Inhibition by Cu^{2+} of amphotericin B induced lysis of erythrocytes

Monique Chéron, Jacques Bolard and Janina Brajtburg*

Département de Recherches Physiques (LA CNRS No. 71), Université Pierre et Marie Curie, 4, Place Jussieu, 75230 Paris Cédex 05, France and **Washington University School of Medicine, Division of Infectious Diseases, Department of Internal Medicine, Box 8051, 660 South Euclid Avenue, St. Louis, MO, USA*

Received 2 October 1984

Amphotericin B-induced lysis of erythrocytes is diminished in the presence of Cu^{2+}, but the prelytic amphotericin B-induced K^+ leakage is unaffected. These results and the weak binding of Cu^{2+} to amphotericin B, demonstrated by circular dichroism and EPR studies, are consistent with the view that Cu^{2+} protects erythrocytes by increasing their resistance to lysis.

Polyene antibiotic *Amphotericin B* *Hemolysis* *Erythrocyte* *Copper*

1. INTRODUCTION

Amphotericin B (AmB) is a polyene antibiotic widely used in the treatment of systemic fungal infections. It has been reported that divalent metal ions affect the interaction of polyene antibiotics with cells, particularly erythrocytes [1]. Because Cu^{2+} is present in serum and erythrocytes, we were interested in determining whether amphotericin B interacts with Cu^{2+} and what effect Cu^{2+} has on amphotericin B cytotoxicity toward erythrocytes.

An additional reason for studying copper effect is that it has been used to solubilize polyene antibiotics in order to facilitate the drug administration [2,3]. It was recently shown [4] that Cu^{2+} polyene derivatives have an in vitro activity similar to that of the parent compounds.

We have studied the influence of Cu^{2+} on AmB-induced K^+ leakage of erythrocytes and hemolysis. We have also studied the Cu^{2+}–AmB complex formation in water, by circular dichroism (CD) and EPR, the complex formation being ascertained either by inducement of optical activity in the d–d transitions of Cu^{2+} in the presence of the chiral AmB or by modification of the Cu^{2+} EPR signal.

In an attempt to better understand the relationship between the Cu^{2+} effect on antibiotic toxicity and antibiotic–Cu^{2+} complex formation we performed some of the experiments with the methylester of amphotericin E (AmE).

2. MATERIALS AND METHODS

AmB was purchased from Calbiochem (San Diego, CA) and used without further purification. The water-soluble derivatives, *N*-glucosyl AmB and AmE, were gifts from Professor E. Borowski (Technical University, Gdańsk, Poland).

AmB was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in sample (0.25%) did not affect erythrocytes. *N*-Glucosyl AmB and AmE were dissolved in distilled water. The experiments were conducted, unless otherwise stated, in 0.155 M NaCl buffered with 10 mM sodium phosphate to pH 7.4 (PBS). The reagents were dissolved in distilled water and filtered on a Milli-Q water purification system. The Cu^{2+} content of PBS was always lower than 5×10^{-6} M.

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

Human venous blood collected in tubes containing EDTA (1 mg EDTA/ml blood) was centrifuged at 1500 × *g* for 10 min and the plasma and buffy coat were removed. Erythrocytes were then washed with PBS, dispersed in equal volumes of PBS and used on the following day. The experiments on permeability and hemolysis of erythrocytes were performed as in [1], with minor modifications. Erythrocytes were dispersed in PBS (5 μl of packed cells/ml buffer) containing various concentrations of $CuSO_4$, antibiotic was added and incubations were performed for the designated times at room temperature with occasional shaking.

For measurements of K^+ or hemoglobin retention, erythrocytes were harvested by centrifugation, rinsed once with PBS and lysed by 15 mM $LiNO_3$ (Fisher Scientific Products, Pittsburgh, PA). The concentration of K^+ was measured in a flame photometer (model 430; Corning Medical and Scientific Medfield, MA) and of hemoglobin in a spectrophotometer at 550 nm. The progress of lysis over time was estimated from the changes in transmittance of erythrocytes dispersion at 650 nm.

For both spectrophotometric determinations, the Coleman Junior II Spectrophotometer was used. Absorption spectra were recorded with a Cary 219 spectrophotometer, CD spectra with a Jobin-Yvon Mark III dichrograph and EPR spectra at −180°C with a Varian CSE 109 spectrophotometer.

3. RESULTS

3.1. *Effect of Cu^{2+} on the permeabilizing and lytic action of AmB on erythrocytes*

Erythrocytes incubated in PBS alone or in PBS supplemented with Cu^{2+} at concentrations of 10–75 μM did not lose K^+ and did not lyse during the experiments. Erythrocytes incubated with AmB at 1.2 μg/ml did not lyse but were almost completely depleted of K^+. Fig.1 shows that the presence of Cu^{2+} in a concentration range of 10–75 μM did not influence this AmB effect in PBS. (The lines illustrating retention of K^+ as a function of AmB concentration in the absence or presence of Cu^{2+} overlapped.) AmB at a higher concentration (e.g., 10 μg/ml) lysed erythrocytes. Line 'a' demonstrates almost total loss of

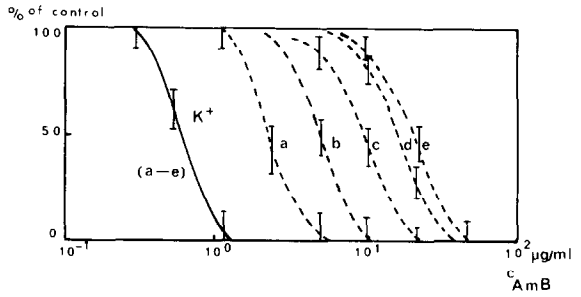

Fig.1. Effect of Cu^{2+} on AmB-induced decrease in retention of K^+ (——) or hemoglobin (– – –) by erythrocytes. Erythrocytes dispersed in PBS without Cu^{2+} (a) or supplemented with 9 μM (b), 18 μM (c), 37 μM (d) or 73 μM (e) Cu^{2+} were incubated 1 h with AmB and harvested. The concentrations of K^+ (——) and hemoglobin (– – –) remaining in erythrocytes were measured and expressed in percentages of control values found in AmB-untreated cells.

hemoglobin in erythrocytes incubated in the absence of Cu^{2+} with AmB at 10 μg/ml. In the presence of Cu^{2+} the AmB was less effective in inducing a decrease in retention of hemoglobin and this inhibitory effect of Cu^{2+} increased with the increase in Cu^{2+} concentrations to the extent that about 10-times higher concentrations of AmB were necessary to induce 50% hemolysis in the presence of 75 μM Cu^{2+} than in the absence of Cu^{2+}.

The inhibitory effect of Cu^{2+} on AmB induced lysis was also measured in time course experiments. Fig.2 shows that lysis in PBS induced by 5.0 μg/ml of AmB occurred much more extensively and rapidly in the absence than in the presence of 37 μM of Cu^{2+}.

The presence of Cu^{2+} during 10 min of incubation of erythrocytes with AmB also protected them from subsequent lysis in hypotonic NaCl solution. Fig.3 shows that erythrocytes incubated in PBS with 1.25 μg/ml AmB in the presence of 37 μM Cu^{2+} had less subsequent lysis in 0.50% NaCl than did those incubated with AmB in the absence of Cu^{2+}. This protective effect of Cu^{2+} was specific for AmB-induced damage; AmB-untreated erythrocytes incubated in PBS with or without 37 μM Cu^{2+} were subsequently equally sensitive to hypotonic hemolysis (inset, fig.3).

The hemolytic action of AmE was also strongly inhibited by Cu^{2+}. AmE at 20 μg/ml caused almost complete lysis of erythrocytes in the absence of

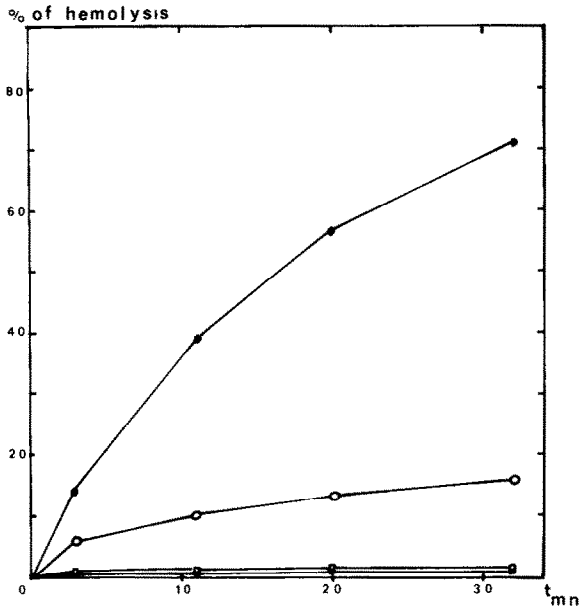

Fig.2. Effect of Cu^{2+} on time course of AmB-induced hemolysis. Erythrocytes were dispersed in PBS (■, ●) or in PBS supplemented with 37 μM of Cu^{2+} (□, ○). At time '0', 5.0 μg/ml amphotericin B in DMSO (●, ○) or only DMSO (■, □) were added. Transmittance of erythrocyte dispersions was measured at time points shown and results were expressed as percentages of hemolysis.

Cu^{2+} and no lysis at all in the presence of 37 μM Cu^{2+}.

3.2. *Complex formation between AmB and Cu^{2+} in aqueous buffer*

3.2.1. Absorption and circular dichroism

Measurements were performed either by preparing concentrated mixtures of reagents in DMSO and then diluting them to the desired concentration

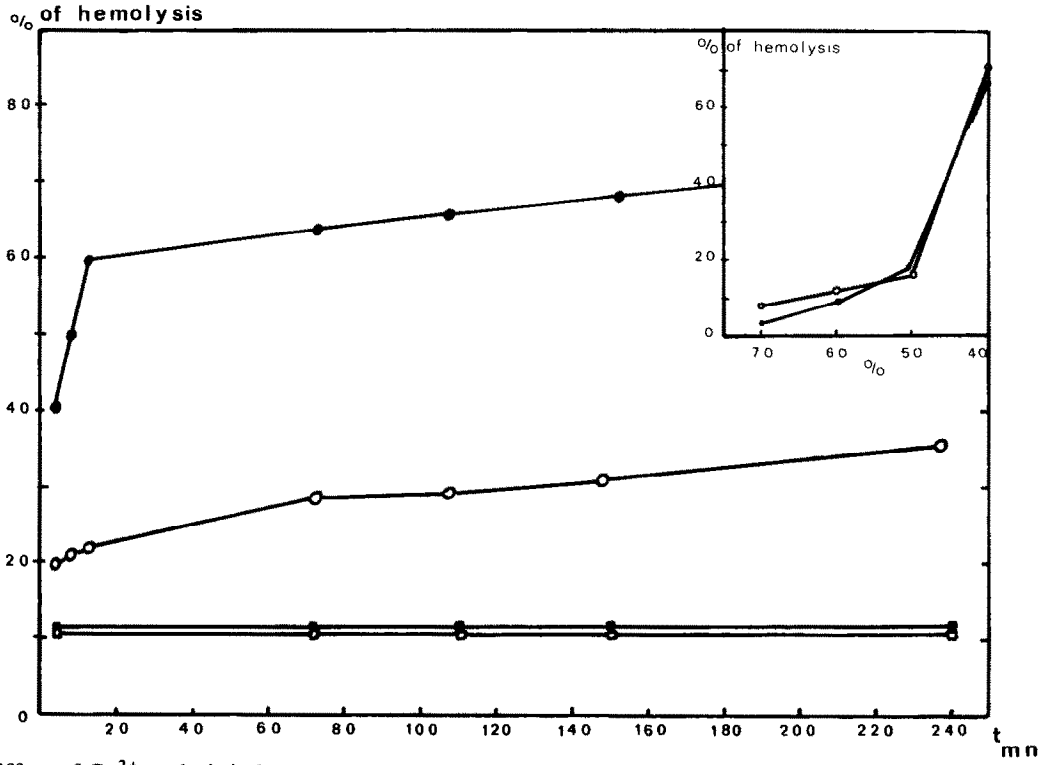

Fig.3. Effect of Cu^{2+} on lysis in hypotonic NaCl solution of erythrocytes pretreated with AmB. Erythrocytes dispersed in PBS were incubated 10 min without any addition (■), with 37 μM Cu^{2+} (□), with 1.25 μg/ml AmB (○) or with 1.25 μg/ml AmB and 37 μM Cu^{2+} (●). Erythrocytes were harvested by centrifugation and dispersed at time '0' in 0.50% NaCl. Transmittance of erythrocyte dispersion was measured at time points shown and results were expressed as percentages of hemolysis. The inset shows hypotonic lysis of AmB-untreated erythrocytes which had been preincubated 10 min in PBS in the absence (●) or presence of 37 μM Cu^{2+} (○) harvested by centrifugation and dispersed in various hypotonic NaCl solutions. 100% represents 150 mM in NaCl. Transmittance was read 4 min after cells were dispersed.

by adding aqueous buffer or by mixing the aqueous suspension of AmB with the Cu^{2+} solution. Similar results were obtained in both cases.

The spectra of the monomeric species of AmB, namely below the critical micellar concentration (CMC) which is approximately equal to 10^{-7} M, were not perturbed by the presence of Cu^{2+}, even in large excess.

The absorption spectra of the aggregates of AmB, above the CMC, were immediately modified by the presence of Cu^{2+} (fig.4A): above 300 nm, a decrease of the spectrum intensity was observed. This decrease depended on the amount of Cu^{2+} added but tended always towards a limiting value. In contrast, below 300 nm there was an increase in the intensity of the whole spectrum. If these mixtures were dissolved in an excess of methanol and their spectra compared to those of AmB without

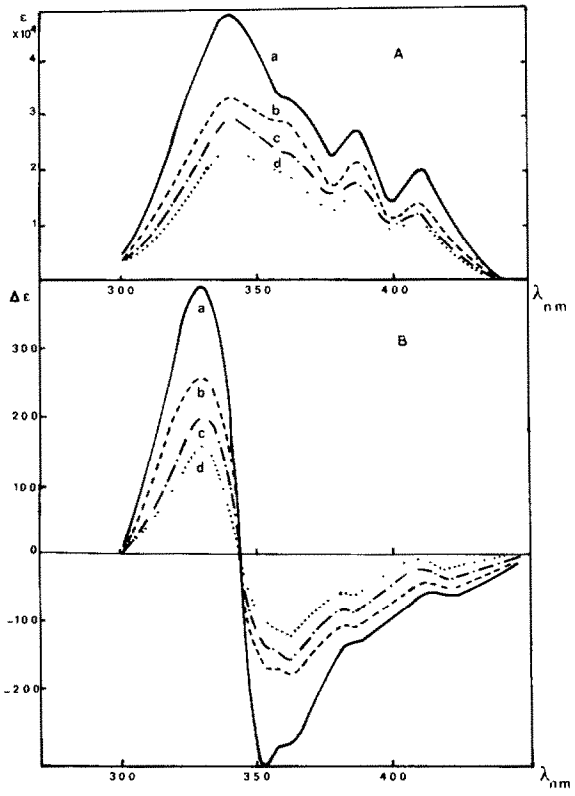

Fig.4. Absorption (A), and CD (B) of a 1.25×10^{-5} M suspension of amphotericin B in phosphate buffer, pH 7.4: (a) without Cu^{2+}; (b–d) with 10^{-5} M and 7.5×10^{-5} M Cu^{2+}. In the absorption spectra the scattering background was subtracted.

Cu^{2+} in the same conditions, no differences were apparent.

The CD spectra of the aggregates of AmB were the superposition of an excitonic doublet centered around 340 nm and of three negative bands at 423, 392 and 368 nm. In the presence of Cu^{2+} a decrease of the whole spectrum was observed, the doublet intensity decreasing more than that of the three negative bands (fig.4B). This general decrease was limited and did not exceed a factor of 7 for a very large excess of Cu^{2+}.

In the wavelength region of the d–d transition (800–450 nm) no CD was observed even with a concentrated solution (10^{-3} M) and a cell path of 10 cm. This was in contrast with what was observed by us and in [5] in organic solvents.

3.2.2. Electronic paramagnetic resonance

The intensity of the Cu^{2+} signal observed at $g = 2.07$ increases in the presence of AmB (more than 4-times for a 10^{-4} M solution of Cu^{2+} in the presence of 10^{-3} M AmB) but did not shift.

3.3. *Complex formation between Cu^{2+} and water-soluble derivatives of AmB*

The addition of Cu^{2+} to the aqueous solution of *N*-glucosyl AmB caused an immediate precipitation. With AmE we observed a decrease of the excitonic CD doublet and of the absorption band at 340 nm but an increase of the bands around 400 nm.

4. INTERPRETATION

Our results (fig.1) show that copper ions have no influence on the first stage of AmB action, K^+ leakage. In contrast, the second stage, hemolysis, is strongly affected: the inhibition is such that in the presence of 75 μM Cu^{2+}, a 10-fold higher concentration of AmB is necessary to induce a 50% level of hemolysis than in Cu^{2+} absence.

The inhibition could result from a chemical degradation of AmB. Although AmB can autoxidize [7–9] and this process could have been accelerated by the presence of Cu^{2+}, the absence of modifications of the AmB spectrum when it was redissolved in organic solvents after 2 h incubation with Cu^{2+}, in water, and the absence of perturbation by Cu^{2+} of AmB-induced K^+ leakage, indicate that the AmB molecule remained unchanged.

Hemolysis could also be inhibited by the binding of AmB to Cu^{2+}. An interaction occurs since the intensity of the AmB absorption and CD spectra decreases in the presence of Cu^{2+}. However, this flattening of the bands may be assigned to the effect described in [6], i.e., a perturbation of Cu^{2+} of the AmB state of aggregation. The fact that the modification of the EPR signals is small confirms that an interaction does exist but is weak.

When AmE was assayed, the addition of Cu^{2+} caused a decrease in the excitonic doublet, which indicates a decrease in self-association. Under the conditions of our experiments (pH 7.4) AmE bears only one charge [10], on NH_3^+, and cannot bind to the positive Cu^{2+}. We attribute, therefore, the decrease of the dichroic doublet to a salt effect, to which AmE is very sensitive.

In summary, the weakness of the AmB–Cu^{2+} complex, the fact that Cu^{2+} does not affect AmB-induced K^+ leakage and the inhibition by Cu^{2+} of AmE-induced hemolysis, even though AmE does not bind to Cu^{2+}, indicate that the decrease in the erythrocyte sensitivity to lysis cannot be attributed to the AmB–Cu^{2+} complex formation. We therefore accept the alternative view that Cu^{2+} interacts with erythrocytes and increases their resistance to AmB- or AmE-induced lysis.

Polyene antibiotic-induced lysis of erythrocytes has often been used as a convenient model in the studies on the toxic effects of these antibiotics. However, the validity of this test has recently been questioned and the direct measurement of ion efflux has been indicated as more advisable to reach a firm conclusion about toxicity [11]. Our results corroborate this proposal since hemolysis is strongly inhibited by traces of Cu^{2+} while K^+ leakage is not affected.

ACKNOWLEDGEMENTS

This work was supported in part by US Public Health Service Grants, CA 15665 and AI 16228.

REFERENCES

[1] Brajtburg, J., Medoff, G., Kobayashi, G.S., Elberg, S. and Finegold, C. (1980) Antimicrob. Agents Chem. 18, 586–592.
[2] Metzger, J. U.S. 3, 928, 570 (Cl.424–118; 461 K).
[3] Aszalos, A. and Vandeputte, J. U.S. 3, 957, 754 (Cl.260–210 AB).
[4] Komarov, E.V., Travkin, O.V., Ivanov, G.G., Mikhailova, M.A., Shneider, M.A. and Amelin, S.V. (1983) Antibiotiki 28, 437.
[5] Beezer, A.E. and O'Brien, P. (1981) J. Inorg. Nucl. Chem. 43, 1669–1670.
[6] Duysens, L.N.M. (1956) Biochim. Biophys. Acta 19, 1–12.
[7] Richards, R.W., Smith, R.M. and Golding, B.T. (1970) J. Antibiot. 23, 603–612.
[8] Bronov, L.V., Kroshilova, T.M., Slonim, V.Z., Ivanov, G.G., Komarov, E.V. and Krunchank, V.G. (1983) Antibiotik 33, 496–501.
[9] Zhdanivich, Y.V., Lokshin, G.B. and Kuzovkov, A.D. (1966) Antibiotiki, 122–126.
[10] Etingov, E.D., Blashko, E.L. and Etingova, N.I. (1974) Antibiotiki 22, 678–680.
[11] Cybulska, B., Mazerski, J., Borowski, E. and Gary-Bobo, C.M. (1984) Biochem. Pharmacol. 33, 41–46.