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ENHANCEMENT OF AMPHOTERICIN B SELECTIVITY BY ANTIBIOTIC INCORPORATION INTO GEL STATE VESICLES. A CIRCULAR DICHROISM AND PERMEABILITY STUDY

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SUMMARY : The permeability induced in cholesterol-or ergosterol-containing phospholipid vesicles by Amphotericin B incorporated into Dipalmitoyl Phosphatidylcholine vesicles has been studied in parallel with Circular Dichroism spectroscopy measurements. In our conditions, Amphotericin B is 5 to 10 times more selective to ergosterol- than to cholesterol-containing vesicles. Such a large difference is not observed when Amphotericin B is directly added to the vesicles suspension as its solution in organic solvent.

The fungicidal selectivity of polyene macrolide antibiotics such as Amphotericin B (AmB) is based upon their greater ability to induce permeability on ergosterol-containing membranes than on cholesterol-containing ones (1). The nature of the sterol is of primary importance for membrane susceptibility, since it is the major component with which polyenes associate to form channels. Recently, the study of the interaction of AmB with lipidic vesicles containing various sterols (2) by circular dichroism spectroscopy, combined with the study of its permeabilizing effect have shown that among the various conformers detectable in these membranes, only one, maybe two, are responsible for permeability inducement. Moreover, these conformers appear to be the same in ergosterol-and cholesterol-containing membrane. The only difference is a quantitative one : the complexes form at lower polyene concentration in ergosterol- than in cholesterol-containing membranes. The greater sensitivity of ergosterol membrane seems to be accounted for by this quantitative difference.

One of the major difficulties encounted in the study of AmB membrane interaction is its poor water solubility which results in its aggregation when added to a membrane suspension as a concentrated solution in organic solvent. The interaction of these large aggregates of AmB with membranes is practically only observable in this condition. This is a massive process, very difficult to analyse in terms of the successive multimolecular

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events which lead to pore formation. Furthermore, it is certainly not really representative of the conditions in which AmB acts in vivo, where it is associated to the various lipidic and lipoproteic plasmatic components (6).

The study of AmB membrane interaction by transfer from a "reservoir" membrane, avoiding completely the presence of aggregated form in the aqueous medium, overcomes this impediment. In the present study the "reservoir" consisted in pure dipalmitoyl phosphatidylcholine (DPPC) small unilamellar vesicles (SUV), loaded with AmB. The choice of DPPC vesicles was dictated by the very high affinity of AmB for this lipid in the gel state (3). Egg yolk lecithin (EPC) SUV containing various proportions of either cholesterol or ergosterol were then mixed with these vesicles. In this condition the antibiotic partitions between the two different membranes and therefore its affinity for ergosterol- and cholesterol-containing membrane may be compared by respect to its affinity for DPPC. As it will be shown, not only the affinity of AmB for ergosterol membrane is much greater than for cholesterol but their sensitivity to the permeabilizing effect is much more important than that classically observed on the basis of the results of experiments involving the aggregated form of AmB.

MATERIALS AND METHODS

EPC was prepared from egg-yolk according to Patel and Sparrow (4). DPPC was from Sigma. Cholesterol and ergosterol were from Fluka and Merck respectively and both were purified by twice recrystallisation in ethanol before use. FCCP (carbonyl cyanide p-trifluoromethoxyphenyl hydrazone) was from Boehringer. AmB was a generous gift from Squibb (France). AmB concentrations were determined from the electronic absorption on the mother liquor in dimethylsulfoxide (DMSO) with $E_{416} = 121400$.

Vesicles were prepared according to the method previously described (2). Permeability studies were carried out by the proton-cation exchange method already described in detail (2, 3, 5). Circular dichroism spectra were recorded with a Jobin-Yvon Mark III dichrograph (2, 3).

To study the interaction of AmB with SUV by C.D. and the inducement of permeability two experimental conditions were used :
a) the "direct addition" of AmB as microliter amounts of a 10^{-3} M solution in DMSO to suspensions of either cholesterol- or ergosterol-EPC SUV.
b) the "transfer method" in which SUV loaded with the desired amount of a 10^{-3} M AmB solution in DMSO were added in equal quantities to the free-AmB SUV. Experimental procedures were already described in details (3).

RESULTS

Circular dichroism study

a) The direct addition of AmB : the data obtained by this method were presented elsewhere (2). The main results of interest will be just summarized here : upon addition to the SUV suspension, the typical spectrum of AmB aggregated in aqueous medium disappears rapidly. Its absence means that the concentration of free amphotericin B in aqueous suspension medium

is $<10^{-7}$ M. At very low AmB/lipids molar ratio (R), that is $R < 5 \times 10^{-5}$, a typical spectrum, so called type I spectrum, characterized by three positive bands is observed. As R increases, a new spectrum, said type II spectrum, takes place. Both spectra are the same in ergosterol- and in cholesterol- containing EPC-SUV. In the present study additional data concerning the appearance of type II spectrum (responsible for permeability (2)) in EPC-SUV containing 20 % of either cholesterol or ergosterol were obtained. This spectrum is totally established at $R = 10^{-4}$ in ergosterol-SUV while it just begins to appear around 5×10^{-4} in cholesterol-SUV. This confirms the quantitative differences between the two types of membranes in their interaction with AmB.

b) The "transfer method". In the following data, EPC-sterol SUV loaded with AmB were mixed with DPPC-SUV. The final spectrum was identical if the experiment was carried out in the reverse way, as in permeability study, but let us measure clearly the kinetics. These kinetics of AmB transfer between the two populations (Fig. 1) were monitored at 394 nm which corresponds to the peak of AmB in DPPC (see Fig. 2 of Ref. 3).

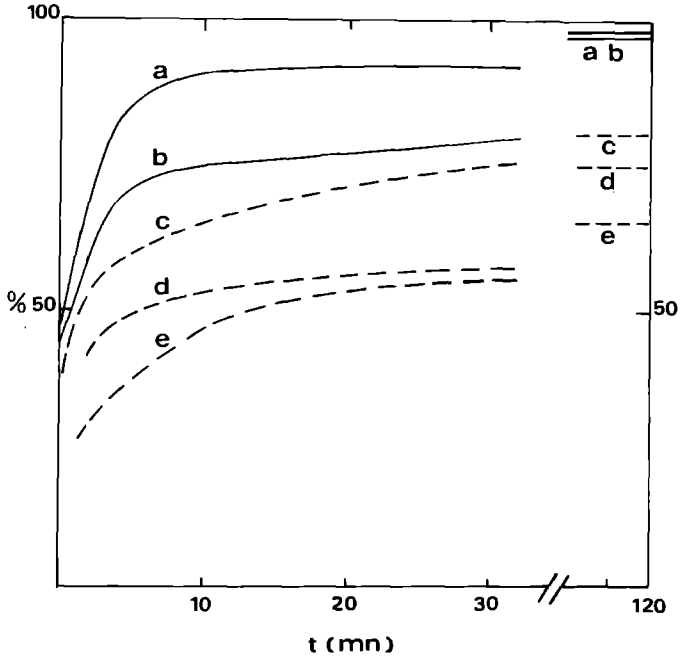

Figure 1 : Kinetics of AmB transfer from AmB incorporated in EPC-sterol vesicles to DPPC vesicles. The percentage of AmB transferred to DPPC vesicles, as determined by CD at 394 nm is given as a function of time.
Sterol content of EPC vesicles (in M/M) :
a) cholesterol 3 % ; b) cholesterol 20 % ; c)ergosterol 2 % ; d) ergosterol 8 % ; e) ergosterol 20 %.

The conclusions which can be drawn are the following :

In the case of the transfer from EPC-cholesterol to DPPC-SUV's :

a) more than 99 % of AmB is transferred to DPPC. There is no significant difference (within the limit of accuracy) between the intensity of the spectrum of 10^{-2} Moles/mole lipids in pure DPPC and of the same AmB concentration in the mixed population ;

b) the kinetics of transfer is slower, the greater the concentration of cholesterol in EPC vesicles.

In the case of the transfer of EPC-ergosterol to DPPC-SUV's :

a) the maximum of AmB transferred is approximatively only 70 to 80 % ;

b) again, the kinetics is slower the greater the ergosterol concentration in EPC-SUV's ;

c) the kinetics of transfer is, in any case, slower in the ergosterol than in the cholesterol case.

Permeability study

The proton flux induced by AmB developed in time until it reaches a plateau, which thereafter remained stable. The percentage of proton release at the plateau was expressed in percent of the total amount of proton titratable. Dose-response curves were obtained by plotting this % of protons releases at the plateau as a function of the antibiotic concentration, expressed in moles per mole of total vesicular lipids.

In Fig. 2 are given the dose-response curves obtained on EPC vesicles containing 20 % of either cholesterol or ergosterol by the "direct method"

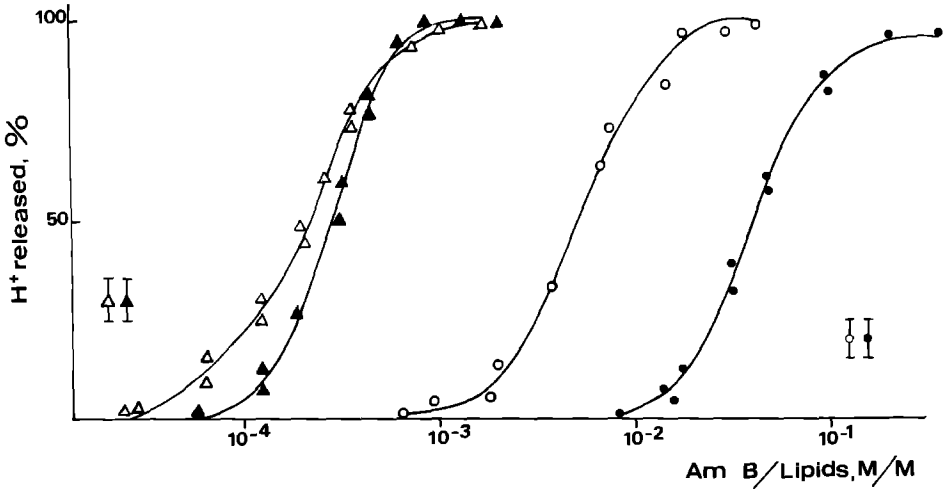

Figure 2 : Protons released (in % of the total intravesicular titratable proton after Triton X-100 addition) induced in EPC vesicles containing : 20 % cholesterol (closed symbols) or 20 % ergosterol (open symbols), by AmB added either directly as DMSO solution (triangles), or transferred from DPPC vesicles (circles) (for explanations, see text).

TABLE I. Permeability induced by AmB in EPC-sterol vesicles

% sterol	P_{50}		Partition ratios $\frac{\text{EPC-sterol}}{\text{DPPC}}$
	direct	transfer	
		CHOLESTEROL	for R = 0.5 x 10^{-2}
0	4.0×10^{-4}	-	-
5	2.8×10^{-4}	$1.2\ 10^{-1}$	<0.01
10	2.4×10^{-4}	$6.0\ 10^{-2}$	<0.01
20	1.4×10^{-4}	$5.0\ 10^{-2}$	<0.01
		ERGOSTEROL	
0	4.0×10^{-4}	-	-
5	2.0×10^{-4}	$2.5\ 10^{-2}$	0.20
10	1.8×10^{-4}	$1.6\ 10^{-2}$	0.25
20	1.0×10^{-4}	$5.0\ 10^{-3}$	0.35

P_{50} : AmB/lipid molar ratio corresponding to 50 % proton release obtained from doses-response curves (Fig. 2).
Partition ratios : obtained from Fig. 1 at equilibrium.

and by the "transfer method". In this latter case the antibiotic was transferred from AmB preloaded DPPC vesicles. In Table I is given as a function of both sterol concentration, the R, corresponding to 50 % proton released in the system (P_{50}) obtained from the corresponding dose-response curves.

By the direct method the sensitivity to permeability inducement by AmB of ergosterol-containing vesicles is about 1.4 times greater than of cholesterol-containing ones. The sensitivity increases with sterol concentration and more so in ergosterol- than in cholesterol-containing vesicles. In contrast with these rather small differences observed by the direct method, the differences observed by the transfer method are much more important. Depending upon sterol concentration, the sensitivity to permeabilization by AmB is between 5 to 10 times greater in ergosterol vesicles than in cholesterol ones.

DISCUSSION

The main conclusion drawn from this study is that AmB exhibits actually a 5 to 10 fold greater ability to induce permeability in ergosterol- than in cholesterol-containing vesicles when added by transfer from DPPC vesicles.

Such a large difference is not obtained when AmB is introduced in the vesicular suspension as DMSO solution.

In a previous paper (3) it has been shown that AmB exhibits a much greater affinity for membrane lipids in the ordered gel state, such as DPPC, than for those in the disordered liquid crystalline state such as EPC containing or not sterols. CD measurements (Fig. 1, Table I, 3rd column) show that at equilibrium in a system containing equal amounts of DPPC and sterol-EPC vesicles and for an AmB/total lipid molar ratio $R = 0.5 \times 10^{-2}$, the partition of AmB in the presence of sterol-EPC vesicles and DPPC vesicles is 20 to 50 times greater in the ergosterol-EPC vesicles than in the cholesterol-EPC vesicles. This in agreement with what is observed by permeability : for $R = 0.5 \times 10^{-2}$ a 50 % release is induced in ergosterol-EPC vesicles and no release in cholesterol-EPC vesicles. The same agreement is not observed for the direct incorporation of AmB : for 20 % sterol-containing vesicles, type II spectrum appears for a much smaller R in the ergosterol case while the permeability dose-response curves are almost superimposed. The reason for this discrepancy may be found in the fact that our CD measurements give data at equilibrium which is not the case with permeability measurements. The brutal incorporation of AmB by the direct process is different of the process of transfer between membranes. As a matter of fact, it appears poorly sensitive to the nature of the sterol and even does not need any sterol to occur (Table 1, 2nd column).

The transfer situation, which proves to be advantageous from the point of view of the selectivity of AmB action on ergosterol-containing membranes, is probably more relevant to the actual situation in chemotherapy, where AmB is introduced in vivo in the blood stream. A recent study (6) shows that in blood, AmB is bound to lipoproteins and other plasma proteins. Therefore, the permeability induced occurs by transfer and experiments on red blood cells (as cholesterol-containing animal cells) and Candida Albicans (as ergosterol-containing infectious microorganisms) show that in the presence of this plasmatic protein the effect on red blood cells is inhibited but not in Candida Albicans. Exactly the same results were observed with liposomal AmB (7). Therefore all these results point out the interest of the transfer method as a more efficient strategy in antifungal therapy.

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