Journal of Cellular Biochemistry 20:127–138 (1982)

Characteristics of Plasma Membrane Isolated From a Mouse T Lymphoma Line: Comparison After Nitrogen Cavitation, Shearing, Detergent Treatment, and Microvesiculation

Janetta G. Culvenor, Thomas E. Mandel, Alison Whitelaw, and Ernst Ferber

The Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria, Australia (J.G.C., T.E.M.), and Max-Planck Institut für Immunbiologie, D-78 Freiburg, Federal Republic of Germany (A.W., E.F.)

Plasma membrane was isolated from the mouse T lymphoma cell line WEHI-22 using four different methods of cell disruption followed by centrifugal fractionation. Disruption by nitrogen cavitation or by shearing with a cell pump produced plasma membrane vesicles of similar buoyant density (1.10 g/ml) and morphological appearance. Few C-type virus particles were present. Cell disruption with 2% Tween-40 produced membrane vesicles of similar morphology but lower density (1.09 g/ml). All of the above preparations resulted in vesicles with aggregated intramembranous particles after freeze fracture. Microvesiculation with a sublytic concentration of a lysophosphatidylcholine analog (ET-12-H) (0.0032% w/v) produced small membrane vesicles which could be isolated without differential centrifugation. However, these had a slightly higher density than vesicles prepared by cavitation or shearing and were contaminated by virus particles. Unlike the other preparations, vesicles prepared with ET-12-H had dispersed intramembranous particles. The enzyme γ-glutamyl transferase was enriched from 20- to 45-fold in the membrane preparations and proved a suitable plasma membrane marker for these cells whose 5′-nucleotidase content is very low.

Key words: electron microscopy, plasma membrane, lymphoma cells

Cultured lymphoma cells which grow rapidly in suspension provide an ideal source of homogeneous cells for membrane studies. Although molecular chracterization of surface molecules is progressing rapidly with immunochemical and labelling techniques using whole cells, isolated plasma membranes are still useful for ultrastructural, physical, and functional membrane studies. Since "purified" plasma membranes are, at best, enriched preparations of varying yield and sometimes doubtfully representative of the intact plasma membrane [1], we considered it useful to compare some of the properties of plasma membrane fractions prepared by different techniques from a single cell line.

Nitrogen cavitation [2] and shearing with a cell pump [3] are established techniques for disruption of cells in suspension and have been compared for calf

Received July 3, 1982; revised and accepted August 27, 1982.

0730-2312/82/2002-0127$03.50

thymocyte plasma membrane isolation [4,5]. Tween-40 (2% w/v) disruption, introduced by Standring and Williams, has been compared with shearing for plasma membrane isolated from rat thymocytes [6]. Sublytic concentrations of detergents cause shedding of membrane components [7,8] by microvesiculation and this effect was utilized by Ferber et al, using a lysophosphatidylcholine analog to isolate plasma membrane from calf thymocytes [9]. This last method has the advantage of obtaining plasma membrane relatively free of intracellular membrane organelles while obviating the need for a series of differential centrifugation steps. Crumpton et al, studied plasma membranes from the mouse T lymphoma cell line WEHI-22 after cell disruption by shearing [10]. We report here further work comparing morphological, ultrastructural, and biochemical properties of plasma membrane preparations from this lymphoma line after disruption by nitrogen cavitation, shearing, 2% Tween-40, and microvesiculation with a lysophosphatidylcholine analog ET-12-H.

MATERIALS AND METHODS

Cell Source

The murine thymoma WEHI-22.1 is a radiation-induced line of BALB/c origin [11]. Batches of 1×10^9–2×10^{10} log-phase cells were harvested from roller bottles as described previously [12], and washed once by centrifugation (300g av × 7 min) and resuspended in 20 mM phosphate/0.15 M NaCl pH 7.4 (PBS) before repeated centrifugation (300g av × 7 min) and resuspension in the selected medium for cell disruption. The preparations were all greater than 90% viable as judged by exclusion of 0.02% Trypan blue.

Isolation of Crude Membrane Pellets

Cell washing, disruption, and all subsequent steps were carried out at 0–4°C, unless otherwise indicated.

Nitrogen cavitation. Cells were suspended at 2×10^7/ml in a HEPES (4-(hydroxyethyl)-piperazinyl-ethane-2-sulfonic acid) buffered salt solution, pH 7.2 [13], equilibrated with 20 atm of nitrogen for 15 min in an Artisan "bomb" (Artisan Metal Products, Waltham, Massachusetts) and disrupted by dropwise release through the small orifice.

After removal of a nuclear pellet at 390g av × 15 min, and a large-granule pellet at 4,800g av × 10 min, a crude membrane pellet was collected at 19,200g av × 30 min. The highest yield of plasma membrane markers was found in this sedimentation fraction rather than in a subsequent "microsomal" fraction after 177,000g av × 60 min as used for calf thymocyte plasma membrane isolation [5].

Shearing with a cell-disrupting pump. Cells were disrupted at 2–5×10^7/ml in 25 mM Tris-HCl pH 7.4/0.15 M NaCl and membranes isolated as described previously [10]. The crude membrane pellet was collected at 19,200g av × 30 min.

Disruption by 2% Tween-40. Cells were stirred for 60 min at up to 2.5×10^8/ml in 2% Tween-40/25 mM Tris-HCl (pH 7.4)/150 mM NaCl/1 mM $MgCl_2$/2 mM $CaCl_2$ and homogenized with four strokes in a tight-fitting glass Dounce homogenizer before isolation of a crude membrane pellet as described by Standring and Williams [6].

Microvesiculation. The lysophosphatidylcholine analog, 1-dodecyl-propane-diol-3-phosphoryl-choline (ET-12-H) was provided by Dr H.U. Weltzien [9]. Cells at 5×10^7/ml were incubated with 75 μM ET-12-H in PBS for 10 min at 24°C. This treatment decreased cell viability by 10% as determined by 0.02% Trypan blue exclusion but did not decrease cell number. Cells were removed (500g av × 10 min) and a membrane pellet collected from the supernatant (177,000g av × 60 min).

Isolation of Plasma Membrane Fractions on Sucrose Gradients

The crude membrane pellets were subjected to two hypotonic washes before gradient centrifugation. This was done by resuspending them in 10 mM HEPES or Tris-HCl pH 7.6 and pelletting at 177,000g av × 60 min, and then with 1 mM buffer followed by resuspension in 10 mM buffer. These samples were layered onto continuous sucrose gradients and centrifuged at 82,500g av × 15 hr in a Beckman SW27 rotor. Gradients were fractionated and sucrose concentration determined with a refractometer. After analysis, fractions containing the peak of plasma membrane marker activity were pooled, washed twice, and resuspended in 10 mM HEPES or Tris-HCl pH 7.6.

Biochemical Assays

Protein was determined with the ninhydrin method [14] for samples in HEPES buffer and otherwise by the Lowry method [15]. γ-Glutamyl transferase (EC 2.3.2.2), succinate dehydrogenase (EC 1.3.99.1), and β-N-acetylglucosaminidase (EC 3.2.1.30) were assayed according to Ferber et al [9]. 5′-Nucleotidase (EC 3.1.3.5) was assayed using a modification of the ATPase procedure described by Ferber et al [9] where each assay used 1,200 nmol adenosine monophosphate (AMP) with 0.4 μCi ^{32}P-AMP, in place of adenosine triphosphate (ATP).

Cell Surface Iodination

Radioiodination of aliquots of washed intact cells with Na ^{125}I-iodide was performed using lactoperoxidase and hydrogen peroxide catalysis as described by Haustein [16] or with 1,3,4,6-tetrachloro-3α, 6α-diphenylglycoluril (IODOGEN) (Pierce, Rockford, Illinois) as described by Burgess et al [17]. Sodium dodecyl sulphate (SDS) gel electrophoresis in 12% polyacrylamide, run as described by Burgess et al [17], of labelled cells or membranes from iodinated cells showed that only components of mol wt greater than 43,000 were labelled with ^{125}I.

Electron Microscopy

For negative staining, unfixed membrane was stained with 2% sodium phosphotungstate (pH 7.0) on coated grids.

For thin sectioning, membrane pellets were fixed with 2.5% glutaraldehyde in PBS and cells in suspension in half-strength Karnovsky fixative for at least 1 hr (4°C) and processed according to Santer et al [18]. Sections were stained with uranyl acetate and lead citrate.

For freeze-fracture, membrane was fixed in suspension in 1.25% glutaraldehyde in PBS for a minimum of 1 hr at 4°C, washed 4 times in distilled water, glycerinated to 25% w/v, frozen in Freon 22 and fractured in a Balzers freeze-etch apparatus (Balzers Ag. Fürstentum, Liechtenstein) at −110°C.

RESULTS AND DISCUSSION

The WEHI-22 lymphoma cells resemble blast-like thymus cells (Figs. 1, 2a) rather than thymus-small lymphocytes. The lymphoma cells are larger, about 10 μm in diameter, than small lymphocytes and have a greater proportion of cytoplasm containing more organelles and many free ribosomes. Endogenous C-type virus particles are frequently found associated with and budding from the plasma membrane, usually over one pole of the cell.

Cell Disruption

Various procedures for membrane isolation and, particularly, cell disruption have been adapted or developed for various tissue types and to the cell numbers available. Nitrogen cavitation and shearing with a cell pump involve mechanical disruption using pressure and shear forces. These techniques have proved effective with large quantities of lymphoid cells in suspension [3,5]. Such disruption necessarily involves cell disorganization and also alteration in plasma membrane morphology as illustrated by the rearrangement of freeze-fracture intramembranous particles [4]. Also, some nuclear breakage is unavoidable and causes contamination of plasma membrane preparations with nuclear membrane, which is difficult to monitor.

The two additional procedures compared here involved detergent treatment. Two percent Tween-40 causes cell lysis, leaving no intact cells, but does not solubilize most membrane components since a membrane fraction enriched in plasma membrane markers could be sedimented at 70,000g av $\times$ 1 hr as found with rat thymocytes by Standring and Williams [6]. The nuclei of the lysed cells were swollen but not

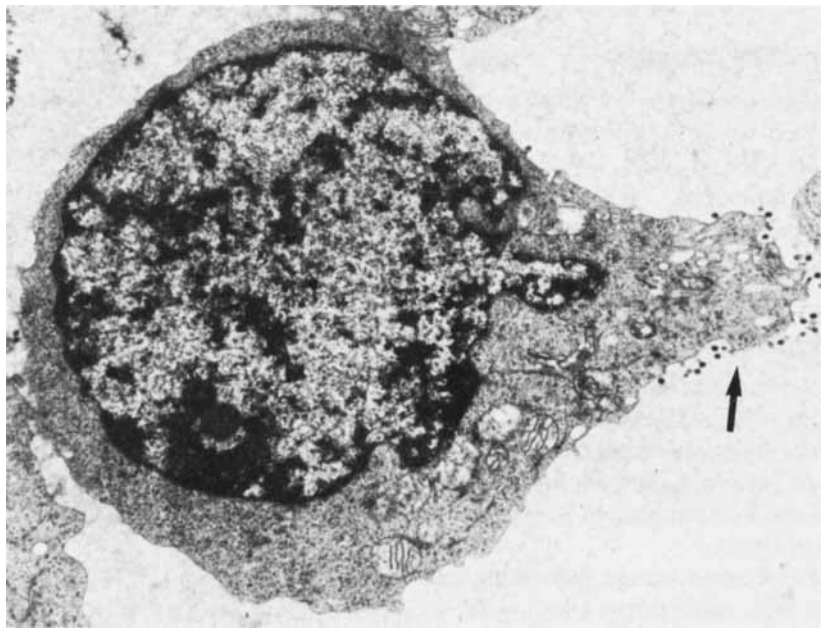


Fig. 1. Electron micrograph of a WEHI-22 T lymphoma cell with C-type virus particles aggregated over the uropod (arrow) $\times$9,140.

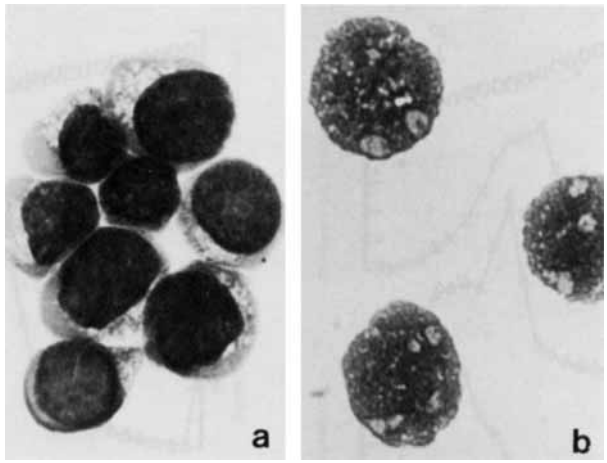


Fig. 2. Light micrographs of WEHI-22 cells (approx ×1,000). Samples were cytocentrifuged and stained with Giemsa, a) after washing in media and (b) after treatment iwth 2% Tween-40 for 1 hr, 4°C, and four strokes of a glass Dounce homogenizer. b) Nuclei are swollen but not disrupted, nucleoli are clearly visible, and there is no evidence of residual cytoplasm.

disrupted (Fig. 2b). Eight other cultured lymphoid cell lines were also completely lysed by 2% Tween-40. On the other hand, about 20% of mouse and human neutrophils remain intact under these conditions (S. Watt, W. Phillips and J.G. Culvenor, unpublished results).

Treatment of the tumor cells with a low concentration of the detergent-like lysolecithin analog caused membrane shedding without cell disruption. By light microscopy cell appearance varied after this treatment for different cell batches, from no apparent change to easily visible surface blebbing. ET-12-H at the concentration used here (75 nmol/5 × 10^7 cells/ml) caused less than 5% release of lactate dehydrogenase (data not shown) into the supernatant, as was also reported with calf thymocytes [9].

Analysis of Washed Crude Membranes on Sucrose Gradients

Representative sucrose density gradient analysis of the crude membranes is shown in Figure 3. Fractions were monitored for density and assayed for the plasma membrane markers γ-glutamyl transferase, 5′-nucleotidase, or ^{125}I surface label, as well as the mitochondrial enzyme, succinate dehydrogenase, and the lysosomal enzyme, β-glucosaminidase.

The activity of γ-glutamyl transferase coincided with the peak of 5′-nucleotidase activity for nitrogen cavitation and shearing membranes (Fig. 3a,b) and with ^{125}I labelling used for the Tween-40 and ET-12-H membranes (Fig. 3c,d). This supported evidence that γ-glutamyl transferase is a useful lymphoid cell plasma membrane marker [5,19]. γ-Glutamyl transferase was particularly useful as a marker with WEHI-22 plasma membrane since 5′-nucleotidase was of very low activity in this cell line as reported previously [10].

The plasma membrane markers peaked in the gradients at 27% w/v sucrose for shearing and N_2-cavitation membranes, at 23% w/v sucrose for Tween-40 mem-

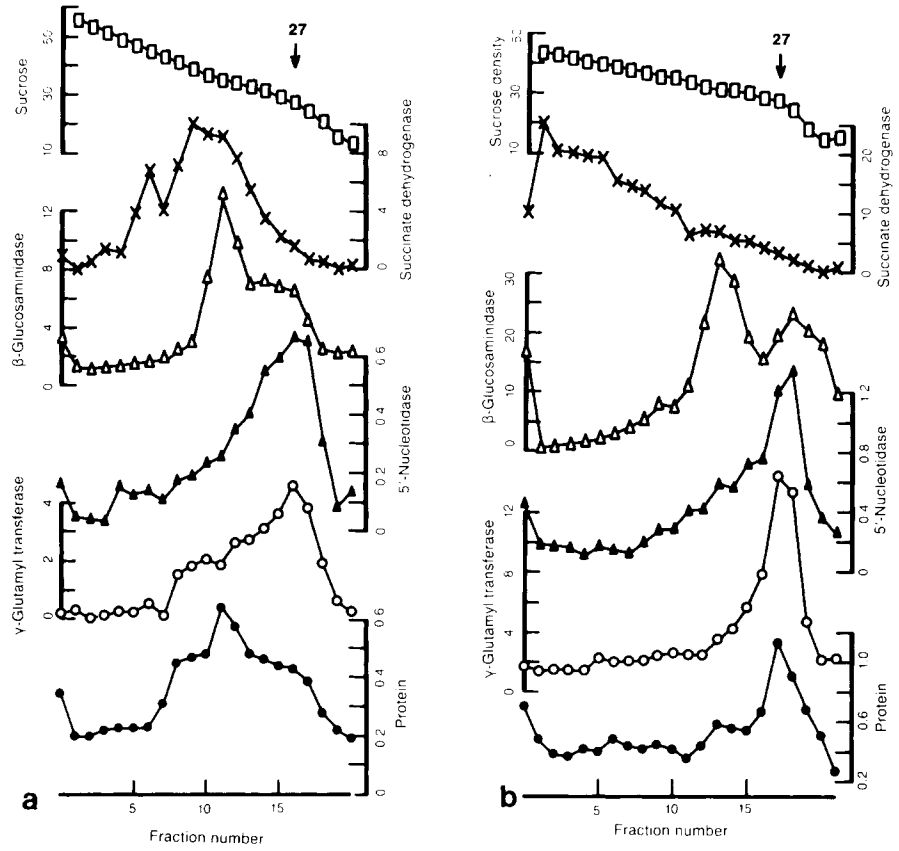
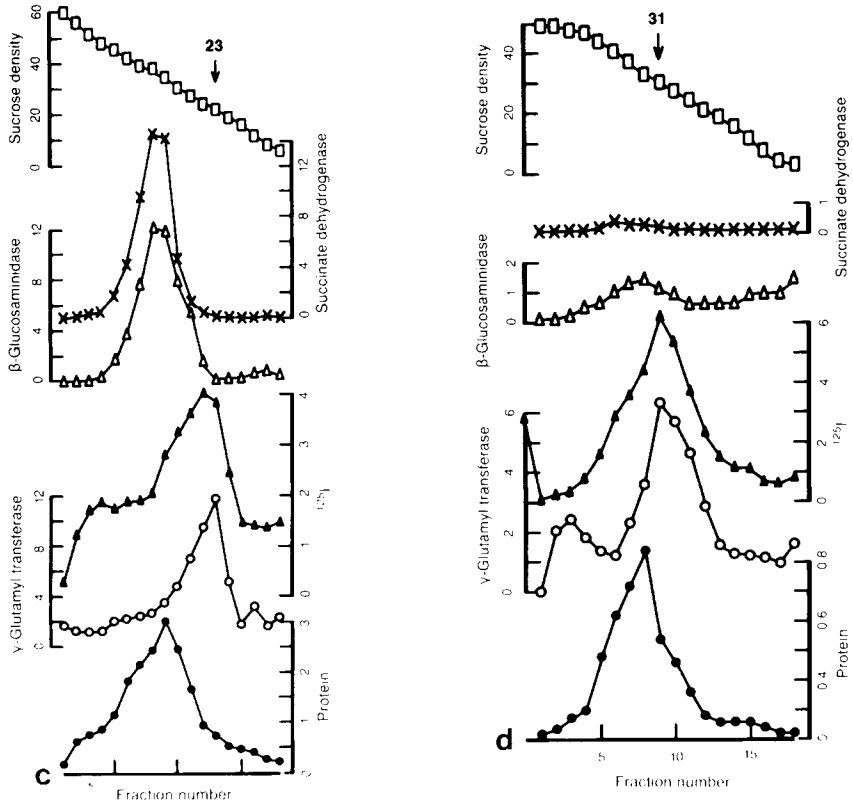


Fig. 3. Sucrose gradient analysis of crude membrane prepared following (a) nitrogen cavitation, (b) shearing, (c) Tween-40, or (d) ET-12-H treatment. Amounts of crude membrane protein applied to these gradients were: (a) 7.5 mg, (b) 12 mg, (c) 21 mg, and (d) 14 mg. Before Tween-40 treatment (c) 3×10^8 cells were treated with 600 μCi $Na^{125}I$-iodide in glass tubes coated with 60 μg IODOGEN, then added to 1.1×10^{10} cells. Before ET-12-H treatment (d) 5×10^7 cells were treated with 2.5mCi $Na^{125}I$-iodide using lactoperoxidase and H_2O_2 catalysis, then diluted in 1.4×10^{10} cells. Fractions were assayed for protein (mg per fraction), ●; γ-glutamyl transferase (nmol/min), ○; 5''-nucleotidase (nmol/min) or ^{125}I activity (cpmx 10^{-4}), ▲; β-glucosaminidase (nmol/min), △; succinate dehydrogenase (nmol/min), X; and sucrose density (% w/v), □. The sucrose density at which the plasma membrane markers peaked is indicated by an arrow.

branes, and 31% w/v for ET-12-H membranes. Membrane density differences reflect variation in composition and, particularly, the protein: lipid ratio. The density for shearing and N_2-cavitation membrane (1.10 g/ml) is similar to that found for other lymphocyte membrane preparations [3]. The lower density of the Tween-40 membrane of 23% w/v sucrose (equivalent to 1.09 g/ml) was also reported by Standring and Williams [6] for rat thymocyte plasma membranes after Tween-40 disruption. The lower density of the Tween-40 prepared plasma membrane facilitates purification in the sucrose gradient, since there is a larger density difference between the plasma membrane markers and other cytoplasmic membrane markers than following the other preparative techniques.



The higher density of the ET-12-H membrane preparations of 31% w/v sucrose (1.12 g/ml) may reflect a real difference in composition due to selective removal of membrane not representative of the total plasma membrane. Ferber et al [9] reported a different enzymic and lipid composition for ET-12-H released membrane compared with nitrogen cavitation plasma membranes from calf thymocytes. Succinate dehydrogenase and β-glucosaminidase were minimal in the ET-12-H gradient fractions showing little contamination of the crude ET-12-H plasma membrane with cytoplasmic membranes. Electron microscopy (described below) showed that these membranes were more contaminated by virus particles than the other preparations. Viral and ribosomal contamination may have contributed to the higher density and may also indicate that ET-12-H membrane is derived from a region of the cell assocated with viral budding or viral binding.

Morphological Appearance of Plasma Membrane Isolated From Sucrose Gradients

Washed plasma membrane peaks from the sucrose gradients were examined by negative staining, thin-section, and freeze-fracture electron microcsopy. All the preparations showed typical membrane vesicles.

As with other lymphoid cells [3] nitrogen cavitation produced vesicles of about 90–300 nm diameter, and shearing a slightly larger size ranging between 100–500 nm diameter. These two preparative procedures produced vesicles relatively free of virus or ribosomes in thin section, and after freeze-fracture, intramembranous particles were aggregated. Micrographs of these preparations are not shown since they were similar to those described after shearing and cavitation of other cell types [3,4].

Figures 4 and 5 compare electron micrographs of the Tween-40 and ET-12-H purified plasma membrane preparations. Tween-40 prepared vesicles were about 100–400 nm in diameter, relatively virus-free, and, in freeze-fracture, intramembranous particles were aggregated. Tween-40 vesicles were thus surprisingly similar to those produced after nitrogen cavitation and shearing. Plasma membrane vesicles from calf thymocytes, isolated following nitrogen cavitation, shearing or osmotic disruption [4], also had aggregated intramembranous particles. This aggregation indicates that the plasma membrane components have undergone some rearrangement, since freeze-fracture of intact untreated cells produces replicas with dispersed intramembranous particles.

ET-12-H membrane vesicles, in contrast, were smaller in size (about 90–200 nm), and thin sectioning revealed considerable contamination by viral particles identifiable by their dense cores (Fig. 5). Freeze-fracture showed that intramembranous particles were not aggregated.

Summary of Properties of the Various Preparations

Table I shows the yield of plasma membrane after typical preparations. A similar percentage (about 0.6%) of starting cell protein was recoverd in the plasma membrane preparations, and this contained 11–23% of the starting γ-glutamyl trans-

TABLE I. Summary of Properties of Representative Plasma Membrane Preparations

Preparative procedure	Nitrogen cavitation	Shearing	Tween-40	ET-12-H
Starting cell number	3.8×10^9	9.2×10^9	1.2×10^{10}	1.4×10^{10}
Total cell protein (mg)	308	942	1,340	1,300
Protein recovered in plasma membrane fraction (mg)	1.8	5.4	4.2	9.6
Percent of starting protein in plasma membrane fraction	0.6	0.6	0. 4	0.7
γ-Glutamyl transferase (nmol/min/mg protein)				
Starting cell sample	0.5	0.7	0.6	0.3
Plasma membrane fraction	10.1(×20)	13.2(×20)	26 (×45)	8.4(×32)
Enzyme recovery (% of total activity)	11	11	16	23
Density on sucrose (g/ml)	1.10	1.10	1.09	1.12
Vesicle size (nm)	90-300	100-500	100-400	90-200
Aggregated IMP (after freeze-fracture)	+	+	+	−
Gross viral contamination	−	−	−	+

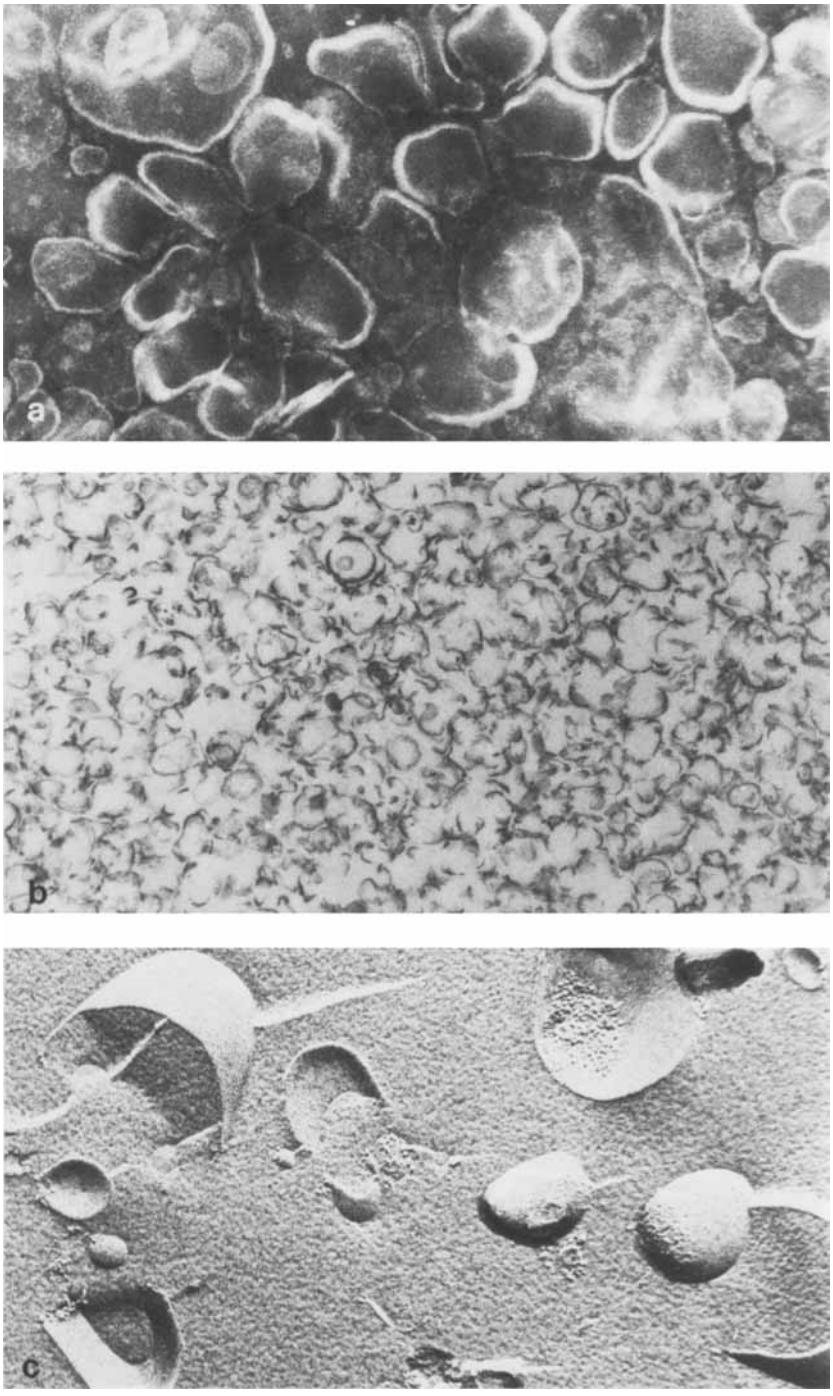


Fig. 4. Electron micrographs of plasma membrane prepared from Tween-40 disrupted cells. a) Unfixed membrane vesicles were stained with 2% w/v sodium phosphotungstate (×74,000). b) Membrane vesicles after thin section (×29,750). c) A freeze-fracture replica of vesicles showing aggregated intramembranous particles on both fracture faces (EF and CF) (×57,500).

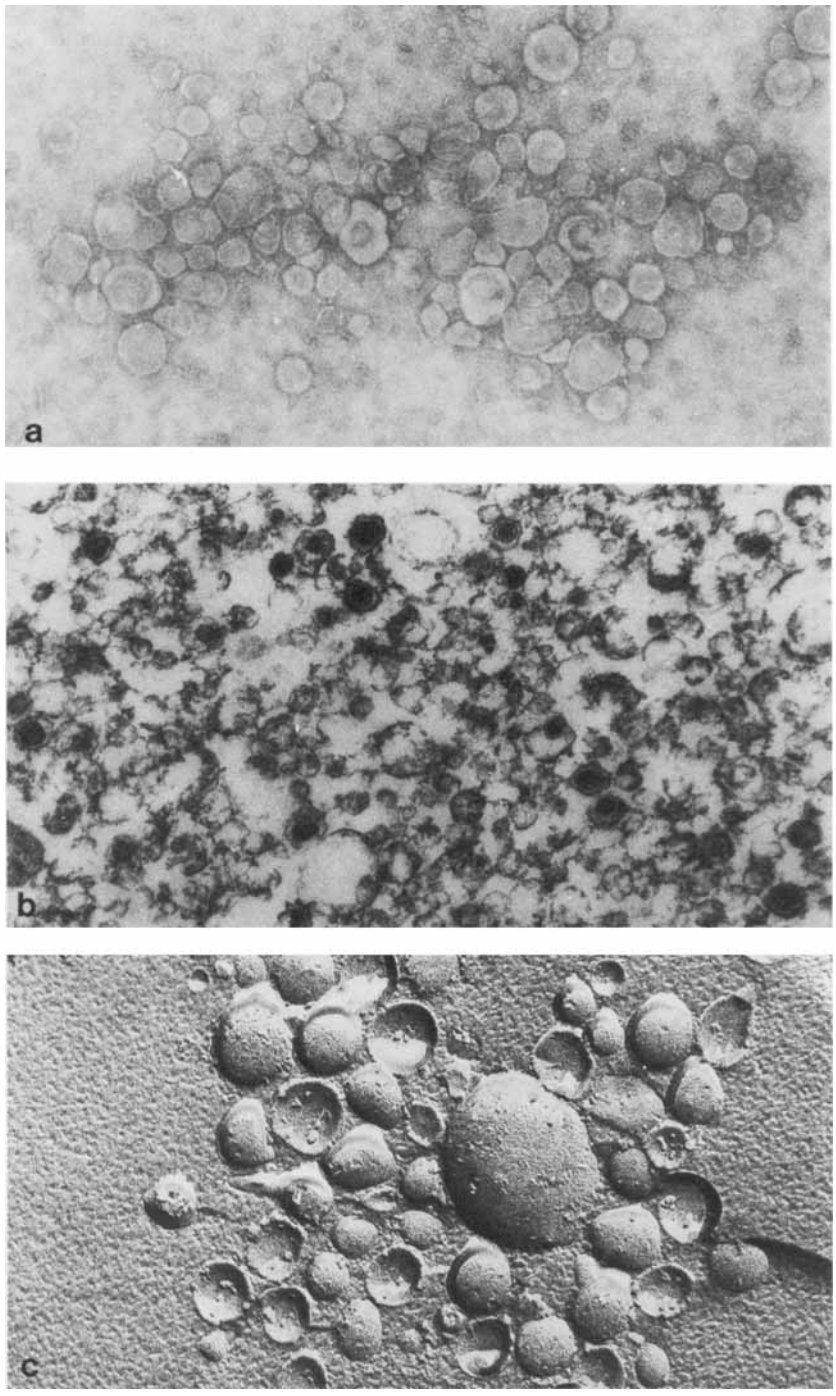


Fig. 5. Electron micrographs of plasma membrane released by microvesiculation induced by ET-12-H treatment. a) Unfixed sample stained with sodium phosphotungstate (×46,250). b) Thin section showing many virus particles in addition to membrane vesicles (×46,250). c) Freeze-fracture replica showing dispersed intramembranous particles in membrane fracture faces (×74,000).

ferase activity. Although recovery was not high, this membrane enzyme was enriched up to 45-fold in the plasma membrane fraction. This yield and purity compares favorably with plasma membrane preparations from other cell types [1,3].

CONCLUSIONS

The four cell disruption methods produced plasma membrane vesicles from one tumor cell line with reasonable yield and enrichment. Comparison with alternate plasma membrane markers indicated that γ-glutamyl transferase is a plasma membrane enzyme in this T lymphoma as found for other T cells (calf thymocytes and human T lines [5,19]). These membrane preparations may prove useful for further study of the properties and functions of this enzyme since its biological function is not known. Recent studies [20] have indicated that it may be involved in receptor-mediated endocytosis. ET-12-H released membrane compared favorably with membrane obtained after complete cell disruption and may be of further interest in investigating plasma membrane association with virus particles. Shedding of plasma membrane vesicles may be analogous to viral budding, perhaps occurring at similar sites on the cell surface associated with similar fluidity properties as suggested by van Blitterswijk et al [21].

The plasma membrane preparations have provided useful complementary information on the nature of the lymphoma cell's surface. Pressure and shear disruption provide preparations suitable for the study of total plasma membrane composition. Disruption with Tween-40 requires no sophisticated equipment and can be easily adapted to small and large cell numbers and is useful for the isolation of integral membrane constituents. Microvesiculation with ET-12-H provides preparations which may prove useful for studying variation in cell surface domains and in particular areas of viral assocation.

ACKNOWLEDGMENTS

We thank June Uren for excellent assistance with cell culture and Dr. A.W. Harris for constructive discussions. The work was supported by the National Health and Medical Research Council (Canberra) and the Stiftung Volkswagenwerk.

REFERENCES

1. Evans WH: In Work TS, Work ES (eds): "Laboratory Techniques in Biochemistry and Molecular Biology." Amsterdam: Elsevier North-Holland, vol 7, 1978.
2. Ferber E, Resch K, Wallach DFH, Imm W: Biochim Biophys Acta 266:494–504, 1972.
3. Crumpton MJ, Snary D: Contemp Top Mol Immunol 3:27–56, 1974.
4. Brunner G, Bauer HC, Suter D, Speth V: Biochim Biophys Acta 507:419–422, 1978.
5. Suter D, Brunner G, Ferber E: Biochem J 175:643–647, 1978.
6. Standring R, Williams AF: Biochim Biophys Acta 508:85–96, 1978.
7. Holdsworth G, Coleman R: Biochem J 158:493–495, 1976.
8. Billington D, Coleman R: Biochim Biophys Acta 509:33–47, 1978.
9. Ferber E, Schmidt B, Weltzien HU: Biochim Biophys Acta 595:244–256, 1980.
10. Crumpton MJ, Marchalonis JJ, Haustein D, Atwell JL, Harris AW: Aust J Exp Biol Med Sci 54:303–316, 1976.
11. Harris AW, Bankhurst AD, Mason S, Warner NL: J Immunol 110:431–438, 1973.
12. Culvenor JG, Harris AW, Mandel TE, Whitelaw A, Ferber E: J Immunol 126:1974–1977, 1981.
13. Brunner G, Heidrich H-G, Golecki JR, Bauer HC, Suter D, Plückhahn P, Ferber E: Biochim Biophys Acta 471:195–212, 1977.

14. Moore S, Stein WH: J Biol Chem 176:367–388, 1948.
15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265–275, 1951.
16. Haustein D: J Immunol Methods 7:25–38, 1975.
17. Watt SM, Burgess AW, Metcalf D: J Cell Physiol 100:1–22, 1979.
18. Santer V, Bankhurst AD, Nossal GJV: Exp Cell Res 72:377–386, 1972.
19. Novogrodsky A, Tate SS, Meister A: Proc Natl Acad Sci USA 73:2414–2418, 1976.
20. Davies, PJA, Davies DR, Levitzki A, Maxfield FR, Milhaud P, Willingham MC, Pastan IH: Nature 283:162–167, 1980.
21. van Blitterswijk WJ, Emmelot P, Hilkmann HAM, Hilgers J, Feltkamp CA: Int J Cancer 23:62–70, 1979.