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SENSITIVITY TO AMPHOTERICIN B AND THE CHOLESTEROL: PHOSPHOLIPID MOLAR RATIOS OF 3T3, L, BHK AND HeLa CELLS*

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Abstract—The effects of amphotericin B (AmB) on four established animal cell lines were compared, measuring cell viability, inhibition of uridine incorporation into RNA, leakage of the enzyme lactate dehydrogenase, and leakage of RNA. In all assays, cell susceptibilities, in decreasing order, were: 3T3, L, BHK and HeLa. The cells, which were more sensitive, had significantly lower cholesterol:phospholipid ratios (c:p) than the less sensitive cells. Furthermore, 3T3 and L cell lines adapted to growth on higher levels of AmB had higher c:p molar ratios than did nonresistant cells. AmB binding to the cell was similar for all of the cell lines tested. These observations suggest a possible relationship between c:p ratios and sensitivity of cells to polyenes.

Polyene antibiotics interact with sterols present in the membranes of eukaryotic cells and increase cell membrane permeability [1, 2]. Considerable evidence indicates that the activity of these agents against cells depends on the type of sterol incorporated into the biomembranes. For example, the development of resistance in yeast to amphotericin B is accompanied by changes in the sterol composition of the cells [3, 4]. Also, fungi are more sensitive to amphotericin B (AmB) than are animal cells, and this is probably linked to the more avid binding of this polyene antibiotic to the ergosterol found in fungal cell membranes than to the cholesterol characteristic of animal cell membranes [5, 6].

Several reports, and a further number of unpublished trials in our own laboratory, have indicated that different animal cell lines containing the same sterol also differ extensively in sensitivity to AmB [7–9]. Here we report a study in which relative sensitivity of four cell lines to AmB was established by four different assays. The order of sensitivity of the cell lines tested, in decreasing order, was consistently 3T3, L, BHK and HeLa cells. An attempt was also made to look for the basis of the different sensitivities. We found that the sensitivity of the cells to AmB appeared to be correlated with the molar ratio of cholesterol:phospholipid in the cells.

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MATERIALS AND METHODS

Chemicals. Amphotericin B in the form of Fungizone was purchased from E. R. Squibb & Sons, Inc., Princeton, NJ. It was dissolved in sterile water before use. The concentrations of the AmB solutions were calculated on the basis of the amounts of AmB in the Fungizone, as stated by the manufacturer. Filipin, in the form of the unfractionated antibiotic complex which was 86 per cent pure, was donated by the Upjohn Co., Kalamazoo, MI. The amounts noted refer to the crude product. Miconazole was a gift from Janssen Pharmaceutical N.V. (Beerse/Belgium). Filipin and miconazole were dissolved in dimethylformamide before use.

[^{3}H]uridine (sp. act. 8 Ci/m-mole) was purchased from Schwarz-Mann, Orangeburg, NY.

Cell culture. HeLa, L 929 and Balb 3T3 cells were maintained in monolayer in Eagle's minimum essential medium supplemented with 0.2 mM of nonessential amino acids, 2 mM glutamine, 10% fetal calf serum, 100 units/ml of penicillin and 100 μg/ml of Kantrex (growth medium). The same medium was used for BHK cells except that the glutamine concentration was doubled and 6% fetal calf serum was used. All experiments were done with cells cultured in the growth medium. Treated cells were removed from monolayer cultures using glass beads or a rubber policeman.

Cell viability determined by three different assays. The viability of L cells after exposure to AmB

for 1 hr at 37° was determined by three different methods:

(1) Trypan blue exclusion [10]. Cells (4×10^5) in 3 ml of growth medium were plated in plastic dishes 35 mm in diameter and grown for 17 hr. The medium was removed and the cells were incubated with 1 ml of fresh medium containing AmB. The incubation was terminated by aspiration of medium. The cells were rinsed twice with 2 ml of fresh medium, incubated for 15 min at 37° with 0.1% trypan blue, removed from monolayers with a rubber policeman and counted in a hemocytometer.

(2) Enzymatic hydrolysis of diacetylfluorescein [11]. This assay is based on hydrolysis of diacetylfluorescein by living cells and an intracellular accumulation of fluorescein which could be visualized under a microscope. Glass slides were placed in plastic dishes 35 mm in diameter and the cells were seeded, grown and exposed to AmB as in the trypan blue assay. After the AmB-containing medium was aspirated, cells were rinsed two times with 3-ml aliquots of phosphate-buffered saline (PBS) and incubated for 5 min at 37° with 1 ml PBS containing 8 μg/ml of diacetylfluorescein. The slides were then removed and rinsed with PBS and the number of fluoresced cells was counted under an inverted fluorescence microscope, Nikon model M.

(3) Growth of clones [12]. Cells were freed from monolayer by 0.25% trypsin, centrifuged for 4 min at 200 *g*, the trypsin was removed, and the cells were dispensed in growth medium at 5×10^5 cells/ml. Aliquots (1 ml) were then incubated with AmB for 1 hr and then diluted with medium to 20 cells/ml. Aliquots (5 ml) were plated on plastic dishes 60 mm in diameter. After 8 days macroscopic colonies were stained with methylene blue and counted.

The sample containing the highest AmB concentration (40 μg/ml) only contained 1.6 μg/ml when diluted to a cell concentration of 20 cells/ml. AmB at this concentration did not affect the growth of clones.

Viability of different cell lines. Cell viability after exposure to AmB was examined by trypan blue exclusion [10]. Cells were assayed in suspension or in monolayer formed by 17 hr growth of 8×10^5 cells seeded in plastic dishes 35 mm in diameter.

Protein measurement. A modified Folin method was used [13], with bovine serum albumin as a standard.

Leakage of lactate dehydrogenase. Lactate dehydrogenase (LDH) (EC 1.1.1.27) was measured colorimetrically [14]. In the presence of NADH, LDH reduced added pyruvate acid to lactic acid; the remaining pyruvic acid was converted to the colored 2,4-dinitrophenylhydrazone. The absorption of the sample depended on remaining pyruvate and was inversely proportional to the amount of LDH released.

The cells (8×10^5) were seeded in 5 ml of growth medium in plastic flasks of 25 cm^2 area and grown for 17 hr. The medium was then removed, and the cells were rinsed three times with 3-ml portions of Hank's solution and incubated in 3 ml Hank's solution with 3.3 μg/ml of AmB at 37°. Samples (50 μl) taken at various time intervals were reacted with pyruvate reagent containing 150 nmoles pyruvate and with hydrazine reagent. The developed color was read at 490 nm on a Coleman Junior II spectrophotometer and compared with a standard curve for pyruvate concentration to determine nmoles of pyruvate reduced.

[^{3}H]*Uridine incorporation.* Incorporation of radioactivity into trichloroacetic acid-insoluble material was measured by a slight modification of a previously described method [15]. Cells (2.5×10^5 cells/plastic dish, 35 mm in diameter) were incubated in 3 ml medium for 18 hr and then re-fed with 3 ml of fresh growth medium containing [^{3}H]uridine (0.04 μCi/ml) and the drugs being studied. Eighteen hr later the medium was removed. Cells were washed three times with 2-ml aliquots of Hank's solution, then removed from the monolayer and put into 5% trichloroacetic acid, filtered onto a glass fiber filter and counted in a liquid scintillation counter.

Leakage of trichloroacetic acid-insoluble material (RNA) from cells. The leakage of prelabeled RNA was determined by comparing the amounts of RNA remaining in AmB-treated cells with amounts in untreated control cells. Cells in monolayer (4×10^5 cells/dish, 35 mm in diameter) were first incubated in 3 ml of growth medium with radioactive uridine (0.3 μCi). After 18 hr the medium was removed, the cells were rinsed two times with 2-ml aliquots of Hank's solution, and 1 ml of fresh growth medium with graded AmB concentrations was added. After 1 hr of incubation at 37° the medium was aspirated, the cells were washed three times with 2-ml aliquots of Hank's solution, and the radioactivity remaining in the cells was measured.

Adaptation of cells to AmB. 3T3 (3×10^6 cells in 15 ml medium) and L cells (2×10^6 cells 15 ml of medium) were seeded in plastic flasks 75 cm^2 in area. After 3 hr on monolayer, AmB was added in the following final concentrations: 2 μg/ml (3T3-R), 6 μg/ml (L-R) and 12 μg/ml (L-R_1). The cells were re-fed every day with fresh medium containing the same concentrations of AmB. 3T3-R cells approached confluency (5.8×10^6 cells/flask) on day 3 and were harvested at this time; L-R cells approached confluency on day 4 (6.0×10^6 cells/flask) and were harvested. The L-R_1 cells were also harvested on day 4 although they were not confluent (3×10^6 cells/flask).

The harvested 3T3-R, L-R and L-R_1 cells were replated in plastic flasks at lower density (0.5×10^6 to 1×10^6 cells/flask) and grown in the presence of AmB concentrations previously noted for another 4–5 days, after which they were harvested and seeded on 35-mm-diameter plastic dishes in 3 ml of growth medium containing AmB. Adaptation of the cells to AmB was measured by determining the amount of radioactive uridine incorporated into RNA in the presence of increasing concentrations of AmB. The cholesterol:phospholipid content of these cells was also measured at this time.

AmB binding. AmB binding to cells was measured fluorometrically, essentially as reported previously for strong binding to yeast cells [16]. Cell suspensions (1 ml) were incubated with AmB in the growth medium, diluted with 10 ml medium and centrifuged for 4 min at 200 *g*. The pellets were rinsed twice with 5-ml aliquots of growth medium, and extracted at 60–65° with two consecutive aliquots of 1 ml of 50% isopropanol (pH adjusted to 10.5 by NaOH). Ad-

ditional rinsing with growth medium or additional extraction with isopropanol did not change the results. The values from mock-assay samples (assay tubes without cells) were subtracted from the results of an actual assay.

Extraction and analysis of lipids. The cells were grown in monolayers almost to confluency in plastic flasks of 75 cm^2 area. The medium was then removed, and the cells were washed three times with 10 ml of phosphate-buffered saline, removed from the monolayer with a rubber policeman, counted in a hemocytometer, and extracted with chloroform–methanol (2:1) at room temperature [17].

The samples were divided and portions of the extract for lipid phosphorus measurements were washed by the method of Folch *et al.* [18] with 0.1 N KCl in the upper phase. Lipid phosphorus was determined in the Biochemical Laboratory of the Jewish Hospital of St. Louis. After the lipid extract was subjected to hot acid digestion, an automated colorimetric molybdic acid–stannous chloride procedure for phosphate was used [19].

For cholesterol and cholesterol ester measurements, portions of the chloroform–methanol lipid extract were concentrated under a flow of nitrogen, applied to Silica gel G thin-layer chromatography plates and developed in a mixture of petroleum ether ethyl ether (7:3). Zones corresponding to free and esterified cholesterol were separated and extracted several times with ethyl acetate. Esterified cholesterol was saponified with 15% ethanolic KOH, extracted with ether and finally subjected to thin-layer chromatography to recover all of the cholesterol by appropriate solvents. Samples of cholesterol were assayed by gas–liquid chromatography and quantitatively compared with a standard cholesterol peak. This procedure enabled us to detect cholesterol or cholesterol ester in amounts as low as 0.1 μg. Duplicates were reproducible to ±1 per cent.

Control experiments. Because Fungizone contains 41% sodium deoxycholate by weight, all experiments designed to investigate AmB effects on cells were repeated with appropriate control amounts of deoxycholate. The control experiments for filipin and miconazole effects were run with dimethylformamide at a concentration equal to that used in experiments with drugs (0.8% or less). We found that deoxycholate and dimethylformamide at these levels produced no detectable effects on cells.

RESULTS

Cell viability studied by different assays. After L cells were exposed to AmB, the number of cells remaining viable was determined by trypan blue exclusion and by enzymatic hydrolysis of diacetylfluorescein. Using the viable cell number after incubation without AmB as the 100 per cent value, the per cent of L cells remaining viable after incubation with 10, 20 or 40 μg/ml of AmB was, respectively, 87, 69 and 49 when assayed by trypan blue exclusion, and 96, 67 and 46 by diacetylfluorescein technique.

AmB also decreased the ability of L cells to form macroscopic colonies on plastic dishes (cloning assay). AmB (10 μg/ml) decreased the number of macroscopic colonies to 67 per cent of control; 40 μg/ml of AmB decreased the number to 58 per cent of controls. The cloning efficiency of controls was 50 per cent.

The similarity of our results on cell viability obtained with trypan blue and with diacetylfluorescein suggests that both measured a similar kind of cell damage. The cloning assay measured the ability of cells to multiply in culture after AmB exposure, and its results were similar to those of trypan blue and diacetylfluorescein. Therefore, in this case, similar concentrations of AmB affected cell viability and ability to multiply in culture.

AmB sensitivity of four cell lines. The effects of AmB on cell viability, RNA synthesis and leakage of lactate dehydrogenase were compared for each of four cell lines. Each assay was done under the same conditions for all the cell types. Cell viability, measured by trypan blue exclusion, was estimated as the per cent of cells remaining viable after incubation with different AmB concentrations in suspension (Fig. 1A) or in monolayer (Fig. 1B). The relative cell susceptibility was in decreasing order: 3T3, L, BHK and HeLa.

Cells incubated with AmB became fragile, and subsequent centrifugation or detachment from monolayer by rubber policeman disrupted cells with the number depending on the extent of previous damage. For example, when 5×10^6 cells/ml were incubated in 100 μg/ml of AmB for 30 min, no pellet was formed. After centrifugation of 7×10^6 cells/ml under the same conditions, the number of cells in the pellet was 70 per cent of the cell number in the control pellet.

The same order of cell sensitivity was found when inhibition of radioactive uridine incorporation into the RNA of actively growing cells was measured (Fig. 2), though RNA synthesis was affected at lower concentrations of AmB than was cell viability. This difference could be caused by experimental differences in the assays such as lower cell number and different incubation times (see Materials and Methods), or it

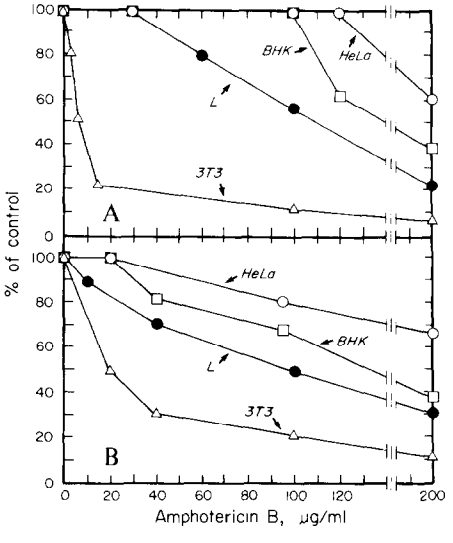

Fig. 1. Toxicity of amphotericin B against (○) HeLa, (□) BHK, (●) L and (△) 3T3 cell lines. (A) Per cent of cells remaining viable after incubation of 3×10^6 cells/ml in suspension with amphotericin B for 15 min at 37°. (B) Per cent of cells remaining viable after incubation of cell monolayer with amphotericin B for 20 min at 37°.

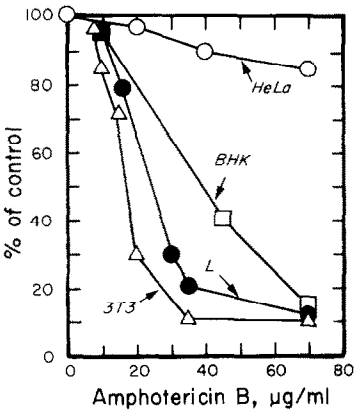

Fig. 2. Effect of different amphotericin B concentrations on [^{3}H]uridine incorporation into RNA of (○) HeLa, (□) BHK, (●) L and (△) 3T3 cells.

is possible that the AmB-induced effects measured occurred with different thresholds. Experiments designed to resolve this question are now in progress.

When cells were incubated in Hank's solution they released a variable amount of LDH into the medium. No pyruvate was released by the cells. The amount of enzyme released could be measured by determining the ability of the medium to reduce added pyruvate. Standardized aliquots of medium from HeLa monolayers reduced 70 or 80 nmoles pyruvate after 1.5 or 2 hr of incubation. For BHK, L and 3T3 cells, the amounts of pyruvate reduced were 10 nmoles after 1.5 hr and 14 nmoles after 2 hr. When the cells were incubated with 3.3 μg/ml of AmB, the nmoles of pyruvate reduced by the medium from each cell type increased to 87 (HeLa), 40 (BHK), 55 (L) and 120 (3T3) after 1.5 hr of incubation and 90 (HeLa), 50 (BHK), 72 (L) and 148 (3T3) after 2 hr of incubation. The difference in the amount of pyruvate reduced after incubation in the presence or in the absence of AmB increased for each cell type in the same order as was observed with other assays (3T3 > L > BHK > HeLa).

We also measured the amount of pyruvate reduced by equal numbers (1×10^5) of each cell type after complete lysis (caused by incubation of cells in Hank's solution with 30 μg/ml of filipin for 30 min). The lysed HeLa, BHK, L and 3T3 cells reduced 2.0, 1.2, 0.9 and 1.2 nmoles pyruvate respectively. When the data on pyruvate reduction after AmB exposure were adjusted by correcting them for these different values, the order of sensitivity did not change.

In order to estimate AmB-induced leakage of RNA from cells preincubated with radioactive uridine, we compared the amounts of acid-insoluble radioactivity remaining in untreated and AmB-treated cells. L and HeLa cells incubated with 60 μg/ml of AmB retained 55 and 87 per cent of radioactivity remaining in untreated cells. Therefore, in this assay the order of sensitivity of the cells to AmB again appeared to be the same.

Effects of another polyene, filipin, and of miconazole on the different cell types. The AmB effects on HeLa and L cells were further compared with the effects of two other membrane-active agents: filipin, another polyene antibiotic, and miconazole, an imidazole derivative. In conditions identical to those in the corresponding assays with AmB, filipin was toxic for both cell lines at lower concentrations than AmB, but L cells were again more sensitive than were HeLa cells. When miconazole was tested, the results were different. For example, HeLa cells were more sensitive to miconazole than L cells, and miconazole was a more potent inhibitor of RNA synthesis in HeLa cells than was AmB (data not shown).

Table 1. Binding of amphotericin B to cells*

	Amphotericin B bound (μg/ml)	
	Expt. A	Expt. B
HeLa	1.7	2.6
BHK	1.6	2.0
L	1.8	2.4

* Cells (3×10^6/ml) were incubated in suspension at 37 for 15 min with two concentrations of AmB in the growth medium: Expt. A, 40 μg/ml; and Expt. B, 80 μg/ml.

Binding of AmB to cells. 3T3 cells were lysed at an AmB concentration that was lower than the limit of our binding assay, and therefore binding could not be measured. The AmB binding to L, BHK and HeLa cells, however, could be assayed under conditions which did not cause cell lysis. The results presented in Table 1 show that the binding of AmB at two different concentrations in the growth medium was similar in all three cell lines.

Cells adapted to AmB. Cells grown in the presence of AmB for 10 days became relatively resistant to AmB as measured by [^{3}H]uridine incorporation into RNA (Fig. 3) and viability (data not shown). In addition to the increased resistance of these cells to AmB, several other changes were apparent. The L cells adapted to growth with the low AmB concentration

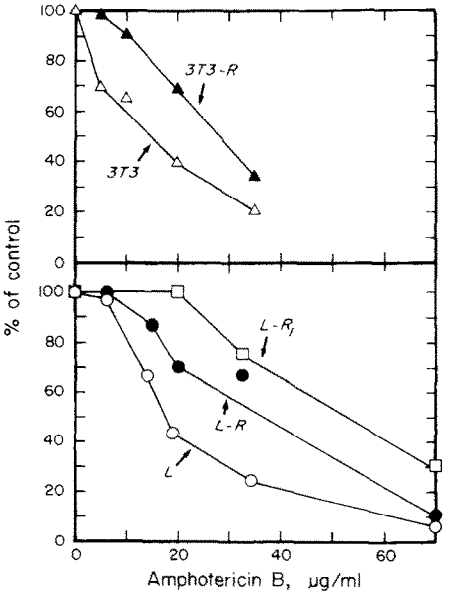

Fig. 3. Effect of different amphotericin B concentrations on [^{3}H]uridine incorporation into RNA of (△) 3T3, (▲) 3T3-R cells, (○) L, (●) L-R and (□) L-R$_1$ cells.

Table 2. Cholesterol, phospholipid (as P) and cholesterol ester content of whole cell extracts

Cell line	Proteins (mg/1 × 10^7 cells)	Replicate experiments	Cholesterol (μg/1 × 10^7 cells ± S. E.)	Lipid phosphorus (μg/1 × 10^7 cells ± S. E.)	Ratio ± S. E.* cholesterol lipid (moles/moles)	Cholesterol ester (μg/1 × 10^7 cells)
HeLa	1.1	6	23.6 ± 1.7	6.3 ± 0.7	0.30 ± 0.02	12.3 ± 5.3
BHK	1.2	4	25.3 ± 6.0	6.0 ± 2.0	0.30 ± 0.02	4.6 ± 1.8
L	2.0	5	18.6 ± 2.5	9.2 ± 0.8	0.16 ± 0.004	0.3 ± 0.10
L-R	1.2	3	15.4 ± 0.9	5.6 ± 1.3	0.24 ± 0.015	2.3 ± 0.66
L-R_1	1.1	3	10.0 ± 1.9	2.5 ± 0.1	0.32 ± 0.04	7.9 ± 1.6
3T3	1.7	7	32.0 ± 1.8	13.0 ± 2.0	0.20 ± 0.017	1.6 ± 0.5
3T3-R	1.3	1	20.4	6.48	0.25	6.3

* S.E. was calculated from paired ratio values.

developed long processes and intracellular vacuoles not seen in normal L cells. The resistant cells also decreased in size, as estimated morphologically by light microscopy and by comparison of protein, cholesterol and phospholipid content.

The resistance of these cells was reversible, for when L-R and L-R_1 cells were transferred to AmB free growth medium, AmB sensitivity returned to the level of control cells after 4 days.

Cellular lipid content. The content of cholesterol, cholesterol ester and phospholipid (as P) in whole cell extracts was determined to see if there was a correlation between the AmB sensitivity of the various cell types and the cellular lipid content.

Table 2 presents the levels of cholesterol, cholesterol ester and phospholipid and the calculated cholesterol:phospholipid molar ratio of the four investigated cell lines and of AmB-resistant cells. We found that HeLa and BHK, the two cell lines relatively resistant to AmB, had a higher cholesterol:phospholipid molar ratio (0.30) than L and 3T3, the two cell lines relatively sensitive (molar ratios 0.16 and 0.20).

The AmB-resistant L and 3T3 cells had an increased ratio compared to control cells, and the extent of the increase correlated with the degree of resistance (see Table 2, L, L-R and L-R_1).

Table 2 also shows the content of cholesterol ester in whole cell extracts of the cells. The cholesterol ester level correlated positively with the cholesterol:phospholipid molar ratio and with cell resistance to AmB. The net loss from the cell of the cholesterol was nearly matched by a gain in cholesterol ester.

DISCUSSION

The four cell lines examined differed significantly in their susceptibility to AmB. In all of the assays, the relative cell sensitivity decreased in this order: 3T3, L, BHK and HeLa cells. These results confirm previous observations of the different AmB sensitivities of cultured cell lines, and the parallel actions of polyene antibiotics on cells as reflected by changes in membrane permeability to the enzyme lactate dehydrogenase, inhibition of nucleoside incorporation into nucleic acids, and the lethal and inhibitory actions of the drugs [15, 20].

The relative sensitivity of the cells was the same for filipin, the other polyene antibiotic studied. Also, as expected, the effect of filipin was always more pronounced than AmB activity. The same order of sensitivity was not observed, however, for another type of membrane-active agent. In two assays, inhibition of uridine incorporation and drug-increased leakage of RNA, miconazole [21, 22] was more potent against HeLa than against L cells. Its inhibition of uridine incorporation into HeLa cells surpassed AmB toxicity, whereas it was less active than AmB against L cells. Another membrane-active agent, croton oil factor TPA, has also been described [23] as a more potent inhibitor of [^{3}H]thymidine incorporation into HeLa than L cells. Therefore, the order of cell sensitivity to AmB and filipin appeared to reflect a characteristic response of the cells to polyene antibiotics.

Neither the cellular content of free cholesterol nor the amount of AmB bound to cells appeared to correlate directly with the sensitivity of the cells to the polyene antibiotics. AmB-resistant HeLa cells bound amounts of AmB similar to the more sensitive BHK and L cells. Similar results were obtained by Amati and Lago [24], who showed that transformed 3T3 cells, AmB resistant and AmB sensitive, bound equal amounts of radiolabeled AmB.

However, cells more sensitive to AmB effects (3T3 and L) had a lower cholesterol:phospholipid molar ratio than did the more resistant cell lines (HeLa and BHK). This suggests that, rather than sterol content itself, the sterol content in relation to phospholipid levels was critical. The hypothesized correlation between AmB sensitivity and cholesterol:phospholipid molar ratio was supported by the experiments with the AmB-adapted cells, which show that AmB resistance increased along with the cellular cholesterol:phospholipid molar ratio.

We cannot explain at present why the differences in sensitivity between 3T3 and L cells or BHK or HeLa cells were not correlated with differences in the cholesterol:phospholipid ratios. It may be that our analytical methods were not sensitive enough to detect small differences in lipid content, or that the cellular cholesterol:phospholipid ratios we measured do not reflect small differences in membrane ratios. The latter is unlikely because when our results on the determination of cellular cholesterol:phospholipid ratios are compared with the membrane cholesterol:phospholipid ratios determined by others [25-29], the same relative order is seen.

We think it is probable, as an alternative, that other factors also play a role in the cell sensitivity to AmB and may explain these discrepancies in our results. For example, Huschen and Feingold [30] have shown that the fatty acid composition of the phospholipids in cells was a determinant of sensitivity to AmB. Plasma or serum also influences cell sensitivity to polyene antibiotics [31, 32]. The extent of influence

in one study [31] depended not only on unesterified cholesterol content in plasma but also on the plasma phospholipid to cholesterol ratio, and various lots of commercial fetal bovine sera have shown a high degree of variability in content of cholesterol and other components [33]. Although each of our assays was conducted under the same conditions using the same serum lots for all cell types, we cannot completely exclude the possibility that some serum components affected AmB action differently for different cell types.

In addition to these variables, the nature of AmB binding, the transport rate of the polyene, and the biochemical characterization of the AmB effects all require further study, and prevent an unequivocal interpretation of the results. However, it is tempting to speculate that the correlation between AmB sensitivity of different cell lines and the cholesterol:phospholipid molar ratio is real, and reflects the relationship between cell sensitivity and the fluidity of the lipid in the surface membrane. Changes in the ratio of cholesterol to phospholipids affect the internal viscosity and molecular motion of lipids within membranes [34, 35] and this may somehow result in the differences in sensitivity of cells to AmB.

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