min) and washed once in phosphate-buffered saline containing 138 mM NaCl, 6.1 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 5 mM KCl, 1 mM $MgSO_4$, and 5 mM glucose, pH 7.4 (NaCl/P_i buffer).

Plasma Membrane Isolation. Cells were brought to a concentration of 10^8 mL^{-1} in buffer containing 140 mM KCl, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 0.25 mM $MgCl_2$ at pH 7.0 (KCl/HEPES buffer) and lysed by freezing in liquid N_2 followed by thawing at room temperature. Phenylmethanesulfonyl fluoride (PMSF) was added to the whole cell lysate from a fresh concentrate (23 mM in dimethyl sulfoxide) to yield a final concentration of 10 μM. Deoxyribonuclease (approximately 5 μg/mL of lysate, diluted from a freshly prepared stock solution containing approximately 5 mg mL^{-1}) was added.

The plasma membrane was isolated by a one-step centrifugation, as described by Maeda et al. (1983). The suspension (3 mL) was layered over an equal volume of 36% sucrose in KCl/HEPES buffer and centrifuged at 200000*g* for 2 h at 4 °C. The plasma membrane was collected from the sucrose-/buffer interface, resuspended in 4 mL of KCl/HEPES buffer, and sonicated briefly to release entrapped cytoplasmic contents. Sonication was omitted in procedures involving measurement of enzyme activities. The plasma membrane was washed twice by resuspension in 5 mL of KCl/HEPES buffer and centrifugation at 200000*g* for 20 min. The pellet was resuspended in KCl/HEPES buffer at a concentration of 1–3 mg of protein mL^{-1}.

Plasma Membrane Analysis. Whole cell and plasma membrane preparations were analyzed for protein (Lowry et al., 1951), phospholipid (Bartlett, 1959), cholesterol (Zlatkis et al., 1953), and DNA (Burton, 1955) and the enzymatic activities of 5′-nucleotidase (Michell & Hawthorne, 1965), succinic dehydrogenase (Earl & Korner, 1965), glucose-6-phosphatase (Swanson, 1950), and cytoplasmic esterase (Rotman & Papermaster, 1966). Lipids were extracted from whole cells and plasma membrane (Bligh & Dyer, 1959) and separated by thin-layer chromatography on silica gel HL plates developed by chloroform/methanol/formic acid (65:25:4), a solvent system that separates the major phospholipid classes cleanly (Daleke & Huestis, 1985). Lipid spots were stained with iodine vapor, scraped from the plates, and quantified by phosphate analysis (Bartlett, 1959). For whole cell phospholipid analysis, cell lysate (to which trace [^{14}C]DPPC had been added to standardize lipid recovery) was extracted with chloroform/methanol (Bligh & Dyer, 1959) and subjected to phosphate analysis, as above. Samples of whole cell lysate and plasma membrane were analyzed by polyacrylamide gel electrophoresis (10% acrylamide); proteins were visualized by Coomassie blue staining (Ames, 1974).

Results and Discussion

Recovery and Purity of Isolated BL/VL3 Plasma Membranes. Centrifugation of whole lymphoma lysate over a 36% sucrose cushion yielded a plasma membrane enriched fraction at the sucrose/buffer interface. This fraction typically contained 1–2% of total cell protein and 20% of membrane-associated activity of 5′-nucleotidase, an ectoenzyme shown by cytochemical studies to be associated with the lymphocyte plasma membrane (Uusitalo, 1981). This membrane yield is comparable with that reported by other workers (Misra et al., 1975; Snary et al., 1976).

Purification of the plasma membrane preparation from nuclear and organelle components is summarized in Table AI. The plasma membrane fraction was free of detectable nuclear material, containing <0.1% of total cell DNA. Succinic dehydrogenase and glucose-6-phosphatase, enzyme markers for mitochondrial and endoplasmic reticular membranes, respectively, were each detectable as 2% of total cell activity. Thus, the preparation was enriched 10-fold in plasma membrane marker relative to specific organelle membrane markers. Less than 0.1% of the cytosolic marker esterase was recovered in this fraction.

Lipid and Protein Composition. The protein and lipid composition of the plasma membrane fraction is compared with the whole cell composition in Table AII. The plasma membrane fraction was enriched in cholesterol, with a cholesterol:protein ratio 17-fold greater and a cholesterol:phospholipid ratio 8-fold greater than in whole cells. Similar cholesterol to phospholipid enrichment has been used as a criterion for the purity of plasma membrane preparations (Coleman & Finean, 1966; Lange & Steck, 1985). Typically, 20% of total cell cholesterol was recovered in the plasma membrane fraction, with the remaining 80% appearing in the nuclear pellet. This is consistent with recovery of membrane-bound 5′-nucleotidase, further evincing a 20% yield of plasma membrane. Lipids were extracted from plasma membrane preparations and separated by thin-layer chromatography, and scraped individual spots were analyzed for phosphate (Table AIII). Phosphatidylcholine was found to be the major phospholipid, accounting for 58.9 ± 0.5% of the total. The aminophospholipids phosphatidylethanolamine and phosphatidylserine made up 29.9 ± 1.3% and 5.1 ± 1.4%, respectively, of total phospholipid. Sphingomyelin (2.9 ± 0.9%) and cardiolipin (2.17 ± 0.06%) were minor constituents.

In comparison with many other types of membranes, the sphingomyelin and phosphatidylserine content of these membranes is low. It is notable that in erythrocytes of many animal species, the choline phospholipids phosphatidylcholine and sphingomyelin account for roughly half of total phospholipid. Individually, they contribute to this total in varying degrees, but their combined population tends to be conserved (Van Deenen & de Gier, 1974). Similarly, the low sphingomyelin content of the BL/VL3 plasma membrane is compensated by an unusually high phosphatidylcholine content. The plasma membranes of porcine mesenteric lymph node lymphoma, in contrast, contain higher proportions of sphingomyelin (18%) and phosphatidylserine (15%) and lower proportions of phosphatidylcholine (46%) and phosphatidylethanolamine (20%) (Levis et al., 1976). Thus, proportions of individual lipids may vary, but the total populations of the choline as well as the aminophospholipids are similar in the two cell systems.

The protein constituents of whole cell lysate and of the plasma membrane fraction are compared in the sodium dodecyl sulfate (SDS)–polyacrylamide gel shown in Figure A1. Both samples contained a complex array of proteins, with over 50 distinct bands detectable upon silver staining (not shown). The protein distribution in the two samples differed significantly, the plasma membrane fraction being enriched in high molecular weight proteins migrating at positions corresponding to 170 and 180 kDa. Omission of the protease inhibitor PMSF during membrane isolation resulted in no obvious differences in polyacrylamide gel patterns.

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Vesicle-to-Cell Protein Transfer: Insertion of Band 3, the Erythrocyte Anion Transporter, into Lymphoid Cells†

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ABSTRACT: Band 3, the erythrocyte anion transporter, transfers spontaneously between human red cells and model membranes. During incubation of intact erythrocytes with sonicated dimyristoylphosphatidylcholine vesicles, the transporter inserts in functional form and native orientation into the liposome bilayer, with the cytoplasmic segment of the protein contacting the lumen of the vesicle [Newton, A. C., Cook, S. L., & Huestis, W. H. (1983) *Biochemistry 22*, 6110–6117; Huestis, W. H., & Newton, A. C. (1986) *J. Biol. Chem. 261*, 16274–16278]. When band 3–vesicle complexes are incubated with erythrocytes whose native band 3 has been inhibited irreversibly, reverse transfer of the protein restores anion transport capacity to the cells [Newton, A. C., Cook, S. L., & Huestis, W. H. (1983) *Biochemistry 22*, 6110–6117]. Here we report the vesicle-mediated transfer of band 3 to human peripheral blood lymphocytes and to cultured murine lymphoma cells (BL/VL3). Subsequent to incubation with protein–vesicle complexes, both lymphoid cell types exhibit a 2–4-fold increase in the rate of chloride uptake. This enhanced permeability is inhibited ≥98% by the exofacial band 3 inhibitor 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid, consistent with right-side-out insertion of functional band 3 into the lymphoid cell membrane.

Spontaneous transfer of membrane proteins between cells and liposomes (Newton et al., 1983; Huestis & Newton, 1986) offers the prospect of altering the transport or antigenic properties of cells. Liposome-mediated insertion of proteins into intact cell membranes has been accomplished with fusogenic agents such as poly(ethylene glycol) (Eriksson et al., 1985; Baumann et al., 1980), viral envelope proteins (Poste et al., 1980; Volsky et al., 1979), and fusogenic lipids (Correa-Frêire et al., 1984). Insertion of foreign protein also has been achieved by fusing biological membranes with cells (Balakrishnan et al., 1983).

An alternative approach to such membrane modification is the exchange of intrinsic proteins between cells and donor membranes that retain their topological integrity (i.e., do not fuse). Das and co-workers have reported the spontaneous transfer of functional epidermal growth factor receptor from

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