THE ACTION OF AMPHOTERICIN B ON MYCOPLASMA LAIDLAWII

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Polyene antibiotics such as amphotericin B, filipin and nystatin are active against many species of cells, causing damage to the cell membrane (Kinsky, 1961; Marini et al, 1961) and, at times, lysis (Kinsky et al, 1962). All sensitive cells thus far examined contain sterols, while bacteria and blue-green algae, which lack significant amounts of sterols (Fiertel and Klein, 1959; Levin and Bloch, 1964), are resistant to the action of the polyenes (Hunter and McVeigh, 1961; Kinsky, 1962). These observations, as well as a body of indirect evidence recently summarized by Kinsky (1963) and by Lampen et al (1962), suggest that the presence of sterols in membrane is required for polyene action.

Mycoplasma laidlawii strain A is a saprophytic PPLO organism that, unlike most mycoplasmas, does not require sterols. When grown in the presence of cholesterol the organism incorporates the sterol into its membrane, but following growth in a sterol-free medium the cells do not contain detectable cholesterol (Razin et al, 1963). In the following experiments M. laidlawii strain A was used to study the action of the polyene antibiotic, amphotericin B. While this paper was in preparation Weber and Kinsky (1965) reported studies using the same organism to investigate filipin action. The results of both studies confirm the critical role of sterols in the action of the polyenes.

Materials and Methods:

M. laidlawii strain A (ATCC #14089) was grown in a partially defined medium (Razin and Cohen, 1963). The bovine serum albumin (Cohn fraction V), the only

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potential source of cholesterol in the medium, was extracted with ethanol-ether (2:1 by volume) until no further cholesterol was detected in the extract as measured by the $FeCl_3$ reaction (Wycoff and Parsons, 1957). Amphotericin B (generously supplied by The Squibb Institute for Medical Research) was dissolved in dimethylformamide at 2 mg/ml and stored at -15°C. Cholesterol was dissolved in the same solvent. The concentration of dimethylformamide in the medium never exceeded 2%, a concentration which had no apparent effect on the organisms. Cell cultures to be compared contained the same amounts of dimethylformamide.

The organisms were grown in the sterol-free medium with and without 20μg cholesterol/ml. Viable counts were performed by spotting 20μl. of appropriate dilutions on growth medium containing 1.5% agar. After 48-72 hours colony counts were done in triplicate. In experiments with cholesterol-4-C^{14} (New England Nuclear Corporation) a Packard Tri-Carb liquid scintillation counter was used.

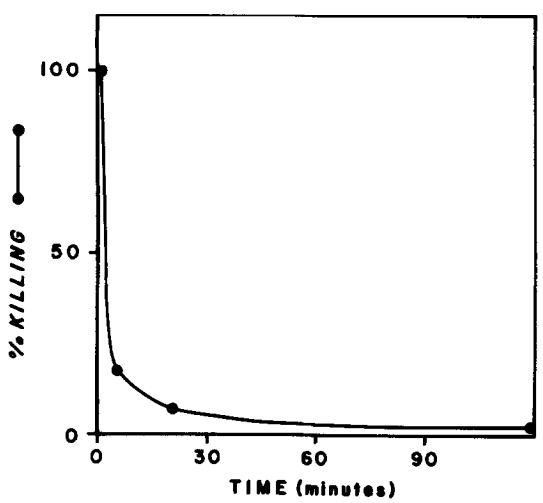

Figure 1: Sensitivity of cholesterol-grown M. laidlawii to amphotericin B. M. laidlawii were harvested after growth at 37°C in medium with 20μg cholesterol/ml, washed and suspended in cholesterol-free medium. Amphotericin B in dimethylformamide was added at time zero at a concentration of 25μg/ml. During the 120 minute test period the untreated (only dimethylformamide added) organisms grew from 9.7×10^7 to 1.8×10^8 organisms/ml.

Results:

The mycoplasmas grown in medium containing 20μg/ml. of cholesterol were treated with amphotericin B. Figure 1 depicts the loss of viability that occurred with 25μg/ml. of the drug. With turbid suspensions, a prompt decrease in turbidity was observed on drug treatment. As little as 2μg of amphotericin B/ml led to significant killing. In contrast, cells grown in the absence of cholesterol were not killed by the drug, but, rather, continued to grow at the normal rate (generation time of about 2 hours). Similarly, digitonin killed by lysis the M. laidlawii grown in the medium with added cholesterol but had no effect in the absence of added sterol.

Table 1: Interconversion of M. laidlawii between amphotericin B sensitivity and resistance.

Cell Type	% Survivors After Two Hour Exposure to Amphotericin B
Cholesterol-grown Cells (S Cells)	2%
Cholesterol-free Cells (R Cells)	200%
S Cells Incubated in Cholesterol-free Medium for 2 hours at:	
37°C	150%
4°C	5%
R Cells Incubated in Cholesterol-containing Medium for 2 hours at:	
37°C	3%
4°C	180%

M. laidlawii were grown in both medium with (sensitive-S cells) and without (resistant-R cells) 20μg cholesterol/ml. Both types of cells were harvested, washed and suspended in cholesterol-free medium. Samples were removed for viable counts with and without exposure to 25μg of amphotericin B/ml for 2 hours. To the R cells 20μg of cholesterol/ml were added in dimethylformamide and the S cells remained in cholesterol-free medium. Each parent culture was divided in half--one incubated at 4°C and the other at 37°C. After 2 hours incubation the 4 batches of cells were harvested, washed, suspended in cholesterol-free medium, and amphotericin B sensitivity was tested as before.

Organisms grown in the presence of cholesterol (amphotericin B sensitive) were unaffected by the antibiotic after incubation for 2 hours at 37°C in cholesterol-free medium, while organisms grown in cholesterol-free medium (amphotericin B resistant) became sensitive on incubation in cholesterol-containing medium. A culture could be alternated between drug sensitivity and resistance by successive incubations in medium with and without cholesterol at 37°C. Similar incubation at 4°C had no effect on amphotericin B sensitivity (Table 1), indicating that either the conversion requires metabolic activity or is temperature dependent.

The kinetics of the change of amphotericin B sensitivity were correlated with the uptake or loss of cholesterol-4-C^{14} from the cells. Figure 2 demonstrates that even after ten minutes of incubation in cholesterol-containing medium the originally resistant cells were already markedly sensitized to amphotericin B. At this time

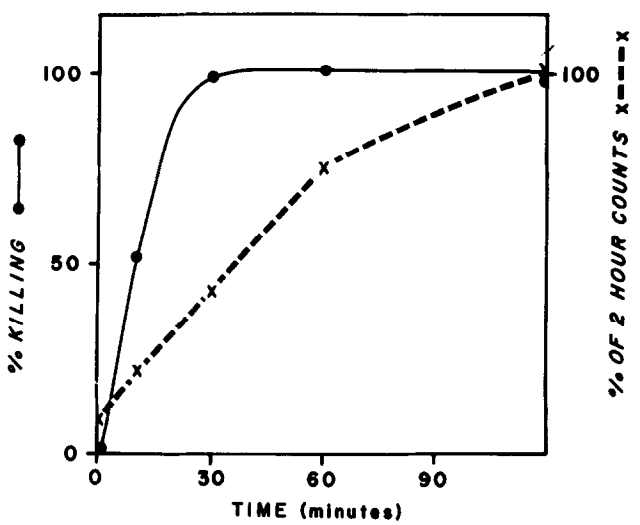

Figure 2: Conversion of M. laidlawii from amphotericin B-resistant to sensitive and the uptake of cholesterol-4-C^{14}.

M. laidlawii were grown in cholesterol-free medium, washed with medium, and resuspended in medium at 37°C containing 20μg cholesterol/ml. and 50 mμC cholesterol-4-C^{14}. Immediately after resuspension and at the indicated intervals thereafter samples were taken both for measurement of the cellular radioactivity and for determination of the sensitivity of the organisms to amphotericin B. The latter was determined as described previously after the organisms were washed at 4°C with cholesterol-free medium. The cells were collected on a Millipore filter (.22μ pore size) washed extensively with ice cold medium, and dispersed with the dried filter in Bray's solution (Bray, 1960) for assay of radioactivity.

the organisms had incorporated only 14% as much cholesterol as in 2 hours. On the other hand, the development of drug resistance by the cells grown in cholesterol and removed to a cholesterol-free medium was not so rapid. It is of interest that drug sensitivity diminished more rapidly than did radioactivity (Figure 3). It is unlikely that the loss of radioactivity was due to lysis during incubation in cholesterol-free medium, since there was an increase in the number of viable particles during the incubation. Continued growth also suggests that the radioactivity per cell at the end of the incubation was actually less than indicated in Figure 3, but, regardless, significant radioactivity remained associated with the organisms. When the M. laidlawii containing labeled sterol were incubated in cholesterol-free medium for 2 hours at 4°C, they remained sensitive to amphotericin B (see Table 1). During this incubation period in the cold less than 5% of the radioactivity was lost to the medium.

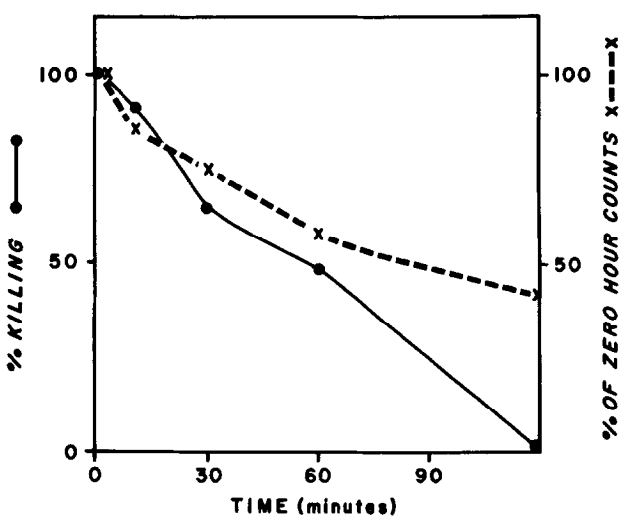

Figure 3: Conversion of M. laidlawii from amphotericin B-sensitive to resistant and the loss of radioactivity from the cells.
M. laidlawii which had been grown in cholesterol-free medium were incubated for 3 hours at 37°C in medium containing 20μg cholesterol/ml. and 100 mμC cholesterol-4-C^{14}. The organisms were washed at 4°C with cholesterol-free medium and then resuspended in this medium. Samples were taken at the indicated times after resuspension and assayed for cellular radioactivity and sensitivity to amphotericin B as described for the experiment depicted in Figure 2.

Discussion:

It is clear that the killing action of amphotericin B on M. laidlawii requires the presence of sterol in the cells, presumably in the cytoplasmic membrane. The killing is rapid, results in lysis, and resembles the action of the sterol-complexing agent digitonin on these organisms. Amphotericin B and cholesterol likely interact directly since the presence of cholesterol in the ambient fluid antagonized the action of the drug on sensitive organisms (Gottlieb et al, 1958).

Sensitive cells grown in the presence of cholesterol-C^{14} became resistant after having lost only a portion of their radioactivity. This may indicate that cholesterol is present in more than one kind of site in the cell, but that its presence in only one locus determines antibiotic sensitivity. Alternatively, it seems possible that the C^{14} from the cholesterol molecule is incorporated into more than one compound in the cell, and that only one is required for the killing action of amphotericin B. This possibility is suggested by the observation of Smith and Rothblat (1960) that M. laidlawii strain B incorporated cholesterol-4-C^{14} into the nonsaponifiable lipid fraction of the organisms, but into a sterol different from cholesterol.

Summary:

M. laidlawii grown in cholesterol-free medium are resistant to amphotericin B; when grown in the presence of cholesterol they are sensitive to the drug. Incubation of the resistant organisms in the presence of cholesterol results in a rapid conversion to sensitivity by a temperature-dependent process involving incorporation of sterol into the cells. Similarly, amphotericin B-sensitive M. laidlawii are rendered resistant by incubation in a cholesterol-free medium, and this change is associated with loss of some of the radioactivity previously incorporated from labeled cholesterol.

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