Revertants of a Chinese Hamster Ovary Cell Mutant Resistant to Suppression by an Analogue of Cholesterol: Isolation and Partial Biochemical Characterization†

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ABSTRACT: A highly efficient selection procedure was developed for isolating revertants of Chinese hamster ovary (CHO) cell mutants resistant to suppression by 25-hydroxycholesterol. The procedure is based on the fact that the specific polyene antibiotic amphotericin B caused a lethal porous complex formation with membrane cholesterol only in cholesterol-rich cells. The wild-type cells and the revertant cells switched to grow from fetal calf serum medium to delipidated fetal calf serum medium for approximately 1 day became deficient in cellular cholesterol content. These cells, unlike the cholesterol-rich mutant cells, became much less sensitive to amphotericin B cytotoxicity. The spontaneous reversion frequency of a previously reported 25-hydroxycholesterol-resistant cell clone, 25-RA [Chang, T.-Y., & Limanek, J. S. (1980) *J. Biol. Chem. 255*, 7787–7795], was found to be approximately 3×10^{-6}, a frequency comparable to other single gene mutations of CHO cells. Biochemical analyses of three of these revertants showed that all defects manifested in 25-RA cells reverted back in parallel, a result suggesting that these observed defects in 25-RA cells are due to a single mutation event, thus supporting the hypothesis (Chang & Limanek, 1980) that a common controlling factor may be involved in mediating the suppressive action(s) of the cholesterol analogue on various cholesterogenic enzyme activities. The function of this common controlling factor is rendered abnormal in 25-RA cells by mutation.

The isolations and biochemical characterizations of cell mutants with specific defects often indicate the existence of specific mechanisms involved in various cellular events, which otherwise could easily be overlooked or not detected in studies using only normal cells. The Chinese hamster ovary (CHO)[1] cell is a valuable model for studying the intracellular mechanisms for regulation of cholesterol metabolism (Chang & Limanek, 1980; Chang et al., 1981a). Various CHO cell mutants with specific defects in lipid metabolism have been isolated and partially characterized [for representative examples, see Berry & Chang (1982), Chin & Chang (1982), Sinensky et al. (1979), Esko & Raetz (1980), and Polokoff et al. (1981)]. In all of these studies, mutagens were used to mutagenize the parental cells before enrichment and isolation of the mutants. It is thus important that revertants of the mutant be isolated, preferably via spontaneous mutation, and be characterized biochemically along with the parental and the mutant cells to assure that the various phenotypic abnormalities observed in the mutant are attributed to the consequence of a single gene mutation.

Previously, this laboratory reported (Chang & Limanek, 1980) the characterization of two CHO cell mutants (clones 25-RA and 25-RB) resistant to suppression by 25-hydroxycholesterol, which is believed to be an intracellular analogue of low-density lipoprotein-bound cholesterol (Brown et al., 1975; Chang & Limanek, 1980; Chin & Chang, 1981). The activities of the first four enzymes in the sterol biosynthetic pathway in 25-RA and 25-RB were all found to be more resistant to suppression by 25-hydroxycholesterol than those found in wild-type cells. In experiments reported in this paper, we made use of the observation that the resistant mutants possessed cellular cholesterol contents 2–3-fold higher than that in the wild-type cells (Chang & Limanek, 1980) to develop a selection procedure for isolating spontaneous revertants from the resistant mutants. The selection procedure is based on the fact that certain polyene antibiotics, such as fillipin or amphotericin B, cause a lethal porous complex formation with membrane cholesterol in cholesterol-rich cells (Kinsky, 1970; Norman et al., 1972; Saito et al., 1977; Hidaka et al., 1978). Furthermore, we report the biochemical characterization of the revertant cells along with the wild-type and 25-RA cells and discuss the implication of our findings.

Experimental Procedures

Materials. Most of the biochemicals were from Sigma Chemical Co. 25-Hydroxycholesterol from Steraloids, with a purity of approximately 98%, was added to tissue culture medium from a stock 2 mg/mL solution in dimethyl sulfoxide (Me_2SO) as previously described (Chin & Chang, 1981). Radioactive chemicals were from New England Nuclear. Amphotericin B was added to tissue culture medium from a stock 30 mg/mL solution in Me_2SO, prepared fresh daily under sterile conditions. 25-Hydroxy[24-^{3}H]cholesterol was from Dr. Albert F. T. Chen as previously described (Chang & Limanek, 1980).

Cells. As previously described (Chang & Limanek, 1980; Chang et al., 1981a,b), CHO cell cultures were grown as monolayers in 25-cm^2 Falcon tissue culture flasks or in 100 × 20 mm Falcon dishes in F-12 medium (linoleic acid deleted) supplemented with either 10% fetal calf serum (FCS–M) or 10% delipidated serum (DeL–M). Fetal calf serum was delipidated once according to the procedure described previously (Chin & Chang, 1981). Cells were grown in a 5% CO_2 tissue culture incubator with nearly 100% humidity (National Appliance Co., Model 3341-4).

Enzyme Assays. For HMG-CoA reductase assay, whole cell homogenates prepared by the method described previously

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[1] Abbreviations: CHO cells, Chinese hamster ovary cells; Me_2SO, dimethyl sulfoxide; FCS–M, 10% fetal calf serum + F-12 medium; DeL–M, 10% delipidated fetal calf serum + F-12 medium; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low-density lipoprotein; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/82/0421-5316$01.25/0

(Chang et al., 1981b) were used. The procedure used for the reductase assay was previously described (Chang et al., 1981a). For HMG-CoA synthase, acetoacetyl-CoA thiolase, and mevalonate kinase assays, the cytosolic fraction was prepared by the digitonin extraction procedure described previously (Chang & Limanek, 1980; Mackall et al., 1979). Appropriate aliquots of the cytosolic fraction were dialyzed at 4 °C for 24 h against two changes of 400-fold excess buffer containing 5 mM potassium phosphate and 0.1 mM EDTA, pH 7.2, and then used to determine HMG-CoA synthase activity (Clinkenbeard et al., 1975; Balasubramaniam et al., 1977). We found that the presence of dithiothreitol during dialysis was unnecessary to obtain optimal synthase activity. We also found that the synthase activity remained linear with time for up to 36 min of reaction time at 30 °C, as long as the formation of the product did not exceed 10% of the total amount of [^{14}C]acetyl-CoA present in the assay mixture. For acetoacetyl-CoA thiolase assay, minor modifications of the procedure previously described were used (Chang & Limanek, 1980). The reaction mixture was preincubated at 27 °C for at least 10 min in a water bath and then preincubated inside the cuvettes at 27 °C for 6 min. The temperature of the cuvette chamber is maintained at 27 °C by a circulating water jacket. The reaction was started by addition of up to 100 μL of cell extract for 3 min. The frozen cell extract, which could be stored at −70 °C for at least 1 week without losing thiolase activity, was thawed and preincubated at 27 °C for approximately 20 min before use to start the reaction. The mevalonate kinase assay was as described previously (Chang & Limanek, 1980). The specific activity of each enzyme was expressed as nanomoles of product formed per minute per milligram of protein under standard assay conditions.

Methods for Estimating Cell Survival after Amphotericin B Treatment. (*A*) *Method 1.* The treated cells were rinsed once with 5 mL/dish of phosphate-buffered saline (PBS), then fed with 8 mL of FCS–M, and grown in the tissue culture incubator for 24 h. The cells were then rinsed with 5 × 10 mL/dish of PBS and scraped into 1.0 mL of PBS; 0.2-mL aliquots of the cell suspension were taken for protein content determination. Control experiments showed that wild-type or 25-RA cells grown in FCS–M treated with amphotericin B at ≥25 μg/mL for 2 h resulted in cell death of more than 99.9%; cell debris were present after the treatment and thus constituted a blank value for estimating the cell survival by measuring the cellular protein content remaining in the dish. For 1 × 10^6 wild-type or 25-RA cells per dish, the blank value was found to be 4.2 ± 1.4 (SD) μg of protein/dish. Since 1 × 10^6 wild-type or 25-RA cells grown in FCS–M or DeL–M were found to contain 0.14 ± 0.005 (SD) mg of total cellular protein, the blank value contributed by the cell debris amounted to 3 ± 1% of the observed cellular protein content which remained in the dish after treatment and was subtracted to obtain the corrected extent of cell survival. The protein content found in nontreated cells without subtracting the blank value was used as the 100% value.

(*B*) *Method 2.* The cells were treated as described for *method 1* but were allowed to grow in FCS–M for 5 days before harvest for cellular protein content analyses. Since the cell doubling time in medium A is approximately 16 h, the sensitivity of *method 2* is amplified 64-fold by cell division as compared to *method 1*; this factor is taken into account to obtain the corrected extent of cell survival.

(*C*) *Method 3.* The cells were treated as described for *method 1* but were allowed to grow without disruption in FCS–M for 7 days. Afterward, the colonies developed in each dish were localized by visually examining the dish opposite a 75-W light source containing an 8.5-in. diameter floodlight projector. Colonies containing 50 or more cells could be detected without difficulty. Identity of colonies is subsequently verified by examining under the phase-contrast microscope with 100-fold magnification. Colonies containing less than 50 cells/colony were ignored. This method was used only when there were less than 100 colonies/dish present. The number of colonies present per dish multiplied by 2 was assumed to be the number of cells that survived after the amphotericin B treatment, since the plating efficiency (number of colonies developed 7 days after plating 100 cells in a 100-mm dish) of CHO cells grown in FCS–M was found to be 50%. The number of cells found in nontreated cells right after the amphotericin B treatment was used as the 100% value to obtain the extent of cell survival by this method.

These three methods differed greatly from one another in sensitivity and limits of detection; however, in combination, they constituted a simple and effective way for measuring cell contents which may differ by as much as 5 orders of magnitude, as exemplified by the experiment described in Figure 2 under Results.

Other Analytical Procedures. Protein was determined by the method of Lowry et al. (1951). Colonies of cells were cloned by using the cloning cylinders (Bellco Glass, Inc., catalog no. 2090-00808) dipped in sterilized vacuum grease (Dow Corning Co., catalog no. 970-V). Trypsin at 0.001% was used to detach cells from the surface. Each valuable clone was recloned once by seeding 0.2-mL aliquots containing 1 cell/0.2 mL of FCS–M into 40 single wells by using the Falcon 96-well plate. After 6 days of undisturbed growth, single colonies were verified under the microscope with a 10-fold magnification. Cellular-free cholesterol and cholesterol ester contents were determined by the following method: Monolayer cultures grown in 25-cm^2 Falcon flasks were rinsed with 3 × 5 mL of buffer containing 50 mM Tris, 0.15 M NaCl, and 2 mg/mL bovine serum albumin (fraction V), pH 7.4, at 4 °C, followed by 3 × 5 mL rinses of PBS at 4 °C. The cells were dissolved by incubating with 1.2 mL of 0.1 M NaOH at room temperature for 30 min and transferred to glass extraction tubes containing 40 μL of 3 M HCl per tube. Aliquots were withdrawn for protein determinations. To the rest of the sample were added 20 000 dpm of [1-^{14}C]cholesteryl oleate and 20 000 dpm of [1,2-$^3H_2$]cholesterol per tube for recovery measurements. The lipids were extracted by the method of Bleigh & Dyer (1959) and separated by thin-layer chromatography using Whatman LK5D plates [solvent system, petroleum ether/ether/acetic acid (90:10:1)]. The sterol ester band and sterol band were scraped off and extracted with ethyl acetate. To each sample was added 3 μg of cholesterol methyl ether as internal standard. The sterol fraction was quantitated by gas–liquid chromatography using an OV-17 column at 255 °C as previously described (Chang et al., 1977, 1979). The sterol ester fraction was saponified; the free sterol fraction was then extracted and quantitated by gas–liquid chromatography as previously described (Chang et al., 1979). The procedural loss of ^{14}C- and ^{3}H-labeled lipid standards during extraction and lipid analysis averaged approximately 15%.

Results

Preliminary studies showed that when wild-type, 25-RA, or 25-RB cells grown in 10% fetal calf serum medium (FCS–M) were treated with amphotericin B at a concentration of ≥25 μg/mL for 2 h, greater than 99.9% cell death occurred. The same results were obtained when the FCS–M-grown cells were switched to grow in 10% delipidated fetal calf serum

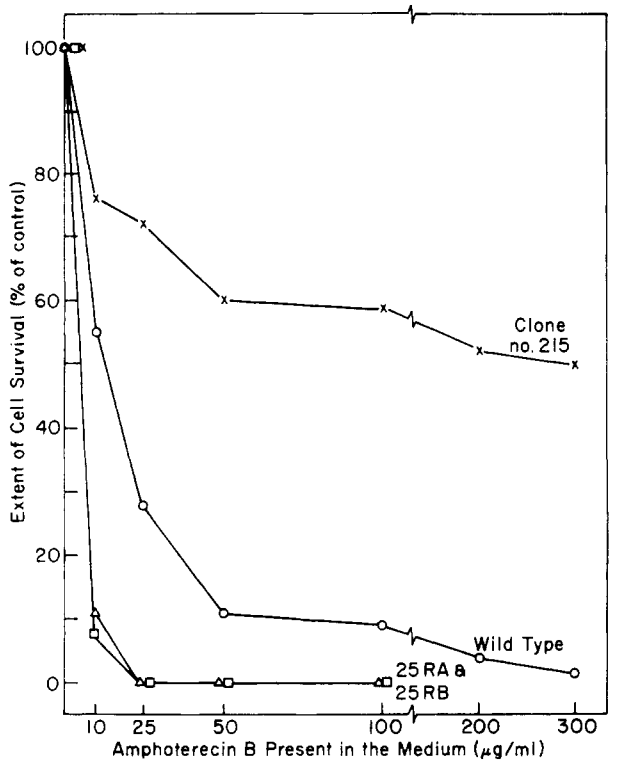

FIGURE 1: Differential cell survival of various CHO cell clones treated with amphotericin B (10–300 μg/mL). Four different cell clones as indicated were plated at 0.4 × 10^6 cells/100-mm dish in 5 mL of FCS–M and grown for 24 h. Afterward, the medium was replaced with 5 mL of DeL–M/dish, with a 5 mL of PBS/dish rinse between the replacement. Cells were grown in DeL–M for 18 h. The medium was replaced; cells were treated with 5 mL/dish of DeL–M containing 1% Me_2SO and amphotericin B at indicated concentrations and incubated in a 5% CO_2 incubator at 37 °C for 2 h. Medium was then replaced with 8 mL of FCS–M/dish, with a 5 mL of PBS/dish rinse between the replacement. The extent of cell survival was estimated according to method 1 as described under Experimental Procedures. The extent of cell survival of 25-RA and 25-RB cells treated with 200 or 300 μg/mL amphotericin B was found to be zero; the result was deleted from the figure for simplicity. Duplicate dishes were employed for each point with the average reported; variation between the duplicate was within 10% of the average.

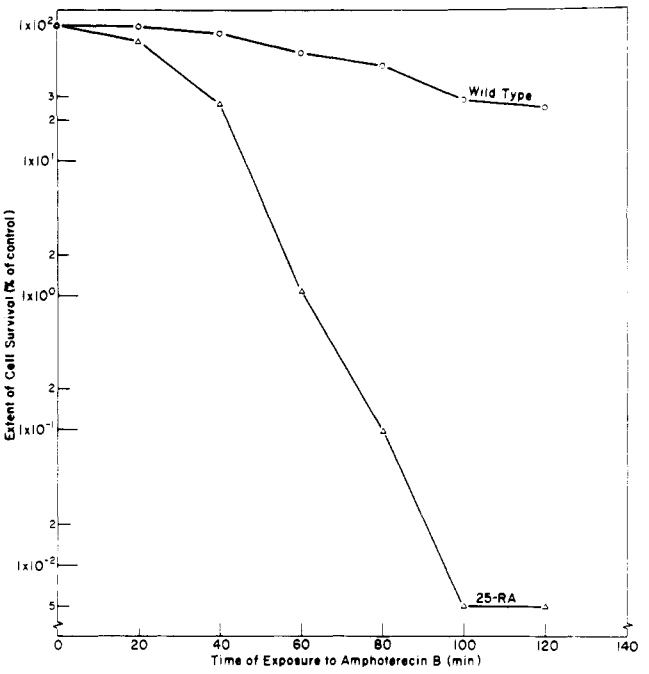

FIGURE 2: Differential cell survival of wild-type and 25-RA cells treated with amphotericin B at 25 μg/mL for various periods of time. Wild-type and 25-RA cells were grown and treated with 25 μg/mL amphotericin B in a manner identical with that described in Figure 1, for the indicated periods of time. After treatment, survived cultures were rinsed with 5 mL of PBS/dish and rescued with 8 mL of FCS–M. For estimation of the extent of cell survival, for wild-type cells, method 1 was employed; for 25-RA cells treated for 20 or 40 min, method 1 was employed; for cells treated for 60 or 80 min, method 2 was employed; for cells treated for 100 or 120 min, method 3 was employed. Duplicate dishes were employed for each point with the average reported. For values obtained from method 1 or method 2, the variation between the duplicates was within 10% of the average. For method 3, the variation between the duplicates was within 25% of the average. See Experimental Procedures for details of methods 1–3.

(DeL–M) for up to 8 h. However, when cells were allowed to grow in DeL–M for 18–24 h, the relatively slow induction of endogenous sterol synthesis coupled with rapid cell division of these cells resulted in large decreases of the cellular cholesterol contents in these cells (Chang & Limanek, 1980), which in turn rendered the wild-type cells much more resistant to killing by amphotericin B than the 25-RA and 25-RB cells (Figure 1). In this experiment, it is seen that CHO cell mutant 215, a cholesterol-requiring auxotroph defective in lanosterol demethylation (Berry & Chang, 1982), is even more resistant to amphotericin B cytotoxicity than the wild-type cell. In an experiment not shown, we found that mutant 1, which is a cell mutant requiring cholesterol and unsaturated fatty acid for growth (Chin & Chang, 1981, 1982), was also more resistant to amphotericin B cytotoxicity than the wild-type cell. The time dependence of amphotericin B (25 μg/mL) killing of wild-type or 25-RA cells is shown in Figure 2. A very large difference in susceptibility toward amphotericin B killing between these two cell types was seen; at the 2-h time point, the ratio of cell survival rates between the two cell types was found to be approximately 10^3. This difference can be seen quantitatively by examining the morphology of these two cell types before (Figure 3A) or after (Figure 3B,C) the treatment. Figure 3C shows that, unlike the 25-RA cells, a significant portion (approximately 30%) of the wild-type cells can be rescued back to exhibit normal morphology by incubating the treated cells in FCS–M for 3 h. We also found that single colonies of 25-RA cells grown in DeL–M for 18 h were much more susceptible to amphotericin B killing than single colonies of wild-type cells, as shown in Figure 4 (top and middle panels).

When approximately (1–2) × 10^6 25-RA cells were grown in DeL–M for 18 h in a single 100-mm dish, treated with amphotericin B at 25 μg/mL for 2 h, and then switched to grow in FCS–M undisturbed for 6 or 7 days, there were colonies invariably found in each dish large enough (≥50 cells/colony) to be seen with the naked eye; the number of colonies varied between 20 and 70/dish. These colonies were then treated with amphotericin B at 25 μg/mL for 2 h to identify potential revertants of 25-RA cells. Those colonies exhibiting morphologies resembling that of wild-type cells after the amphotericin B treatment (Figure 4, top panel) were localized by marking their positions with a magic marker at the exterior of each dish. After 1 or 2 days of incubation in FCS–M to restore normal growth, they were cloned by using the cloning rings and recloned once (see Experimental Procedures for details). In a single experiment, from a clone of 25-RA cells (cloned immediately before the experiment) grown to 40 × 10^6 cells in 20 100-mm dishes, 45 candidates were found. Each clone was then tested according to the experiment described in Figure 3; 18 clones were found to be false candidates (they were clones of 25-RA cells which survived the amphotericin B killing). The remaining 27 clones were all

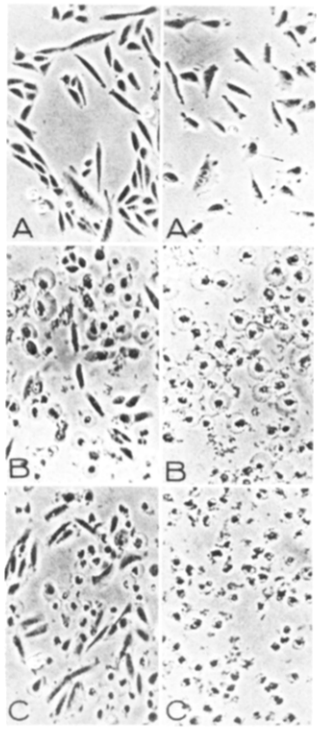

FIGURE 3: Morphology of wild-type (left panels) and 25-RA (right panels) cells before and after amphotericin B treatment. Cells were grown and treated with amphotericin B at 25 μg/mL for 2 h as described in Figure 2. Random sections of the monolayers were chosen and photographed by a Polaroid camera after 100-fold magnification with a Nikon phase-contrast microscope. (A) Immediately before treatment; (B) immediately after treatment; (C) incubated in FCS–M at 37 °C for 3 h after treatment.

much more resistant to killing by amphotericin B than the 25-RA cells. They could be classified into two main types. The first type (18 clones) of cells grew well in DeL–M without any lipid supplement; they were all shown to be sensitive to 25-hydroxycholesterol at 1 μg/mL. As shown in Figure 5A, the three representative clones (Am-R2, Am-R7, and Am-R13) grown in DeL–M exhibited cell division rates similar to those of wild-type and 25-RA cells. In DeL–M + 1 μg/mL 25-hydroxycholesterol, they were shown to be sensitive to 25-hydroxycholesterol cytotoxicity (Figure 5B). Unlike 25-RA cells which grew to cell confluency, when wild-type cells and amphotericin B resistant clones were incubated with 1 μg/mL 25-hydroxycholesterol for 4 days, the protein contents per flask of these cultures became less than 10% of those found in the zero-time cultures (data not shown). This experiment shows that they are revertants of 25-RA cells. The morphology of a representative clone of revertants (Am-R2) before and after the amphotericin B treatment is shown in Figure 4 (bottom panel). The apparent spontaneous reversion frequency is thus $18/(40 \times 10^6) = 0.45 \times 10^{-6}$. If one takes into account the fact that the survival rate of wild-type cells after amphotericin B treatment (2 h at 25 μg/mL) is approximately 30% (Figure 2), and that the plating efficiency of CHO cells in FCS–M is approximately 50%, the corrected spontaneous reversion frequency is 3×10^{-6}. The second type (nine clones) of cells grew in DeL–M for only 1 day before cell division stopped. These cells were found to be auxotrophs requiring cholesterol and unsaturated fatty acids for growth;[2] i.e., the phenotypic abnormalities of these cells resemble those of the CHO cell mutant 1 cells (Limanek et al., 1978; Chin & Chang, 1981, 1982). In a result not shown, we were able to isolate spontaneous revertants from 25-RB cells by the same procedure described above.

[2] Result to be published.

Table I: Relative Various Cholesterogenic Enzyme Activities of Cells Grown in FCS–M[a]

cell clone	relative cytosolic aceto-acetyl-CoA thiolase activity	relative HMG-CoA synthase activity	relative HMG-CoA reductase activity	relative mevalonate kinase activity
wild type	1.0 (50)	1.0 (0.53)	1.0 (0.064)	1.0 (1.15)
25-RA	3.8	15.0	2.5	2.2
Am-R2	1.0	1.4	0.8	1.1
Am-R7	0.9	2.3	1.0	1.2
Am-R13	1.1	1.3	1.0	1.0

[a] Cells were plated at 0.03×10^6/25-cm² flask and grown in 3 mL of FCS–M for 5 days, with a 3 mL/flask or a 5 mL/flask medium replacement at the end of the third or fourth day, respectively. Afterward, cells were harvested for various indicated enzyme activity determinations as described under Experimental Procedures. Values shown represent the mean of results from duplicate flasks in a single experiment. Values shown in parentheses represent the mean of the enzyme specific activity found in duplicate flasks of wild-type cells, with a variation of less than 7% from the mean in each case. Enzyme activities were expressed as $nmol \cdot min^{-1} \cdot mg^{-1}$.

Table II: Cellular Sterol Content of Various CHO Cell Clones[a]

	cellular sterol content (μg of sterol/mg of protein)			
	free[b]		esterified	
cell clone	FCS–M	DeL–M	FCS–M	DeL–M
wild type	19.2	11.1	8.3	ND[c]
25-RA	28.4	19.0	41.2	8.9
Am-R2	18.6	9.1	7.3	ND
Am-R7	21.9	10.0	8.2	ND
Am-R13	19.5	9.6	4.8	ND

[a] Cells were plated at 0.03×10^6/3 mL of FCS–M for 3 days. Afterward, certain flasks were harvested for cellular sterol content determination at zero time. The remaining flasks were rinsed once with 5 mL of PBS, fed with 3 mL of DeL–M/flask, and grown for 24 h before being harvested for cellular sterol content determinations. Cellular sterol contents were determined as described under Experimental Procedures. Values shown represent the average of results from duplicate flasks. Deviation of the duplicate was within 10% of the mean in each case. [b] For all five clones, cells grown in FCS–M contained cholesterol as the only sterol found. For cells grown in DeL–M for 1 day, cholesterol constituted approximately 90–95% of the total sterol; the rest was found to be lanosterol (0–5%) and a sterol (5–8%) with a retention time very similar to that of desmosterol on an OV-17 column; the identity of this sterol was not pursued further. [c] The sensitivity of the method employed would have detected the sterol at 0.1 μg/mg of protein. ND, not detectable.

It was previously shown (Chang & Limanek, 1980) that 25-RA cells grown in FCS–M had elevated levels of activities of the first four cholesterogenic enzymes as compared with those found in the wild-type cells. We now find that the activities of all of these enzymes reverted back to near-normal levels in three different revertant clones (Table I). The much elevated level of cellular cholesterol content in the 25-RA cells grown in FCS–M (Chang & Limanek, 1980) is now found to be mainly in the form of cholesterol ester (Table II), which formed droplets in intact cells large enough to be seen under a phase-contrast microscope with 100-fold magnification. When wild-type and 25-RA cells were switched to grow in DeL–M for 24 h, the rapid cell division of these cells (Chang

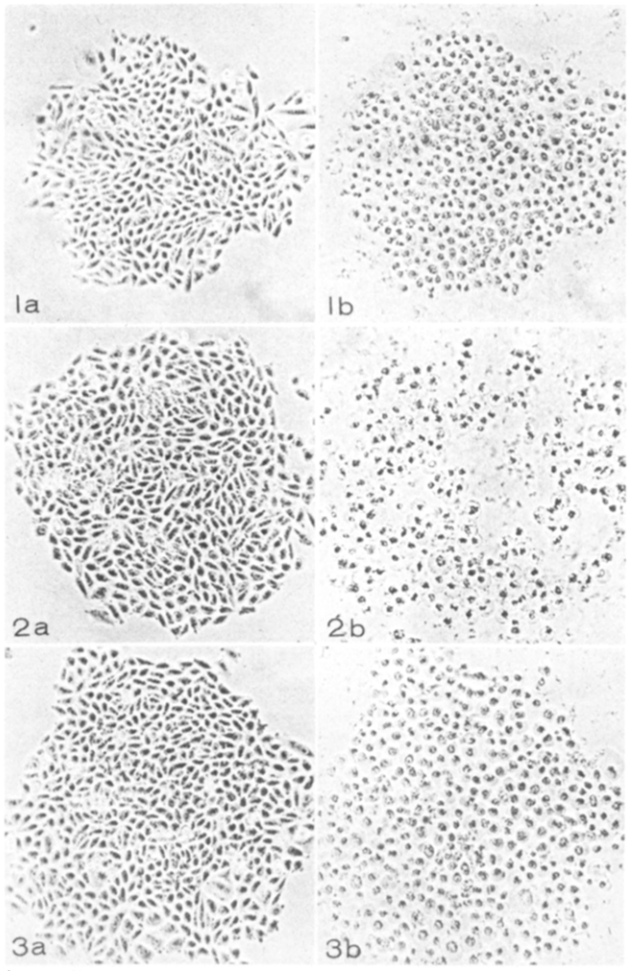

FIGURE 4: Morphologies of single colonies of wild-type (1a,b), 25-RA (2a,b), or Am-R2 (3a,b) cells before and after amphotericin B treatment. Colonies of cells were developed by plating approximately 200 cells in a 100-mm dish in 6 mL of FCS–M, incubated in a 5% CO_2 incubator at 37 °C without disruption for 5 days. Afterward, cells were rinsed with 5 mL of PBS/dish, switched to 6 mL of DeL–M/dish and grown for 18 h, and then treated with amophotericin B at 25 μg/mL for 2 h as described in Figure 2. A representative colony for each cell type was chosen and photographed by the method described in Figure 3 before (a) and immediately after (b) amphotericin B treatment.

Table III: Esterification of Cell-Bound 25-Hydroxy[^{3}H]cholesterol Incorporated into Cells Grown in DeL–M[a]

	% esterification of 25-hydroxy[^{3}H]cholesterol	
incorpn time (h)	wild type	25-RA
3	15.5	14.1
6	24.0	23.3
8	17.0	15.8

[a] Cells plated at 0.2 × 10^6/75-cm^2 flask in 10 mL of FCS–M were grown for 63 h, rinsed once with 10 mL of PBS, and switched to grow in 10 mL of DeL–M for 46 h. Medium was replaced once at the 32nd hour. Afterward, the medium was replaced with 8 mL/flask of DeL–M containing 0.3 μg/mL 25-hydroxy[^{3}H]cholesterol (15.4 × 10^4 dpm) and 0.3% Me_2SO for the indicated periods of time at 37 °C. Afterward, cells were harvested for total ^{3}H incorporation, percent esterification of 25-hydroxy[^{3}H]cholesterol, and protein determinations as previously described (Chang et al., 1981a). The total amount of ^{3}H incorporation into wild-type and 25-RA cells was found to be very similar at these three time periods, confirming our earlier finding described previously (Chang & Limanek, 1980). Values shown represent the mean between duplicate flasks. Variations between the duplicates were within 5% of the mean.

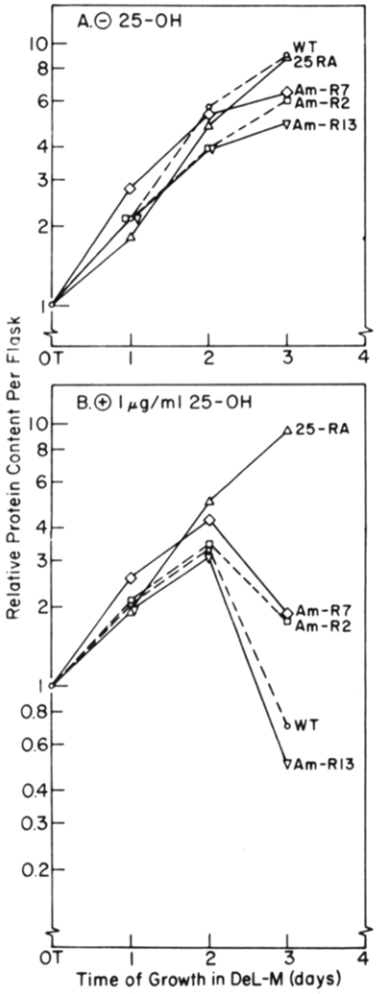

FIGURE 5: Growth of wild-type, 25-RA, or various revertant cells in DeL–M without (A) or with 1 μg/mL 25-hydroxycholesterol (B). Various cell clones were plated at 0.04 × 10^6/25-cm^2 flask and grown in 3 mL of FCS–M for approximately 3 days. Duplicate flasks were harvested for cellular protein content measurements at zero time. The remaining cell cultures were rinsed with 5 mL of PBS/flask and were grown in 3 mL of DeL–M containing 0.33% Me_2SO with or without 1 μg/mL 25-hydroxycholesterol. Medium was replaced once per day, with 4 mL/flask for the second-day cultures and 5 mL/flask for the third-day cultures. Growth was monitored by measuring the cellular protein content per flask as described previously (Chang & Limanek, 1980). Values from duplicate flasks were determined, with the average values reported. Deviations between duplicates were within 10% of the averages.

& Limanek, 1980) caused extensive dilution in the cellular-free cholesterol and cholesterol ester contents of these cells; however, mainly due to the large amount of cholesterol ester stored at zero time (grown in FCS–M), the 25-RA cells were able to maintain a certain level of cholesterol content such that these cells, unlike the wild-type cells, were still very susceptible to amphotericin B killing. It is found that (Table II) the free cholesterol and cholesterol ester contents of the three revertant cells grown in FCS–M and in DeL–M are very similar to those found in the wild-type cells, thus providing the basis for the enrichment procedure employed for isolating the revertants from 25-RA cells.

Previously, we found that when wild-type and 25-RA cells were switched to grow in DeL–M for approximately 2 days, significant activity increases of the first four cholesterogenic enzymes were observed; all of these increased enzyme activities were susceptible to suppression by 25-hydroxycholesterol (Chang & Limanek, 1980). We now find that in wild-type cells, the concentration of 25-hydroxycholesterol required to

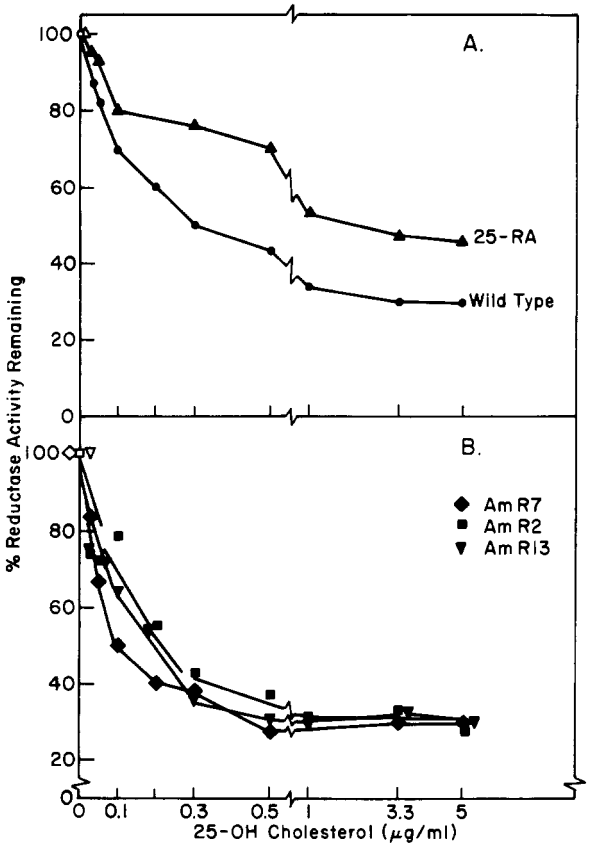

FIGURE 6: Suppression of HMG-CoA reductase activity in various cell clones by different concentrations of 25-hydroxycholesterol present in the medium. Various cell clones were plated at 0.03 × 10^6/25-cm^2 flask, grown in 3 mL of FCS–M for 63–66 h, rinsed with 5 mL of PBS/flask, and grown in 3 mL of DeL–M for 30 h; medium was then replaced, and cells were refed with 4 mL of fresh DeL–M/flask and grown for another 16 h. Afterward, the medium was replaced, and cells were treated with 6 mL/flask of DeL–M containing 0.33% Me_2SO and the indicated concentrations of 25-hydroxycholesterol and incubated at 37 °C in a 5% CO_2 incubator for 6 h. Afterward, cells were harvested to prepare cell extracts for HMG-CoA reductase specific activity determinations as previously described (Chang et al., 1981a,b). Duplicate cultures of wild-type and 25-RA cells were employed; reported values were averages between the duplicates; variations between duplicates were within 5% of the averages. Single cultures of various revertant cells were employed. The specific activities of HMG-CoA reductase in nontreated cell cultures were the following (in nmol·min^{-1}·mg^{-1}): wild type, 0.81; 25-RA, 0.53; Am-R2, 0.79; Am-R7, 0.66; Am-R13, 0.84. For each cell clone, the percent activity remaining was determined by dividing the specific activity of reductase found in the treated culture by that of the nontreated culture.

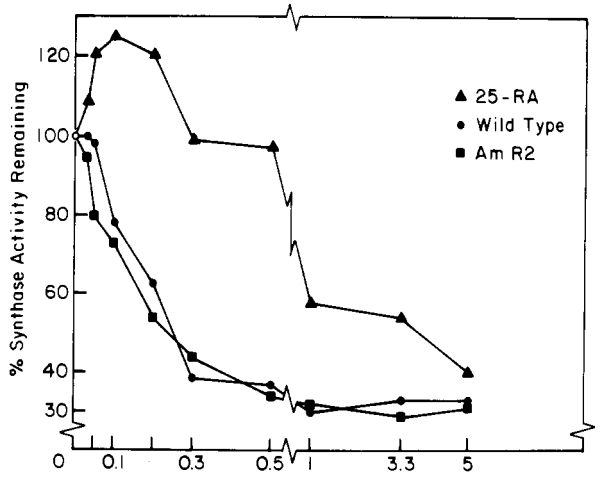

FIGURE 7: Suppression of HMG-CoA synthase activity in various cell clones by different concentrations of 25-hydroxycholesterol present in the medium. Various cell clones were plated and grown as described in Figure 6. Cells were then treated with 25-hydroxycholesterol at the indicated concentrations in 7 mL of DeL–M for 15 h at 37 °C in a 5% CO_2 incubator. Afterward, cells were harvested in 0.5 mL of digitonin buffer/flask and used for synthase specific activity determinations as described under Experimental Procedures. Duplicate cultures of wild-type and 25-RA cells were employed; reported values were averages between the duplicates; variations between duplicates were within 7% of the averages for synthase determinations. Single cultures of various revertant cells were employed. The specific activities of synthase in nontreated cultures were the following (in nmol·min^{-1}·mg^{-1}): wild type, 7.2; 25-RA, 20.2; Am-R2, 8.2. The suppression curves of synthase activities in Am-R7 and Am-R13 cultures yielded results very similar to those shown for Am-R2 cells and were deleted from the figure for simplicity.

be present in the medium to cause half-maximal suppression on the activities of HMG-CoA reductase (Figure 6), or HMG-CoA synthase (Figure 7), was approximately 0.1–0.2 μg/mL; in the 25-RA cells, the concentrations required for half-maximal suppression were shifted upward by (2.5 ± 0.5)-fold[3-5] for HMG-CoA reductase (Figure 6) and by 5- or 7-fold[6] for HMG-CoA synthase (Figure 7). In the revertant cells, these values were found to have reverted to near-normal (Figures 6 and 7). In three separate experiments, we found that the concentration of 25-hydroxycholesterol required to cause half-maximal suppression on the activity of acetoacetyl-CoA thiolase in the 25-RA cells was consistently higher than that in the wild-type cells and those in the revertant cells (data not shown); however, since the extent of suppression of thiolase activity by 25-hydroxycholesterol in each cell type was not very severe (in a typical experiment, after 15-h incubation with 5 μg/mL 25-hydroxycholesterol, the observed suppression in each cell type was found to be between 20 and 25%), it was not feasible to quantitate the difference between these cell types. A 25-hydroxy[^{3}H]cholesterol incorporation experiment revealed that at three different time points examined (3, 6, or 8 h), the percent esterification of cell-bound ^{3}H-labeled

[3] Result obtained from the average of four separate experiments; Figure 6 represents one of these experiments.

[4] It could be calculated from this figure that the absolute specific activities of HMG-CoA reductase in the presence of 1 μg/mL 25-hydroxycholesterol were approximately the same in the 25-RA cells and in the wild-type cells (0.28 nmol·min^{-1}·mg^{-1}), yet the 25-RA cells were not killed by 25-hydroxycholesterol (Figure 5B). At present, we cannot offer a satisfactory explanation to this observation. It may be because the experiment in Figure 6 was rather acute in nature (performed in 6 h), while the growth experiment (Figure 5B) was a chronic one (performed as a 3-day time course); thus, the direct comparison between the two experiments may not be valid.

[5] When the data points presented in Figure 6A were replotted as percent activity suppressed (percent activity suppressed being 1 – percent activity remaining) vs. concentration of 25-hydroxycholesterol present in the medium, two hyperbolic curves were obtained. Statistical analyses employing the computer program MINITAB (written by Thomas A. Ryan, Jr., Statistics Department, The Pennsylvania State University, 1981) showed that the double-reciprocal plots of these two curves conformed to two straight lines intercepting at the vertical axis with a correlation coefficient of 0.984. The analyses also showed that the percent maximal suppression of reductase activity was 77% for both cell types; the concentration of 25-hydroxycholesterol required to cause half-maximal suppression was 0.17 μg/mL for wild-type cells and 0.5 μg/mL for 25-RA cells; the latter two values differed from each other at a confidence level which exceeded 99.9%. The same analyses showed that the concentration for half-maximal suppression in three different revertants ranged from 0.08 to 0.14 μg/mL. Copies of the MINITAB computer program may be available on request.

[6] Result obtained from the average of two separate experiments; Figure 7 represents one of these experiments.

sterol was found to be very similar between wild-type and 25-RA cells (Table III), indicating that the differential suppression of various cholesterogenic enzyme activities by the sterol in these two cell types was not due to the difference in percent esterification of the cell-bound sterol.

Discussion

In this report, we demonstrate that the wild-type CHO cells grown in DeL–M for approximately 1 day become partially resistant to killing by amphotericin B, a specific polyene antibiotic known to interact with plasma membrane bound cholesterol in intact cells. A very large difference in the extent of cell survival between the treated wild-type cells and the 25-hydroxycholesterol-resistant mutants grown in DeL–M was found (Figures 1 and 2), probably because the DeL–M-grown wild-type cells contain less free cholesterol in the plasma membrane than those of the resistant mutant cells. It is not exactly clear why a difference of approximately a factor of 2 in the cellular free cholesterol contents between these cells (Table II) would form such a large difference in susceptibility toward amphotericin B killing. It may be that a given threshold of cholesterol content is needed to be present in the cell membrane to cause the amphotericin B to be cytotoxic. By using this highly selective killing procedure, we have isolated revertants of 25-RA cells via spontaneous mutation, at a frequency of approximately 3×10^{-6}, a frequency comparable to or greater than other single gene mutations of these cells (Chasin et al., 1974; Patterson & Carnright, 1977; Davidson & Patterson, 1979). Biochemical analyses of three of these revertants show that all defects manifested in 25-RA cells (Chang & Limanek, 1980) have reverted back in parallel, a result suggesting that these observed defects in 25-RA cells are due to a single gene mutation, thus supporting the hypothesis (Chang & Limanek, 1980) that a common controlling factor may be involved in mediating the suppressive action(s) of a specific analogue of cholesterol on various cholesterogenic enzyme activities. The function of this common controlling factor is rendered abnormal in 25-RA cells by mutation. Previously, biochemical characterization of a different CHO cell mutant (mutant 1; Limanek et al., 1978; Chin & Chang, 1981, 1982) also suggests that a common regulatory mechanism may exist in controlling the activity increases of various cholesterogenic enzymes, as well as LDL-binding activity. We have not examined whether the LDL-binding activity in 25-RA cells grown in FCS–M is elevated or not; if it is so, it would explain why 25-RA cells grown in FCS–M would accumulate so much more cholesterol ester than the wild-type cells (Table II). The unexpected finding was that, along with isolation of the revertants, we were able to isolate several clones which were shown to be cholesterol and unsaturated fatty acid requiring auxotrophs. Partial characterization[2] of these mutants showed that they possessed a defect in the induction of various cholesterogenic enzymes upon removal of serum lipids from the growth medium; i.e., these mutants seem to possess the same phenotypic abnormalities as those found in mutant 1 cells (Chin & Chang, 1981, 1982). The relation between the altered genetic locus in these mutants and the one in 25-RA cells awaits further biochemical and genetic analyses.

In addition, we found in wild-type cells the concentration of 25-hydroxycholesterol required to be present in the medium to cause half-maximal suppression on HMG-CoA reductase and HMG-CoA synthase activities to be approximately 0.1–0.2 μg/mL; in the 25-RA cells, the concentrations for half-maximal suppression were found to be shifted upward (Figures 6 and 7). The magnitudes of the upward shifts were found to be different for each individual enzyme, which may simply reflect the limitation of using these results in trying to probe the rate-limiting step quantitatively, since these experiments were performed in intact cells. For each enzyme, there may be several steps involved which constitute the overall process of enzyme suppression by the incoming sterol. Nevertheless, these data do suggest that a near-equilibrium process or a steady-state process involving association of the specific cholesterol analogue (or its derivative) and the putative common controlling factor may constitute part of the rate-limiting step leading to the overall processes of suppression on various cholesterogenic enzymes. This information should be useful for future studies in attempting to isolate the putative common controlling factor and study its molecular properties in vitro.

Acknowledgments

We thank Dr. Raymond K. Neff, director of academic computing at Kiewit Computation Center of Dartmouth College, for generous help in statistical analysis using the MINITAB computer program and Mildred Farnham for her help in preparing this paper.

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Synthesis and Properties of a Nonexchangeable Radioiodinated Phospholipid†

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ABSTRACT: An efficient method for the synthesis and purification of *N*-[3-(3-[^{125}I]iodo-4-hydroxybenzyl)propionyl]-phosphatidylethanolamine (^{125}I-phenylpropionyl-PE), a nonexchangeable iodinated lipid of high specific radioactivity, is described. The technique involves acylation of phosphatidylethanolamine with *N*-succinimidyl 3-(3-[^{125}I]iodo-4-hydroxyphenyl)propionate (monoiodinated Bolton–Hunter reagent) and purification by thin-layer chromatography. Quantitative incorporation of the iodinated lipid into vesicles prepared by a variety of techniques was observed to occur. With these vesicles, no transfer of the labeled lipid to other vesicles or cells occurred, irrespective of vesicle composition or the presence of other transferable lipids in the same bilayer membranes. This, ^{125}I-phenylpropionyl-PE appears to be an accurate liposome tracer and can be used in a variety of in vitro and in vivo assays that require high levels of sensitivity unobtainable with most other lipid-labeling techniques.

It is generally accepted that lipid vesicles can interact with cells by various mechanisms, notably by adsorption to cell surfaces, by fusion, and/or by lipid transfer. Although the definition of these various events is straightforward, experimental evidence that unambiguously demonstrates these phenomena is difficult to obtain (Pagano & Weinstein, 1978; Poste, 1980; Pagano et al., 1981b).

Most of the available information on the mechanism involved in vesicle–cell interactions has come from experiments in which vesicles are "labeled" with aqueous space markers or with radiolabeled and/or fluorescent phospholipids. Encapsulated markers used for this purpose include compounds such as sugars (Batzri & Korn, 1975; Huang & Pagano, 1975; Poste & Papahadjopoulos, 1976), antibiotics (Gregoriadis & Neerunjun, 1975; Tyrrell et al., 1977), proteins (Magee et al., 1974; Weissmann et al., 1977), carboxyfluorescein (Weinstein et al., 1977), and technetium (Dunnick et al., 1976) or indium (Mauk & Gamble, 1979). Although these methods are straightforward, definitive interpretation of results is, for the most part, complicated since vesicles are invariably leaky, and the loss of entrapped material varies considerably with a multitude of diverse factors such as lipid and cell type, temperature, and medium employed. Similar problems are also encountered with certain radiolabeled and fluorescent phospholipid markers, especially when one considers that phospholipids can exchange with other vesicles and cell membranes (Pagano & Huang, 1975; Sandra & Pagano, 1979; Struck & Pagano, 1980; Pagano et al., 1981a,b).

A reliable radiolabeled nonexchangeable phospholipid marker could resolve some of these problems. In line with observations that the addition of a cyclical moiety to the primary amino group of phosphatidylethanolamine abrogates its exchange properties (Struck & Pagano, 1980), we describe here the synthesis, purification, and applications of an iodinated nonexchangeable phosphatidylethanolamine derivative, *N*-[3-(3-[^{125}I]iodo-4-hydroxybenzyl)propionyl]dipalmitoylphosphatidylethanolamine (^{125}I-phenylpropionyl-PE)[1] (Figure 1), of high specific activity.

Experimental Procedures

Materials and Routine Procedures. DOPC, DOPE, DPPC, DPPE, *N*-NBD-PE, and C_6-NBD-PC were purchased from Avanti Biochemicals (Birmingham, AL). *N*-Rh-PE was prepared by reacting lissamine rhodamine B sulfonyl chloride (Molecular Probes, Plano, TX) with DPPE as previously described (Struck et al., 1981). ^{125}I-BHR (sp act. ~2000 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Phospholipase A_2 (hog pancreas) was a product of Boehringer Mannheim and was used as described by Kates (1972). Radiation was measured with an Amersham/Searle Model 1185 automatic γ counter. Fluorescence was quantified with a Farrand MK II spectrophotofluorometer.

Cells. Monolayer cultures of the UV-radiation-induced UV-2237 fibrosarcoma syngeneic to C3H mice were grown to confluency in Eagle's minimum essential medium supplemented with L-glutamine and 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cell sus-

† From the Cancer Metastasis and Treatment Laboratory, NCI-Frederick Cancer Research Facility, Frederick, Maryland 21701. *Received April 28, 1982.* This work was sponsored by the National Cancer Institute, Department of Health and Human Services, under Contract NO1-CO-75380 with Litton Bionetics, Inc. The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commerical products, or organizations imply endorsement by the U.S. Government.

[1] Abbreviations: ^{125}I-phenylpropionyl-PE, *N*-[3-(3-[^{125}I]iodo-4-hydroxybenzyl)propionyl]dipalmitoylphosphatidylethanolamine; ^{125}I-BHR, *N*-succinimidyl 3-(3-[^{125}I]iodo-4-hydroxyphenyl)propionate, monoiodinated Bolton–Hunter reagent; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; C_6-NBD-PC, 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)-amino]caproyl]phosphatidylcholine; *N*-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine; PBS, Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, pH 7.2; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles.

0006-2960/82/0421-5323$01.25/0