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Interaction of amphotericin B and its N-fructosyl derivative with murine thymocytes: a comparative study using fluorescent membrane probes

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The polyene antibiotics amphotericin B (AmB) and *N*-(1-deoxy-D-fructos-1-yl)amphotericin (*N*-Fru-AmB) have different activity towards murine thymocytes (*N*-Fru-AmB is less toxic but is a potent immunomodulator). The interactions of the drugs with these cells have been studied by fluorescence methods. Fluorescence energy transfer from 1-[4-(trimethylammonio) phenyl]-6-phenylhexa-1,3,5-triene, *p*-toluenesulfonate (TMA-DPH) to polyenes was used to follow the binding of the two drugs to the plasma membrane. The results, confirmed by circular dichroism measurements, indicate that at saturation the ratio AmB bound/plasma membrane lipid is low (less than 1 molecule of polyene for 170 lipids). The slightly higher binding of AmB as compared to *N*-Fru-AmB demonstrates that affinity of the antibiotic for plasma membrane does not account for the activity of the polyenes towards lymphocytes. The effect of the two polyenes on membrane fluidity was studied by steady-state fluorescence anisotropy. The results suggest that AmB strongly perturbs the structure of the membrane whereas only a slight decrease of the anisotropy is observed with *N*-Fru-AmB in the range of concentration where the biological activity has been demonstrated. Polyene location was further investigated by comparing the energy transfer efficiency obtained with TMA-DPH and with the parental compound 1,6-diphenylhexa-1,3,5-triene, *p*-toluene sulfonate (DPH). While AmB binds to plasma membrane, as well as to intracellular structures, *N*-Fru-AmB seems to accumulate into the cell and bind to intracellular membrane structures.

Introduction

Amphotericin B (AmB) is an efficient antifungal agent used in the treatment of systemic and deep-seated mycotic infections (for a review, see Ref. 1). However, negative side effects are known, such as toxicity for host cells, which are counterbalanced by positive effects at sublethal doses such as synergism with antitumor compounds (see, for example, Refs. 2–5) or stimulation of the immune response (see, for example, Refs. 6–11). Amphotericin B and its derivatives may therefore have therapeutic value apart from its antibiotic activity. Yet the mechanisms underlying these immunomodulating or antitumor properties remain poorly understood (for a review, see Ref. 12).

In this report, we were interested in elucidating some aspects of the interaction of polyenes with lymphoid cells. Previous work on the effects of AmB on the immune system has shown that thymocytes are extremely sensitive to the cytotoxic effect of AmB both in vivo and in vitro in mice [13]. Murine thymocytes were thus selected as a cellular model to investigate AmB interaction with lymphoid cells. The effect of AmB was compared with that of an interesting derivative, the *N*-Fru-AmB, which was previously shown to be less toxic than AmB for thymocytes (unpublished results) and less haemolytic for human erythrocytes [14]. Moreover, we have shown that this derivative is a very potent polyclonal activator for peripheral T and B lymphocytes in mice [15]. It is thus a good candidate for a comparative study of polyenes interactions with lymphocytes.

The first step in the cell–polyene interaction is the binding to plasma membrane. The chemical modification brought to AmB by introducing a fructosyl group leads to a decreased hydrophobicity of the molecule which correlatively increases its solubility. We were first interested in elucidating the consequences of this modification on the binding efficiency to cells and to determine whether binding differences could explain dif-

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ferences observed in toxicity and stimulating activities of the two compounds. The quantitation of polyene binding to cells raised some methodological problems since classical methods based on the separation of the bound fraction from the free one could not be used due to the formation of sedimentable aggregates. Circular dichroism of polyenes has been previously used to follow their binding to model membranes [16–19] but since it monitors the free fraction this method is of poor help in case of low binding extent. Hence in the present study the binding was evaluated using the 1-[4-(trimethylammonio)phenyl]-6-phenylhexa-1,3,5-triene, *p*-toluenesulfonate (TMA-DPH) fluorescent probe. This one rapidly incorporates into cell membranes when added to intact living cells, according to a partition equilibrium [20,21]. The quantum yield of the probe inserted in the membrane leaflet is high whereas the fluorescence of the form in the aqueous medium is negligible [22,23]. The overlap of the absorption spectrum of the polyene with the emission spectrum of TMA-DPH allows to anticipate singlet-singlet energy transfer between the two molecules in the membrane according to the photophysical process originally described by Förster [24]. The amount of polyene bound to cells can be deduced from the experimental energy transfer efficiency according to the theory of Fung and Stryer [25].

Changes in membrane dynamics have been shown to be involved in the modulation of several membrane functions [26–30]. Considering the interactions of polyenes with sterols and the involvement of these molecules in the rigidity of the membrane, we then explored their effect on membrane fluidity. The membrane fluidity was followed using TMA-DPH as a steady-state anisotropy probe [31,32]. The measurements revealed a clear difference in membrane fluidity when thymocytes were incubated with *N*-Fru-AmB as compared to AmB.

Finally we took advantage of the particular behavior of TMA-DPH and DPH to further explore the possible interaction of the two polyene antibiotics with the intracellular membranes. Indeed, although they have the same photophysical properties, the two probes exhibit interesting differences in the labelling of the cell membranes. First their location in the membrane leaflet is slightly different: because of its positive charge, TMA-DPH is anchored at the lipid/water interface with the DPH moiety intercalated between the upper portions of the fatty acyl chains, probing essentially the fatty acyl chains regions as far down as C_8–C_{10}. In contrast, DPH is slightly deeper embedded in the hydrophobic core of the leaflet [21]. Second, the distribution of these probes in the cell yields to different patterns: TMA-DPH exclusively labels the plasmic membrane when incubated less than 60 min with the cells [33], whereas DPH, in the mean time, binds to plasmic and internal membrane [34–36]. These differences were used to investigate the location of polyenes. The results indicate that AmB and its derivative have intracellular locations.

Materials and Methods

Chemicals

The fluorescent probes TMA-DPH and DPH were purchased from Molecular Probes (Oregon, U.S.A.). Dimethylformamide (DMF) and dimethylsulfoxide (DMSO) were from Merck first grade purity.

Polyenes

Amphotericin B was a generous gift of Squibb France. The *N*-Fru-AmB derivative was prepared according to the method of Falkowski et al. [37]. Stock solutions were obtained by dissolving 1 mg of polyene in 20 μl of dimethyl sulfoxide (DMSO) and suspended in 1 ml of Dulbecco's modified phosphate-buffered saline (DPBS).

Cellular systems

Thymocytes were freshly prepared from the thymus of 8–10-week-old DBA/2 mice. Cell suspensions were prepared in DPBS according to standard methods (see, for example, Ref. 38). Living cells were purified by sedimentation over Ficoll gradient (Lymphoprep Nyegaard and Co., Norway) and kept on ice for the duration of the experiment. Viability was checked by Trypan blue exclusion.

Cells labelling and fluorescence measurements

A 10^{-6} M TMA-DPH solution was prepared in DPBS buffer from a $2 \cdot 10^{-3}$ M stock solution in DMF. After vigourous stirring, 2 ml of this solution was allowed to equilibrate at 25 °C in the cuvette holder of a JY3D Jobin Yvon spectrofluorimeter. The excitation monochromator was set up to 365 nm. For kinetics recordings, the emission wavelength was 427 nm which corresponds to the maximal intensity of the emission spectra of TMA-DPH in membranes. Cells were added as 20 μl of packed cells to achieve a final concentration of $1.5 \cdot 10^6$ cells per ml. Gentle stirring ensured the homogeneity of the suspension during recordings. Upon cell addition intensity increased and a steady state was reached after 2 min, indicating that the TMA-DPH was incorporated into cell membranes. Further addition of polyenes was done at this point. The same experiments were performed with DPH. In that case, polyenes were added 25 min after mixing cells with the probe. Steady-state anisotropy measurements were performed with TMA-DPH under same conditions as above except that the fluorimeter was equipped with one polarizer on the excitation beam and another one on the emision-beam (polarizers Jobin Yvon). Each polarizer was able to rotate to achieve parallel or perpendicular polarization directions. The background contribution of cells was

subtracted by measuring the polarization components of an unlabelled cell suspension.

Calculations methods

Energy transfer to the singlet level. Singlet-singlet energy transfer was analyzed according to the theory of Förster [24]. The transfer efficiency E was defined as the fraction of donor that is deexcited by transfer to the acceptor. In our system a non radiative transfer occurs. the polyene antibiotic which is the acceptor do not emit and all the fluorescence comes from the donor. Then, if quenching is only due to singlet-singlet transfer, the efficiency of energy transfer can be calculated from the quenching of the energy donor using

$$E = 1 - (F/F_0) \tag{1}$$

where F and F_0 are the fluorescence intensities emitted in the presence and in the absence of the acceptor, respectively. A requirement for resonance energy transfer to occur is that the absorption of the acceptor must overlap the donor fluorescence. Therefore an additional quenching occurs which consists in trivial photon reabsorption but which is distinguishable from energy transfer at the singlet level. Consequently the value of F has to be corrected for this inner filter effect in the following manner.

$$F = F' \cdot 10^{(A_1 + A_2)/2} \tag{2}$$

where F' is the observed value after addition of the acceptor and A_1, A_2 are the absorbances at the excitation and emission wavelengths, respectively.

The parameter which characterizes the donor-acceptor system is R_0, called the characteristic transfer distance. It is the distance at which the transfer efficiency is equal to 0.5 and it is given by

$$R_0(\text{Å}) = 9.79 \cdot 10^{-3} (J K^2 \Phi n^{-4})^{1/6} \tag{3}$$

where K^2 is a complex geometric factor that depends on the relative orientation of the transition dipoles of the donor and acceptor. Assuming that donor and acceptor molecules rotate rapidly as compared to the fluorescence lifetime of the donor, we used an average value of K^2 equal to 2/3 [39]. This value is somehow uncertain since it cannot be measured directly but the R distance depends only on $(K^2)^{1/6}$ and the error on K^2 does not severely alter the value of R. Φ is the fluorescence quantum yield of the donor, equal to 0.8 [22], n is the refractive index of the medium taken equal to 1.4, J is the normalized spectral overlap integral defined as

$$J(\text{M}^{-1} \cdot \text{cm}^{-1}) = \frac{F_D(\lambda) \epsilon_A(\lambda) \lambda^4 \, d\lambda}{F_D(\lambda) \, d\lambda} \tag{4}$$

where $F_D(\lambda)$ is the donor fluorescence intensity and $\epsilon_A(\lambda)$ $(\text{M}^{-1} \cdot \text{cm}^{-1})$ is the absorption coefficient at the wavelength λ (cm).

The theory of Fung and Stryer [25]. The theory of Fung and Stryer has been established for donor-acceptor pairs randomly distributed in synthetic membrane vesicles. The authors have shown that the efficiency of the energy transfer depends only on three variables R_0, σ the surface density of the acceptor, and a the distance of closest approach of donor and acceptor. They were then able to calculate for several donor-acceptor pairs characterized by R_0 values from 20 to 70 Å, the energy transfer as a function of the surface density of the acceptor [25]. We will use these results to determine the corresponding value of surface density of the acceptor from the R_0 and transfer energy values that we obtained experimentally in our system.

Steady-state anisotropy. The polarization P is theoretically given by

$$P = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp}$$

where $I_\parallel$ and $I_\perp$ are the intensities of light emitted parallely and perpendicularly to the direction of the polarization of incident light, respectively. It has been corrected for the instrumental asymmetry and the value of P is finally obtained as follows

$$P = \frac{I_{VV} - I_{VH}(I_{HV}/I_{HH})}{I_{VV} + I_{VH}(I_{HV}/I_{HH})}$$

The two subscripts referred to the orientation of the excitation and emission wavelength, respectively. For instance I_{HV}, corresponds to the intensity of vertically polarized emission with an horizontally polarized excitation. The excitation and emission wavelengths were set to 365 and 427 nm, respectively.

Results will be given in terms of steady-state anisotropy (r_{ss}) calculated from P as follows:

$$r_{ss} = 2P/(3 - P)$$

Results

Polyene binding to thymocytes

The absorption and emission spectra of TMA-DPH in membranes are shown in Fig. 1 together with the absorption spectra of the polyenes. From the overlap of the emission spectra of the probe and the absorption of the polyenes, an energy transfer between the two molecules is expected.

The polyene absorption spectra have been shown to be sensitive to aggregation state and environment [18,19] of the molecules. The aggregation which occurs in aqueous medium depends on the polyene concentration. When polyenes bind to cells their environment is

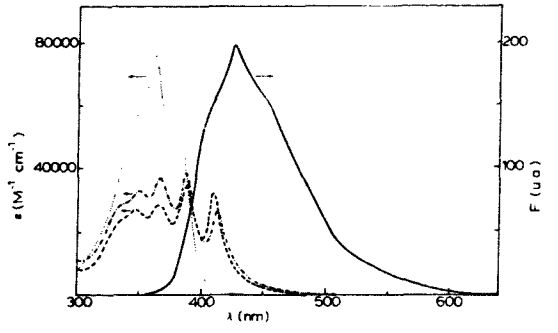

Fig. 1. Absorption spectra of AmB recorded with a polyene concentration equal to $5 \cdot 10^{-6}$ M in PBS (------) and in the presence of vesicles of DPPC 0.1 mM (·–·–·) and TMA-DPH fluorescence (———) and absorption spectra (·····) recorded in the presence of vesicles of DPPC 0.1 mM.

changed and the absorption spectra are expected to be consequently modified. These spectral properties changes have been taken into account to calculate the value of R_0 characterizing our system since the energy accepting form is actually the membrane-bound polyene. The spectral characteristics of the membrane-bound form has been obtained by mixing AmB with dipalmitoyl/phosphatidylcholine (DPPC) small unilamellar vesicles in the gel state (25°C) under AmB to lipid ratios such that AmB is totally bound to the lipid leaflet [19]. The spectra of the bound form is shown Fig. 1 in comparison with the free one at the same concentration. R_0 was calculated for AmB and *N*-Fru-AmB in the DPPC-bound and free forms for concentrations corresponding to the range used in our energy transfer experiments.

Results in Table I show that spectral changes induced by polyene concentration and environment did not significantly modified R_0 values. Absorption changes due to membrane binding reduced of 1 Å the value of R_0 but polyene concentration had virtually no effect. For further application of the Fung and Stryer formalism, a R_0 value of 40 Å was considered.

TABLE I

Characteristic transfer distances between TMA-DPH and polyenes free or bound to DPPC membranes, at various concentrations

Polyene	Concn. (M)	Environment	R_0 (Å) [a]
AmB	$1 \cdot 10^{-6}$	PBS	40.3
AmB	$1 \cdot 10^{-6}$	DPPC	39.3
AmB	$5 \cdot 10^{-6}$	PBS	41.2
AmB	$5 \cdot 10^{-6}$	DPPC	40.7
N-Fru-AmB	$2 \cdot 10^{-6}$	PBS	40.1
N-Fru-AmB	$2 \cdot 10^{-6}$	DPPC	39.2

[a] The incertitude on R_0 values have been calculated and found equal to 0.5 Å.

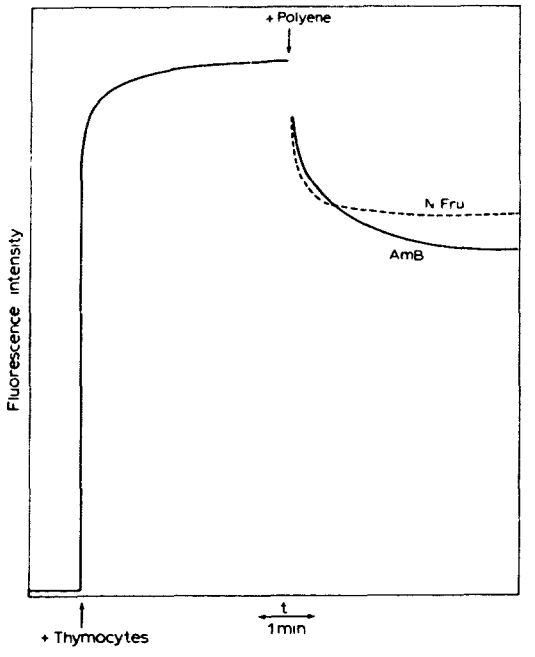

Fig. 2. Kinetics of TMA-DPH incorporation by thymocytes and quenching by polyenes. Final cell concentration is equal to $1.5 \cdot 10^6$ cells per ml. AmB (———) and *N*-Fru-AmB (– – –) were added to a final concentration equal to $2 \cdot 10^{-6}$ M.

Fig. 2 shows the typical variation of fluorescence emitted at 427 nm by a 10^{-6} M TMA-DPH solution when thymocytes and then polyenes were added. After polyenes addition, fluorescence quenching was observed. Rather similar profiles were obtained with the two polyenes although the kinetics of the quenching were faster with the *N*-Fru-AmB derivative. The quenching amplitude was measured after about 5 min, as soon as a steady level of fluorescence was reached. The absorbance of the sample was measured and the contribution of the inner filter effect, resulting from the reabsorption of the emitted photons was subtracted as indicated in Methods. The remaining quenching was interpreted in terms of electronic energy transfer at the singlet level from the probe to the polyenes.

Fig. 3 shows the energy transfer efficiency as a function of the concentration of polyene added to TMA-DPH treated thymocytes for AmB and its *N*-Fru-AmB derivative. A saturation of the polyene binding was observed at a concentration around $6 \cdot 10^{-6}$ M. The Fung and Stryer formalism which allows to connect E with the acceptor surface density when R_0 is known, was applied here for $2 \cdot 10^{-6}$ M polyene and a R_0 value of 40 Å. The polyene surface density obtained with AmB and *N*-Fru-AmB were $3.7 \cdot 10^{-3}$ and $2.8 \cdot 10^{-3}$ acceptors per lipid, respectively. These values correspond to 1 AmB per 270 lipids and 1 *N*-Fru-AmB per

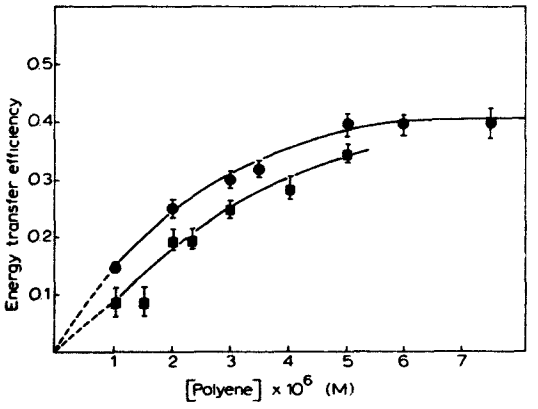

Fig. 3. Energy transfer efficiency (from TMA-DPH to polyene) as a function of AmB (●) or *N*-Fru-AmB (■) concentration.

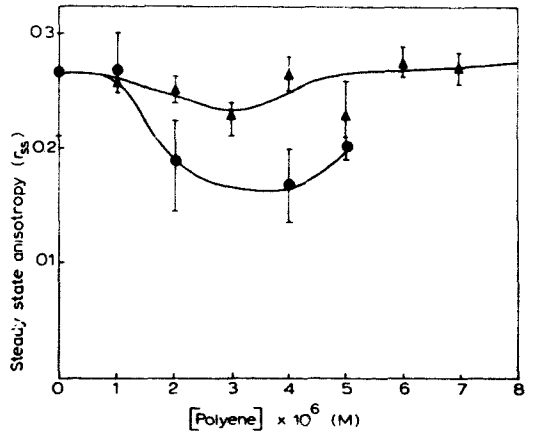

Fig. 4. Steady-state anisotropy of TMA-DPH in thymocytes plasma membrane as a function of AmB (●) or *N*-Fru-AmB (▲) concentration.

370 lipids. According to the data of Hoessli and Runger-Brändle [40] on murine T lymphocytes the number of plasma membrane lipids (cholesterol/phospholipid ratio approximately equal to 1) per cell is about $3.4 \cdot 10^9$. Consequently, for a total polyene concentration equal to $2 \cdot 10^{-6}$ M, we calculated a bound fraction of 1.6% for AmB and 1.2% pour *N*-Fru-AmB. Details of the results are given Table II. At saturation there is 1 molecule of AmB bound for 168 lipids and 1 molecule of *N*-Fru-AmB for 231 lipids.

These calculated concentrations of bound polyene have been controlled using circular dichroism (CD) in the case of a total polyene concentration equal to $2 \cdot 10^{-6}$ M. Indeed, the CD spectrum of polyenes, still more than the absorbance spectrum is very sensitive to conformation and environment of the molecule. Very different profiles are obtained when polyenes are free or bound to membranes as already shown [18,19]. The amplitude of the dichroic doublet which characterizes the free polyene was used to estimate the amount of bound polyene. 4 ml of the same polyene solution were divided in two samples and $1.5 \cdot 10^6$ cells per ml were added in one of them. The evolution with time of the CD spectrum of each sample was followed (data not shown). The decrease of the dichroic doublet of AmB with cells as compared to AmB alone never exceeded 2%. This totally corroborated the low binding rate calculated from the fluorescence results. However, it should be pointed out that even whether CD measurements allowed to verify the consistancy of the fluorescence results, they give poorly accurate values, due to the low binding extent of the polyene to cells. Indeed only small differences in free polyene concentration can be seen in the presence and in the absence of cells. This underlines the necessity to use a parameter related to the bound form rather than to the free one to evaluate the binding in the case of low binding extents.

TABLE II

AmB and N-Fru-AmB binding to thymocytes as determined from the energy transfer (E) from TMA-DPH

	Total concn. (M)	*E*	σ [a]	Bound concn. (M)	Binding extent (%)
AmB	$2 \cdot 10^{-6}$	0.25	$3.7 \cdot 10^{-3}$	$3.13 \cdot 10^{-8}$	1.57
N-Fru-AmB	$2 \cdot 10^{-6}$	0.20	$2.8 \cdot 10^{-6}$	$2.37 \cdot 10^{-8}$	1.19

[a] Polyene per lipid.

Membrane fluidity changes

Steady-state anisotropy (r_{ss}) values were obtained as indicated in methods. Fig. 4 shows the evolution of this

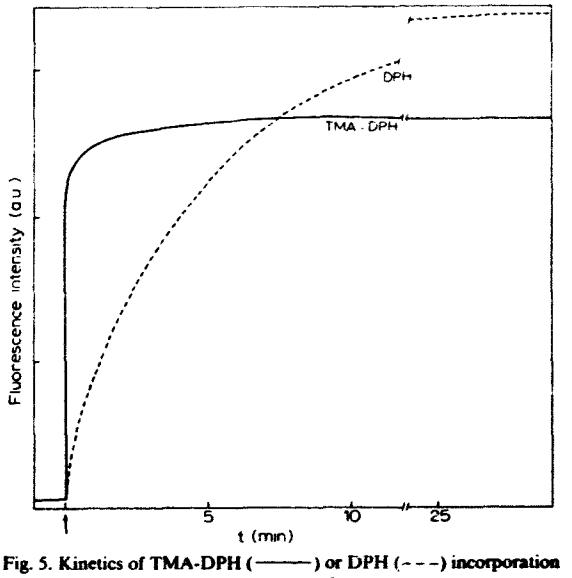

Fig. 5. Kinetics of TMA-DPH (———) or DPH (- - -) incorporation in thymocytes membranes.

TABLE III

Energy transfer efficiencies from TMA-DPH or DPH to polyenes

	E	
	AmB (10^{-6} M)	*N*-Fru-AmB (10^{-6} M)
TMA-DPH	0.12 ± 0.03	0.11 ± 0.04
DPH	0.13 ± 0.02	0.28 ± 0.05

parameter after the addition of increasing concentrations of polyene to thymocytes. A drastic decrease of r_{ss} was observed when AmB concentrations reached $2 \cdot 10^{-6}$ M whereas no such modification of anisotropy was induced even by *N*-Fru-AmB concentration of $7 \cdot 10^{-6}$ M. Only a low decrease of the r_{ss} value was observed around $3 \cdot 10^{-6}$ M. Surprisingly, the value of r_{ss} increased again when higher AmB concentrations were added. This will be discussed below.

Polyene cellular localization

TMA-DPH has been shown to incorporate rapidly and to reside in the plasma membrane. In contrast the parental DPH compound needs around 30 min to reach a plateau of incorporation and was shown to label intracellular membranes as well as plasma membranes [34–36]. This is consistent with the fluorescence profiles that we obtained when TMA-DPH or DPH were incorporated into cells at the same concentration (Fig. 5). Despite these differences, the photophysical properties of the probes embedded in lipidic environment are identical [23]. Table III gives the energy transfer efficiency values obtained using either DPH or TMA-DPH as the donor for AmB and its *N*-Fru-AmB derivative. The energy transfer to AmB was as efficient when occuring from DPH than from TMA-DPH, that is as efficient when the probe was located in the plasma membrane than when it labelled all the cellular membranes. Since the energy transfer efficiency does not depend on the donor concentration [25], it means that the AmB density was the same in all membranes and suggests therefore that AmB does not only bind to plasma membrane but also to internal membranes. In contrast with the *N*-Fru-AmB derivative, the transfer was more efficient from DPH than from TMA-DPH, strongly suggesting drug accumulation inside the cell.

Discussion

Fluorescence energy transfer appeared to be a sensitive and adequate method to follow the binding of polyenes to cells. Its measurement does not require the usual separation of free and bound fraction which has the disadvantage to disturb the binding equilibrium and is difficult to perform with polyene antibiotics which form aggregates sedimenting during centrifugation [12]. Compared to CD, the energy transfer method offers the advantage of yielding experimental data related to the bound fraction and not to the free one which is of importance when the binding extent is low, which was the case here. The shape of the binding curves suggests that AmB and its *N*-Fru-AmB derivative interact with cellular membranes according to a saturable equilibrium with a definite number of sites and not only according to a partition equilibrium. It must also be pointed out that their binding extent is low and corresponds at most to 1 AmB per 170 lipids or 1 *N*-Fru-AmB per 230 lipids. This result is in good agreement with those obtained with cholesterol-containing egg yolk phosphatidyl choline vesicles [41] or with hepatocytes [42]. Surprisingly, we observed that the binding curves of *N*-Fru-AmB was very close to that of AmB although the two compounds exhibit very different toxic and immunostimulating behaviors [15]. These results indicate that the affinity of the two polyenes for cell plasma membranes cannot account alone for their biological activity since quasi equivalent bindings lead to different biological effects. In support Szponarski et al. [43] reported that the binding of several polyenic derivatives is disconnected from their haemolytic activity on red blood cells. The slightly lower binding of *N*-Fru-AmB observed in our experiments could likely be related to its higher solubility in aqueous medium.

Results have been quantified according to Fung and Stryer's theory which has been developped from studies on synthetic lipid vesicles. It is based on the consideration of the membrane as a plane two-dimensional system. This, which has been demonstrated as being valid in the case of vesicles, can accurately be extrapolated to the case of cell membrane since its curvature is low as compared to that of vesicles. The point to be discussed is the fact that we conserved the same surface density unit as Fung and Stryer, that is 'acceptors per lipid' Indeed, the vesicle membrane is constituted of a homogeneous lattice of phospholipids with a surface area per molecule which has been taken equal to 70 Å^2 by Fung and Stryer. In the case of cell membranes, the membrane lattice is constituted by the association of proteins and lipids. The proteic domain has been left out of account in our calculations considering that the fluorescent probes used labelled the lipidic phase of the membrane and that the polyene antibiotics are believed to interact with lipids. The lipidic domain of the mammalian cell membrane is mainly constituted by phospholipids, glycolipid and cholesterol. Experiments performed with monolayers at 30 mN · m^{-1} by Evans et al. [44] have shown that the surface area occupied by 1-palmitoylphosphatidylcholines ranges from 50 to 90 Å^2-depending on the saturation of the fatty acyl chain. Under the same conditions, cholesterol occupies a surface area of 40 Å^2. According to their structure glycolipids can be assumed to have surface areas in the same range than phospholipids. For the sake of simplic-

ity we considered, in the present study, a mean lipid surface area equal to 70 $Å^2$ and expressed the acceptor surface density in the same unit as Fung and Stryer.

The steady-state anisotropy value (r_{ss}) is usually considered as being determined mainly by the degree to which the fluorophore rotations are restricted by the molecular packing of the lipids (a static factor) rather than its rotational rate (a dynamic factor). The static part is related to the lipid order parameter while the dynamic part is related to the microviscosity. Whatever is the origin of the AmB-induced changes in r_{ss}, they indicate strong changes in the probe environment. The fluidity of the lipid matrix is certainly one of the important factors that regulate the cell membrane feature by modulating for instance the degree of exposure of membrane functional proteins (see, for example, Ref. 27). Yet the amplitude of the r_{ss} decrease that we have observed with AmB in this study does not seem compatible with the conservation of the probe environment structure. One hypothesis is that AmB would induce the formation of micelles or aggregates within the membrane as suggested by O'Neill et al. [45] for nystatin, another polyene antibiotic or by Maher and Singer [46] for small amphipathic molecules. Nystatin was shown to form such micelles in L cells at a nystatin to lipid ratio greater than 1 to 300. Our experiments performed with $2 \cdot 10^{-6}$ M AmB, could lead to a comparable situation. Micelle formation can promote a gross redistribution of components in the plane of the membrane. Furthermore detergent-like effects could lead to the extraction of membrane components and explain the re-increase of r_{ss} for AmB concentration above $5 \cdot 10^{-6}$ M.

With *N*-Fru-AmB, only a low decrease of r_{ss} was observed. A small contamination of the derivative by the parental AmB molecule might also account for this small decrease. Yet, it is clear that AmB and its *N*-Fru-AmB derivative have different effects on the membrane even though they similarly bind to the membrane. The induction of AmB micelles in the membrane and not by *N*-Fru-AmB remains a possible explanation of their differential effect on thymocytes. The membrane disorganisation induced by AmB could be related to its high toxicity.

It is now clearly established that DPH penetrates inside the cells and binds to intracellular membranes and the plasma membrane as well [33–36]. On the other hand, it is admitted that the cationic derivative TMA-DPH selectively stains the plasma membrane at least during short term incubations. The fact that the energy transfer efficiencies of DPH to AmB and its derivative are similar or even greater in the case of *N*-Fru-AmB to those to TMA-DPH suggests that AmB can cross the plasma membrane and acceed to the intracellular membranes in a relatively short time. Furthermore, *N*-Fru-AmB would accumulate in the intracellular compartment more than AmB. AmB has generally been considered to only bind to plasma membrane. This was not based on experimental evidence but on the assumption that cholesterol is the target of AmB and the ratio cholesterol/phospholipid is lower in intracellular membranes. However, model calculations suggest that as a matter of fact a high fraction of the cholesterol is present in the membranes of intracellular organelles [47]. For instance, if we consider Roozemond and Urli's data on rabbit thymocytes [48], it appears that only 34% of the cholesterol molecules and 14% of the phospholipids are in the plasma membrane. It is evident that the large amount of remaining lipids, located inside the cells, cannot be neglected the more as AmB binds also to phospholipids [12,41].

In conclusion, it is tempting to postulate that the two polyenes are forming different complexes with plasma membrane components for similar molecular ratios. AmB-membrane complex would lead to high disorganisation of the membrane, whereas *N*-Fru AmB will only slightly modify it allowing the expression of physiological activities such as stimulation of the cells. We plan to test this hypothesis by studying the interaction of the two polyenes with model membranes such as lipidic vesicles. In other respects we have shown that the plasma membrane might not be the only important cellular target of the polyenes. Their intracellular location has to be taken into account in the analysis of their biological effects. Experiments are in progress to determine some cytoplasmic target of the polyenes.

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