NO EVIDENCE OF OXIDANT EVENTS IN AMPHOTERICIN B CYTOTOXICITY VERSUS *L. INFANTUM* PROMASTIGOTES

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Summary :

Amphotericin B is used for the treatment of systemic mycoses and visceral leishmaniasis. The objective of our study was to evaluate the impact of catalase, ascorbic acid and ketoconazole on the amphotericin B toxicity towards *Leishmania* promastigotes membrane by two flow cytometric tests, the membrane potential assay using a cationic dye, [$DiOC_5$ (3)], and the membrane permeability test using propidium iodide. The collapse of membrane potential appeared at amphotericin B concentrations weaker than those assessed by the membrane permeability test. The binding of amphotericin B to membrane sterol was not modified by catalase or ascorbic acid whereas amphotericin B-induced growth inhibition could be modulated by these products. The permeabilizing effect of amphotericin B on parasite membrane was strongly reduced in the presence of ketoconazole. These results confirmed the pore hypothesis of amphotericin B action and suggested that flow cytometric methods constituted a valuable alternative to conventional methods for assessing the effect of drugs on cellular membrane and evaluating parasite susceptibility to polyene antibiotics.

KEY WORDS : *Leishmania infantum*, membrane potential, antibiotic susceptibility, membrane permeability, amphotericin B, ketoconazole, ascorbic acid, catalase.

Résumé : CYTOTOXICITÉ DE L'AMPHOTÉRICINE B SUR LA FORME PROMASTIGOTE DE *L. INFANTUM* : AUCUNE PREUVE DE L'INTERVENTION DE PHÉNOMÈNES OXYDANTS

L'amphotéricine B est utilisée pour le traitement des mycoses systémiques et des leishmanioses viscérales. L'objectif de notre étude était d'évaluer l'impact de la catalase, de l'acide ascorbique et du kétoconazole sur la toxicité membranaire de l'amphotéricine B envers les formes promastigotes de Leishmania *par deux test de cytométrie en flux, le test du potentiel de membrane utilisant un marqueur cationique, le [$DiOC_5$ (3)], et le test de la perméabilité membranaire basé sur l'utilisation de l'iodure de propidium. La diminution du potentiel de membrane est apparue pour des concentrations d'amphotéricine B plus faibles que celles déterminées par le test de perméabilité membranaire. La fixation de l'amphotéricine B aux stérols membranaires n'a pas été modifiée ni par la catalase ni par l'acide ascorbique alors que son activité anti-prolifératives pourrait être modifiée par ces produits. L'effet perméabilisant de l'amphotéricine B sur la membrane parasitaire a été fortement réduit en présence de kétoconazole. Ces résultats ont confirmé que la formation de pores membranaires consituaient le principal mécanisme dans l'action létale de l'amphotéricine B. Ainsi, les méthodes de cytométrie en flux constituent une alternative intéressante aux méthodes conventionnelles pour l'évaluation de l'effet de drogues sur la membrane cellulaire et pour l'évaluation de la sensibilité des parasites aux antibiotiques polyéniques.*

MOTS CLÉS : Leishmania infantum, *potentiel de membrane, sensibilité aux antibiotiques, perméabilité membranaire, amphotéricine B, kétoconazole, acide ascorbique, catalase.*

INTRODUCTION

Protozoa of the genus *Leishmania* are obligate intracellular parasites identified as the causative agents of leishmaniasis, parasitic disease widely distributed through the world and considered since 1984 as one of the major public health problems by the World Health Organization. The generally accepted therapy for all forms of leishmaniasis consists of pentavalent antimonial agents (Berman, 1988; Herwaldt, 1992), however clinical resistances and therapeutic failures in immunocompromised patients have been increasingly recognized in recent years, leading to the need of secondline drugs (Faraut-Gambarelli *et al.*, 1997). The pore forming polyene antibiotic amphotericin B, generally used to cure serious systemic fungal infections, has been shown efficient against various *Leishmania* species and successfully applied to the treatment of both cutaneous and visceral leishmaniasis (Croft *et al.*, 1991; Davidson *et al.*, 1994). Moreover, recent developments have shown a significant improvement of amphotericin B therapeutic index by the use of new delivery systems in which the antibiotic is bound to liposomes (Paul *et al.*, 1997; Mullen *et al.*, 1997).

It is generally accepted that the damaging action of amphotericin B to cells originates from its binding to sterols incorporated in cellular membranes, with the immediate consequence of membrane disorganization and depolarization, formation of aqueous pores and increased permeability to protons and monovalent

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cations (Beggs *et al.*, 1994; Ramos *et al.*, 1996). However, experiments performed on unicellular fungi and mammalian cells have suggested that possible oxidative stress in relation to amphotericin B autooxidation could also complete amphotericin B toxicity, since protection against amphotericin B could be achieved by antioxidant compounds such as catalase and enhanced toxicity could be produce by prooxidants such as ascorbic acid (Sokol-Anderson *et al.*, 1986, 1988; Bratjburg *et al.*, 1990).

In a preceding study, we demonstrated that the flow cytometric techniques based on the measurement of membrane potential and permeability developped by Ordonez in the 1990s on bacteria and fungi could be applied to the assessment of amphotericin B susceptibility in *Leishmania* promastigotes (Azas *et al.*, 1997b). For this purpose, we showed that the effect of amphotericin B on *Leishmania* membrane was illustrated by a dose-dependent potential drop that reached its maximal level at the same concentrations that inhibited *in vitro* parasite growth. Although these results supported clearly the sterol hypothesis of amphotericin B-membrane interactions, additional experiments were necessary for investigating the effects of oxydant-dependent events on amphotericin B-membrane interactions.

In the present work, we planned to explore the oxidative hypothesis of amphotericin B action on cellular membrane. Therefore we measured the effects of prooxidant and antioxidant compounds such as ascorbic acid and catalase on amphotericin B-induced membrane potential and permeability variations, as compared to those of sterol synthesis inhibitors such as ketoconazole.

MATERIAL AND METHODS

Amphotericin B-induced membrane depletion and antiproliferative activity on *Leishmania* promastigotes were studied by three series of experiments realised in triplicate using flow cytometry: the immediate effect of amphotericin B on membrane permeability was assessed by a dye exclusion test using propidium iodide, the action of amphotericin B on membrane potential was estimated by using [$DiOC_5$ (3)], a carbocyanine dye which is positively charged and accumulates inside the cell according to the Nernst equation, rending it fluorescent, the antileishmanial activity of amphotericin B was established by determining parasite growth. Influence of oxidative events and membrane sterol amounts on amphotericin B-membrane interactions was performing respectively by a 15 minutes pre-treatment with subtoxic concentrations of ascorbic acid or catalase and by a 72 hours pre-treatment with subtoxic concentrations of ketoconazole. Drugs were incorporated in triplicate cell cultures.

PARASITES AND REAGENTS

Experiments were performed on the registred strain *L. infantum* (MHOM/FR/LEM75), promastigotes were grown in RPMI medium (Eurobio, Paris, France) supplemented with 15 % foetal calf serum (Sigma Chemical Compagny, St.-Louis, MO, USA), incubated at 25° C and replicated every five-seven days. 3,3'-dipenthyloxocarbocyanine iodide [$DiOC_5$ (3)] was obtained from Molecular Probes (Eugene, OR, USA). Amphotericin B, propidium iodide (PI), ascorbic acid and ketoconazole were purchased by Sigma Chemical Compagny (St.-Louis, MO, USA).

FLOW CYTOMETRIC TECHNIQUES

Amphotericin B-induced membrane permeability changes were measured by the flow cytometric technique described by Green *et al.* in 1994. This method was based on the capacity of propidium iodide to infiltrate damaged membranes and accumulate inside nuclei rending them fluorescent. Propidium iodide 50 µg/ml final concentration was incorporated into *Leishmania* exponentially growing cultures after addition of a range of amphotericin B concentrations, then 10,000 parasites were immediately analysed by flow cytometry. Cells with damaged membranes could be identified according to their high fluorescence compared to non-altered promastigotes and the action of amphotericin B on membrane permeability was expressed as the percentage of non fluorescent cells (intact cells) compared to the control culture.

Membrane potential was estimated by a flow cytometric technique previously adapted to *Leishmania* parasites (Azas *et al.*, 1997): [$DiOC_5$ (3)], 0.5 µM final concentration was added to log-phase cultures and incubated at 25° C during three hours. At the end of the incubation period promastigotes were analysed for their green fluorescence: membrane potential was expressed as the average fluorescence intensity observed in a distribution of 10,000 cells.

The antiproliferative activity of amphotericin B was measured in promastigotes treated with various amphotericin B concentrations for a 48 hours incubation period, promastigotes growth was estimated by numbering promastigotes by flow cytometry.

FLOW CYTOMETRIC PARAMETERS

Membrane permeability, membrane potential and parasite growth were analysed on a FACScan analytical flow cytometer (Beckton Dickinson, Paris, France) equipped with a 15 mW, 488 nm air cooled argon ion laser. Mul-

tiparametric data were acquired using FACScan resaerch software. The optimized instrument parameters were: Forward scatter (Voltage E-1, gain 1, mode Log), Side scatter (voltage 250, gain 1, mode Log), Fluorescence 1 (voltage 418, gain 1, mode Log) and Fluorescence 2 (voltage 550, gain 1, mode Log). Comparison between amphotericin B-induced membrane permeability or potential changes and parasite growth inhibition was achieved by determining the Inhibitory Concentrations 50 % (IC_{50}) or 90 % (IC_{90}), corresponding to the concentrations of antibiotic that induced respectively 50 % or 90 % membrane-damaged parasites, a 50 % or 90 % membrane potential drop and a 50 % or 90 % growth inhibition compared to the control culture.

RESULTS

The effects of amphotericin B on membrane permeability, membrane potential and promastigote growth are reported in figures 1 and 2 and resumed in table I. The mathematical model used to calculate the inhibitory concentrations (IC_{50}, IC_{90}) was shown to be statistically acceptable in all experiments ($r > 0.90$). Figure 1 shows effects of amphotericin B on membrane permeability after various incubation periods (1a) and with increasing amphotericin B concentrations (1b). Amphotericin B-induced membrane damage was apparent after only 20 min of treatment with high concentrations of antibiotic (0.27 µM) and was achieved after 30 min. However, the effect of lower concentrations of antibiotic ($< IC_{90}$) on parasite membrane required more than 45 min of treatment to be maximal. The percentage of intact cells related to the PI membrane permeability decreased gradually in promastigote cultures treated with increasing concentrations of amphotericin B and reached a value of 10 % at the amphotericin B concentration IC_{90} of 0.246 µM (Table I). Figure 2 shows membrane potential, membrane permeability and parasite growth variations after amphotericin B treatment. Membrane permeability was evaluated after 45 min of treatment. Membrane potential expressed as the percentage of fluorescence compared to the control culture, dropped with increasing concentrations of antibiotic and reached a minimum (about 60 %) at the concentrations that inhibited cell growth (CI_{90} = 0.126 µM).

The inhibitory concentrations obtained in the membrane potential assay were highly related to the antiproliferative activity of amphotericin B.

The action of catalase on the amphotericin B-induced activity versus membrane permeability at various time intervals with increasing concentrations of antibiotic is presented in figure 3 and resumed in table II. After 30 min of treatment, the maximal action of amphotericin B on membrane permeability was not modulated by catalase treatment whatever the concentrations tested. We just observed a slight effect on the velocity of membrane- pores formation permeable to PI. The same results were found for the combination of amphotericin B and ascorbic acid as seen in table II. However, the leishmanicidal activity of amphotericin B on *Leishmania* promastigotes treated by ascorbic acid and catalase was enhanced after 48 h of incubation. The inhibitory concentration IC_{90} was divided by two after treatment with subtoxic concentrations of catalase or ascorbic acid. The effects of ketoconazole pretreatment on amphotericin B toxicity in promastigotes are presented in figure 4 and summarized in table II. Ketoconazole-treated promastigotes responded slightly to amphotericin B. The inhibitory concentrations IC_{50} of amphotericin B calculated by the mathematical model

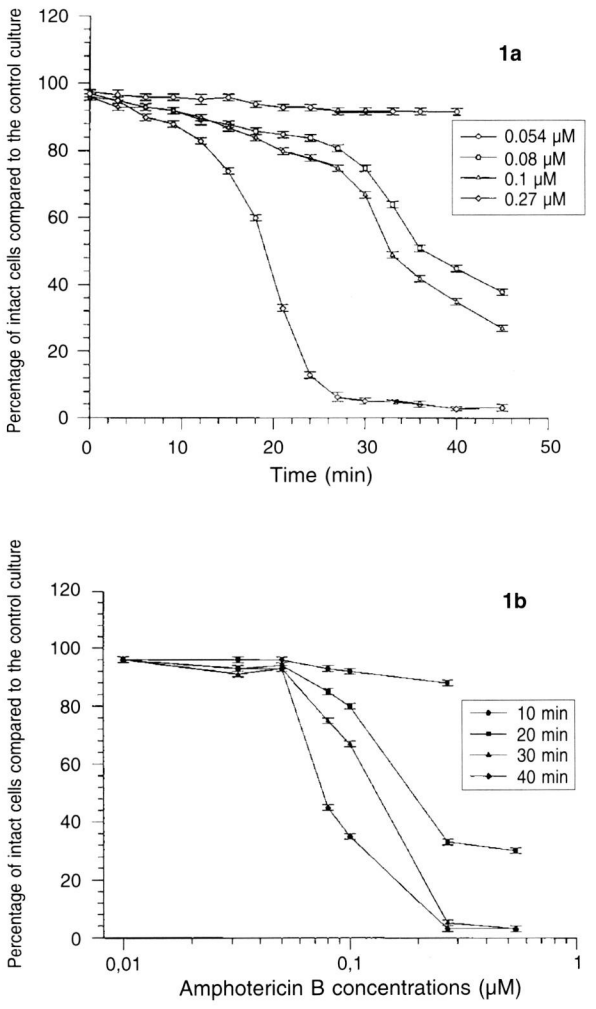

Fig. 1. – Amphotericin B activity on promastigote membrane permeability.
1a: at various time intervals.
1b: at various amphotericin B concentrations.

Parameters	Model	Coefficients a, b, c	Correlation Coefficient r^2	IC_{50} (µM)	IC_{90} (µM)
Growth	y = a + bexp (-x/c)	a = −1.08 b = 110 c = 0.04	0.94	0.034	0.109
Membrane integrity	y = a + bexp (-x/c)	a = 0.89 b = 105.2 c = 0.09	0.91	0.075	0.246
Membrane potential	y = a + bexp (-x/c)	a = 66.2 b = 38.42 c = 0.05	0.96	0.040	0.126

IC_{50} = Amphotericin B concentration that induced a 50 % decrease in growth, membrane potential or in percentage of viable cells compared to the control culture.
IC_{90} = Amphotericin B concentration that induced a 90 % decrease in growth, membrane potential or in percentage of viable cells compared to the control culture.

Table I. – Action of amphotericin B on the growth, membrane integrity and membrane potential of *Leishmania* promastigotes.

used in the two flow cytometric techniques increased gradually with increasing concentrations of ketoconazole. The inhibitory concentration IC_{90} in the membrane potential assay was multiplied by 50 after treatment with subtoxic concentrations of ketoconazole and was distributed from 0.126 to 6.34 µM while in the membrane permeability test the IC_{90} was 28 fold higher (from 0.246 to 6.845 µM). However, the azole action on amphotericin B antiproliferative activity was

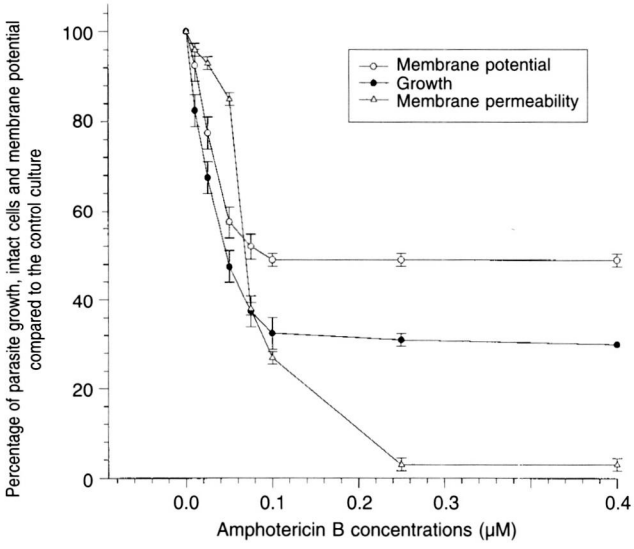

Fig. 2. – Effects of amphotericin B on membrane potential, membrane permeability and parasite growth.
The determination of membrane permeability was based on the capacity of PI to infiltrate damaged membranes rending the cells fluorescent. The results were expressed as the percentage of non fluorescent cells (intact cells) compared to the control.

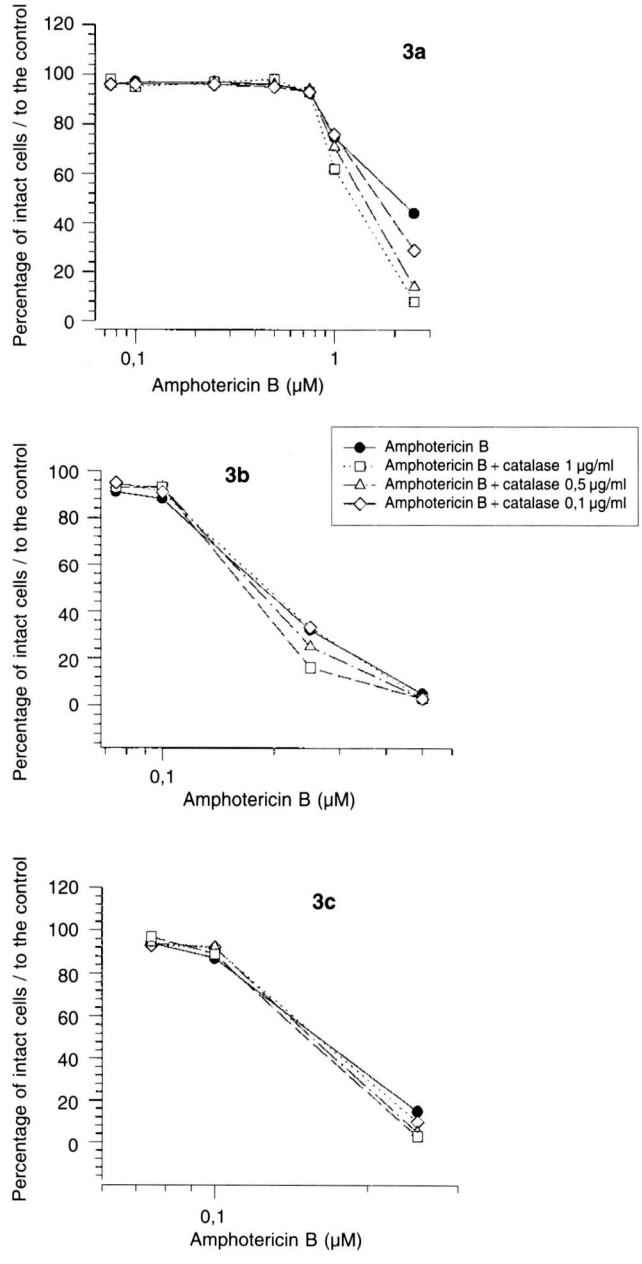

Fig. 3. – Effects of catalase on the amphotericin B-induced activity versus membrane permeability at various time intervals.
3a: treatment: 15 min.
3b: treatment: 30 min.
3c: treatment: 45 min.

Products	Growth			Membrane permeability after 1 h of treatment			Membrane potential		
	r^2	IC_{50}	IC_{90}	r^2	IC_{50}	IC_{90}	r^2	IC_{50}	IC_{90}
Amphotericin B (µM)	0.93	0.034	0.109	0.91	0.075	0.246	0.94	0.040	0.126
Amphotericin B+									
Catalase 0.01 µg/ml	0.95	0.034	0.115	0.94	0.086	0.246	0.90	0.030	0.106
Catalase 0.05 µg/ml	0.94	0.027	0.090	0.95	0.081	0.233	0.92	0.037	0.124
Catalase 0.1 µg/ml	0.95	0.021	0.070	0.91	0.080	0.227	0.96	0.027	0.110
Amphotericin B+									
Ascorbic acid 1 µg/ml	0.96	0.025	0.083	0.95	0.084	0.241	0.90	0.032	0.103
Ascorbic acid 5 µg/ml	0.90	0.011	0.048	0.92	0.087	0.248	0.91	0.030	0.102
Ascorbic acid 10 µg/ml	0.91	0.010	0.041	0.91	0.078	0.244	0.90	0.025	0.085
Amphotericin B+									
Ketoconazole 0.001 µg/ml	0.91	0.037	0.129	0.94	0.110	0.714	0.95	0.256	0.446
Ketoconazole 0.005 µg/ml	0.90	0.048	0.157	0.90	0.610	4.170	0.90	0.595	2.74
Ketoconazole 0.01 µg/ml	0.92	0.054	0.188	0.91	1.010	6.845	0.91	1.060	6.34

r^2 = Correlation coefficient related to the mathematical model.
IC_{50} = Amphotericin B concentration (µM) that induced a 50 % decrease in growth, membrane potential or in percentage of viable cells compared to the control culture.
IC_{90} = Amphotericin B concentration (µM) that induced a 90 % decrease in growth, membrane potential or in percentage of viable cells compared to the control culture.

Table II. – Effects of catalase, ascorbic acid and ketoconazole on *L. infantum* amphotericin B toxicity.

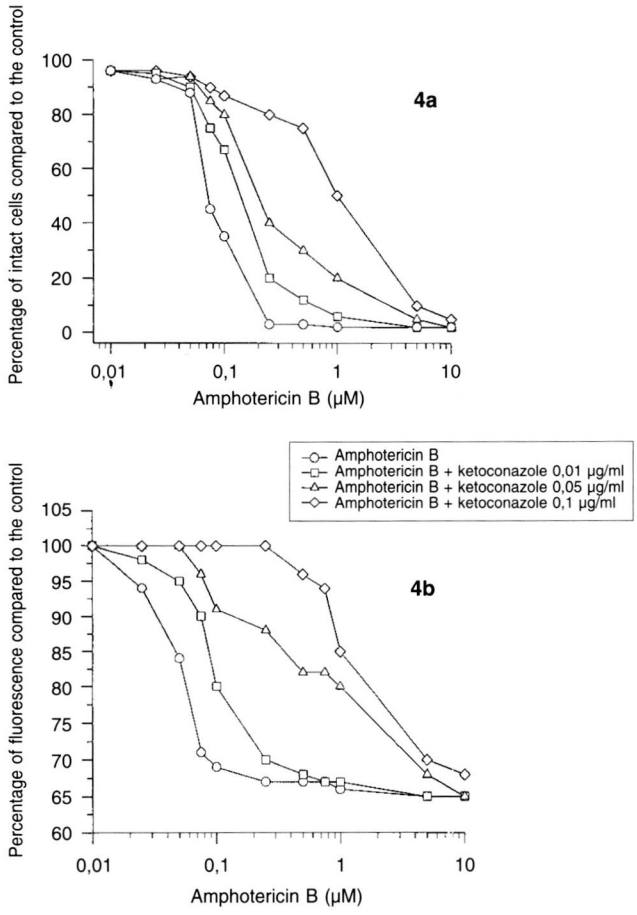

Fig. 4. – Effects of ketoconazole on membrane toxicity of amphotericin B.
4a: membrane permeability.
4b: membrane potential.

weaker on promastigotes incubated for 48 h since the inhibitory concentration IC_{90} was only multiplied by 1.7 (0.109 to 0.188 µM).

DISCUSSION

In sterol-containing model membranes, amphotericin B forms aqueous pores that modify membrane permeability (Bratjburg *et al.*, 1990). In parasitic protozoa such as *Leishmania* spp, the pore theory of amphotericin B action has been supported by the finding that a total depletion of desmethyl sterols by ketoconazole reduced the lytic activity of amphotericin B, by depriving the polyene antibiotic of its capacity to form aqueous pores (Ramos *et al.*, 1994, 1996).
However, this hypothesis has been challenged by the fact that amphotericin B permeabilizing effect on sensitive cells was dissociated from its lethal effect. This observation led to the conclusion that other mechanisms such as chemical degradation of membrane or physicochemical detergentlike membrane damage may be primarily responsible for the fungicidal action (Ramos *et al.*, 1996).
Indirect support for this hypothesis is provided by the evidence of protection against amphotericin B-induced toxicity afforded by antioxidant compounds such as catalase while prooxidant compound like ascorbic acid could enhanced the fungicidal effects of amphotericin B (Sokol *et al.*, 1988). This modulation of antibiotic action could be related to two kinds of amphotericin B-binding to membrane sterol and some authors have

proposed that the membrane- amphotericin B binding could be dependent of the type of cations and anions included in the external aqueous solution (Osaka *et al.*, 1997).

In our study, we used flow cytometric techniques to investigate the impact of three compounds on amphotericin B-treated *Leishmania*: catalase, ascorbic acid and ketoconazole. PI is an intercalator of double and single stranded nucleics acids. This impermeant probe stain only organisms wich have damaged membrane and was used in direct viability assays. However, the measurement of membrane permeability based on the uptake of the fluorescent probe via the antibiotic damaged membrane could be adapted to *L. infantum* promastigotes for assessing the effects of pore forming antibiotics on the parasite (Pore *et al.*, 1994).

The length of time required for the membrane to be damaged is an important consideration. It is likely that more time would be required for antibiotic penetration of the cell wall of yeasts. Concerning *Leishmania*, we found that a 30 min exposure to the antibiotic was all that was required. The membrane potential assay was based on the use of a cationic dye which accumulates inside the cell according to the Nernst equation. In a preceding study, we demonstrated that this technique could be adapted to unicellular parasite such as *Leishmania*: the maximal membrane potential decrease was obtained for the same concentration of amphotericin B that inhibited parasite growth (Azas *et al.*, 1997a; Azas *et al.*, 1997b). In the present study, the collapse of membrane potential appeared at amphotericin B concentrations weaker than those assessed by the membrane permeability test. This result was fully consistent with recent data described by Ramos *et al.* (1996): low concentrations of amphotericin B enhanced the cation permeability across ergosterol-containing liposomes leading to a drop in membrane potential while higher concentrations induced the formation of aqueous pores permeable to both cations and anions, responsible for cell death.

Our results demonstrated that amphotericin B-membrane damaged is achieved after a 60 min antibiotic treatment at concentrations that inhibited parasite growth. During this time the toxicity of amphotericin B on membranes evaluated by the increase of membrane permeability, was not modified by the addition of antioxidant or prooxidant. The binding of amphotericin B to membrane sterol was not modified by catalase or ascorbic acid which have been shown to modulate the number of $H_20_2^-$ molecules in the external aqueous solution. However, after a 48 h incubation period, catalase enhanced antibiotic effects on growth inhibition. These results are similar to those reported by Beggs *et al.* (1979) or Sokol-Anderson *et al.* (1986) on yeasts. These authors postulated that catalase might inhibit amphotericin B autooxidation and extend antibiotic effects.

Results observed with ketoconazole agreed with the pore theory of amphotericin B action. A total depletion of desmethyl sterols by the azole derivative effectively reduced the antiproliferative activity of amphotericin B and deprived the polyene antibiotic of its capacity to form aqueous channels in the cell membrane (Ramos *et al.*, 1994). The permeabilizing effect of amphotericin B on parasite membrane measured by increase of membrane permeability and decrease of membrane potential, was strongly reduced in the presence of ketoconazole. Nevertheless, the effect of ketoconazole on amphotericin B-related growth inhibition after a 48 h incubation period was greatly weaker, resulting probably from membrane sterol depletion as well as perturbation of lipid metabolism.

In conclusion, our results corroborated earlier reports demonstrating that the main mechanism of antileishmanial action of amphotericin B consisted at the formation of aqueous pores permeable to small cations and anions (Ramos *et al.*, 1996, 1994). This mechanism appeared to be independant from oxidant and antioxidant events, and was strongly related to the amount of lipid in the cell membrane (Mbongo *et al.*, 1998). On this basis, flow cytometric methods constituted a valuable alternative to conventional methods for assessing the effect of drugs on the cellular membrane and evaluating parasite susceptibility to polyene antibiotics: results obtained by these methods could not be modified by modulation of external culture conditions.

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