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Selective Membrane Toxicity of the Polyene Antibiotics: Studies on Natural Membranes

CHUEN-CHIN HSUCHEN AND DAVID S. FEINGOLD

Infectious Disease Unit, Beth Israel Hospital-Children's Hospital Medical Center, and Departments of Medicine, Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts 02215

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The effect of polyene antibiotics on *Candida albicans*, human erythrocytes, and *Acholeplasma laidlawii* was studied. The results sustain the observations made with lecithin-sterol liposomes. The distribution of double bonds in the membrane sterol nucleus appears to be of major importance in conferring polyene susceptibility; those sterols with the ergosterol nucleus are far more effective than those with a nucleus similar to cholesterol. Different polyenes vary in their membrane selectivity. The clinical implications of these observations are discussed.

The most widely held view of polyene action is that these antibiotics interact with the membranes of susceptible cells to alter essential membrane permeability characteristics, and the presence of sterol is a prerequisite for this interaction (5, 6). One line of evidence was derived from the study of *Acholeplasma laidlawii* (formally called *Mycoplasma laidlawii*). These organisms do not require sterol for growth, but are capable of incorporating into their cytoplasmic membranes sterols presented in the medium (1, 7). Cholesterol was shown to be required for conferring filipin (10) and amphotericin B (2) susceptibility on *A. laidlawii* strain A. More recently, by using freeze-etch electron microscopy techniques, it was shown that filipin induced the formation of aggregates in *A. laidlawii* B only after cultures were grown in the presence of cholesterol (9).

In this laboratory, we have shown that the incorporation of cholesterol at high molar ratios can suppress nystatin and amphotericin B susceptibility (3), that ergosterol, the major sterol in most yeasts, when compared with cholesterol, the major sterol in most mammalian cells, is more effective in conferring and does not suppress polyene sensitivity, and that the distribution of double bonds in the sterol nucleus can be related to the selective toxicity of clinically employed polyenes toward sterol-containing model membranes (4). These observations were made with lecithin liposome model membrane systems. In this report, supporting data are presented based on studies with *Candida albicans*, human erythrocytes, and a strain of *A. laidlawii* B cells.

MATERIALS AND METHODS

C. albicans, a clinical isolate, was identified by standard techniques. It was grown out with rotary shaking at 37 C in rich medium (1% tryptone, 0.5% yeast extract, 0.5% glucose, and 0.5% NaCl adjusted to pH 7.0 with 1 N NaOH) to approximately 5×10^7 colony-forming units per ml and diluted into 10 volumes of growth medium; 2 ml of this dilution was added to tubes containing 0.1 ml of the polyenes at various concentrations in dimethylformamide, and control tubes contained 0.1 ml of dimethylformamide. The organisms were incubated at room temperature for one h, after which the number of viable colony-forming units was determined by plating 0.1 ml of appropriate dilutions onto agar plates (1.5% agar in growth medium). Colonies were counted after overnight incubation at 37 C.

Freshly drawn human erythrocytes were washed three times in saline (0.15 M NaCl) at 4 C and diluted into 125 volumes of buffered saline (10 mM phosphate buffer, pH 6.8), and 2 ml of this suspension was added to tubes containing 0.1 ml of polyenes at various concentrations. After incubation at room temperature for 1 h, the samples were centrifuged, and the extent of hemolysis was determined by the absorbancy of the supernatant fluid at 540 nm. The blank control contained 0.1 ml of dimethylformamide, and the 100% control contained 10 μg of digitonin per ml. The 100% control gave an absorbancy of 1.0, whereas the corresponding value in the blank control was below 0.02.

Two strains of *A. laidlawii* were studied. Strain A was originally obtained from the American Type Culture Collection; strain B was generously supplied by Paul Smith. The organisms were grown at 37 C to about 10^8 colony-forming units per ml in lipid-depleted basic medium (8) supplemented with 0.5 μM oleic acid. They were diluted with 10 volumes of medium, and 2 ml of this dilution was added to test tubes containing 0.1 μmol of sterol. Dimethylformam-

ide was present in all samples (including the control) to a final concentration of 2.5% (vol/vol). After incubation at 37 C for the indicated times (1 or 4 h), antibiotic susceptibility was determined by diluting samples of inoculum into 50 volumes of basic medium (sterol-free, lipid-depleted), incubating them with the given polyene for 1 h at 37 C, and enumerating the survivors by plating appropriate dilutions onto agar plates (3.4% Difco PPLO agar, 0.5% glucose, 1.0% Difco PPLO serum fraction, 200 units of penicillin G per ml) after about 72 h of incubation at 37 C.

The sources of the polyenes and sterols are listed in the accompanying paper (4). Filipin complex was contributed by the Upjohn Company (Kalamazoo, Mich.).

RESULTS

The fungicidal and hemolytic potency of amphotericin B and nystatin were tested against *Candida albicans* and human erythrocytes, respectively (Table 1, Fig. 1); filipin complex was included for comparison. Nystatin at 25 μg/ml gave 2 logs of killing against the fungus, with practically no effect on human erythrocytes at concentrations up to 50 μg/ml. The filipin complex, in contrast, appeared to have more potent hemolytic than fungicidal activity.

The data presented in Table 2 compares the polyene sensitivity of the 2 strains of *A. laidlawii* without and with the incorporation of the sterols, cholesterol, or ergosterol. Strain B is more discriminating in its requirement for sterol incorporation; cholesterol does not confer nystatin susceptibility on this strain, whereas ergosterol does. Both sterols confer susceptibility to both antibiotics in strain A. Strain B was selected for further study of the quantitative aspects of the polyene sensitivity and the structural requirements of the sterol for sensitizing ability, because it does differentiate better between the polyenes and among the sterols.

TABLE 1. *Fungicidal effect of polyenes on Candida albicans*[a]

Polyene added	Concn (μg/ml)	CFU[b]/ml
None (control)		7.5×10^6
Amphotericin B	1.0	4.9×10^4
	2.5	1.5×10^4
	5.0	1.2×10^4
	10.0	4.0×10^3
Nystatin	2.5	7.7×10^6
	10.0	1.3×10^5
	25.0	9.1×10^4
	50.0	5.0×10^3
Filipin	2.5	6.3×10^6
	50.0	1.8×10^5

[a] *Candida* with the test polyenes were incubated at room temperature. After 1 h, the number of survival cells was determined by plate counting.

[b] Colony-forming units.

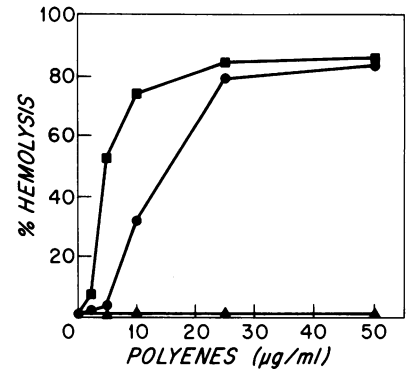

FIG. 1. *Effect of amphotericin B (■), nystatin (▲), and filipin (●) on hemolysis of human erythrocytes. Samples with the test polyenes were incubated at room temperature for 1 h and centrifuged, and the extent of hemolysis was determined by measuring the supernatant fluid absorbancy at 540 nm as compared with digitonin-lysed controls.*

TABLE 2. *Sensitivity of cholesterol- and ergosterol-grown A. laidlawii toward polyenes*[a]

Condition of preincubation	Polyene added	Concn (μg/ml)	Survivors (CFU[b]/ml)	
			Strain A	Strain B
Control[c]	Blank control[c]		7.4×10^6	3.8×10^6
	Amphotericin B	50.0	6.7×10^6	3.3×10^6
	Nystatin	125.0	5.9×10^6	3.3×10^6
Cholesterol	Blank control		6.2×10^6	3.0×10^6
	Amphotericin B	50.0	0×10^3	2.6×10^4
	Nystatin	125.0	4.6×10^4	3.1×10^6
Ergosterol	Blank control		6.1×10^6	3.2×10^6
	Amphotericin B	50.0	6.8×10^4	0×10^3
	Nystatin	125.0	0×10^3	0×10^3

[a] Cells were preincubated in the presence of sterol for 4 h. Strain A was obtained from the American Type Culture Collection (Rockville, Md.) Strain B was obtained from P. F. Smith of the University of South Dakota.

[b] Colony-forming units.

[c] Sample was incubated in the absence of sterol (or polyenes) with 2.5% dimethylformamide. (vol/vol).

The preincubation with ergosterol for 1 h led to about 90% killing by both amphotericin B (at 25 μg/ml) and nystatin (at 125 μg/ml) (Fig. 2). Further incubation (4 h) led to even greater sensitivity, over 5 logs of killing by amphotericin B and nystatin at concentrations of 25 μg/ml and 125 μg/ml, respectively. In comparison, 1 h of incubation with cholesterol gave no appreciable polyene response. Upon prolonged incubation (4 h), the cells remained insensitive to nystatin, but yielded slightly over one log of killing with amphotericin B at 50 μg/ml.

Table 3 shows the results of studies of the effects of various sterols on the polyene susceptibility of *A. laidlawii* B. Ergosterol differs structurally from cholesterol in that it has two more double bonds (Δ7, Δ22) and an additional 24β-methyl group. 5,7-Cholestadien-3β-ol, which has a nucleus structure identical to ergosterol and a C-17 side chain structure identical to cholesterol, was as effective as ergosterol in sensitizing the cells to nystatin and amphotericin B. On the other hand, stigmasterol, which has a nuclear structure identical to cholesterol and a C-17 side chain structure similar to ergosterol, had no sensitizing effect at all. Epicholesterol, with a 3α-hydroxyl group, and dihydrocholesterol (cholestanol), which contains no double bond in the sterol nucleus, were both ineffective. 4,6-Cholestadien-3β-ol had an impressive effect in conferring polyene susceptibility to this strain of *A. laidlawii* B.

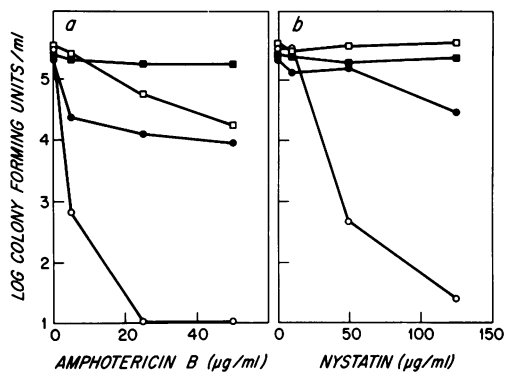

FIG. 2. *Susceptibility of A. laidlawii strain B to amphotericin B (a) and nystatin (b). Cells were preincubated in the presence of cholesterol* (■, □) *or ergosterol* (●, ○) *for 1* (■, ●) *and 4* (□, ○) *h.*

DISCUSSION

Studies of the polyene susceptibility of model membranes reported in the accompanying paper (4) have led to certain principles which we find confirmed, in general, with naturally occurring membranes. One would expect that the ergosterol-containing membranes of *C. albicans* would be susceptible to both amphotericin B and nystatin; this is indeed the case (Table 1). With the cholesterol-containing erythrocyte membranes, one might expect variable results, depending on the molar percentage of cholesterol, because at high concentrations this sterol can inhibit polyene action. Our data with model membranes suggest that nystatin action may be more strongly inhibited by cholesterol than by the action of amphotericin B. The results with the cholesterol-containing erythrocytes may reflect this. Erythrocyte hemolysis occurs with low concentrations of amphotericin B and not at all with 50 μg of nystatin per ml (Fig. 1).

Because the apolar aspects of the phospholipid clearly influence the polyene action on membranes, our studies with *A. laidlawii* strain B are particularly revealing in isolating the influence of the sterols per se. With these organisms, there is no killing by amphotericin B

TABLE 3. *Effect of sterols on the polyene susceptibility of A. laidlawii strain B*[a]

Condition of preincubation	Polyene added	Concn (μg/ml)	Cells were preincubated with sterol for	
			1 h (CFU/ml)	4 h (CFU/ml)
Control	Blank control		1.92×10^5	1.88×10^5
	Amphotericin B	50.0	1.86×10^5	1.22×10^5
	Nystatin	125.0	1.55×10^5	1.09×10^5
5,7-Cholestadien-3β-ol	Blank control		1.80×10^5	1.68×10^5
	Amphotericin B	50.0	2.50×10^3	0×10
	Nystatin	125.0	5.51×10^4	0×10
Stigmasterol	Blank control		1.90×10^5	1.96×10^5
	Amphotericin B	50.0	1.61×10^5	2.24×10^5
	Nystatin	125.0	1.38×10^5	1.86×10^5
Epicholesterol	Blank control		1.61×10^5	2.18×10^5
	Amphotericin B	50.0	1.83×10^5	1.38×10^5
	Nystatin	125.0	1.56×10^5	1.21×10^5
Dihydrocholesterol (cholestanol)	Blank control		1.68×10^5	1.98×10^5
	Amphotericin B	50.0	1.81×10^5	1.69×10^5
	Nystatin	125.0	1.69×10^5	2.16×10^5
4,6-Cholestadien-3β-ol	Blank control		1.75×10^5	1.28×10^5
	Amphotericin B	50.0	0×10	0×10
	Nystatin	125.0	2.01×10^4	0×10

[a] Refer to notes under Table 2.

or nystatin in the absence of sterol. Cholesterol sensitizes strain B only to amphotericin B; ergosterol has a much more potent sensitizing effect which applies to both polyenes. The other 3β-hydroxysterols containing two double bonds in the nucleus, 5,7-cholestadien-3β-ol and 4,6-cholestadien-3β-ol, also sensitize dramatically to the lethal action of both polyenes.

The prolonged systemic use of amphotericin B is often accompanied by dangerous toxicity, especially to the kidneys. It is likely that this damage is caused by polyene damage to the cytoplasmic membranes of the renal tubular cells. Our data suggest that nystatin may be much less toxic to man and his cholesterol-containing membranes while retaining efficacy against ergosterol-containing fungal membranes. Amphotericin B and filipin complex are both fungicidal and hemolytic, but their relative potencies differ for those activities. It has generally been assumed that the potential toxicity of the many polyenes antibiotics isolated would be similar and that their effects on different sterol-containing organisms would be comparable. This assumption clearly is not valid, and the pharmacology of the various polyenes deserves careful investigation or reinvestigation.

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