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Involvement of Oxidative Damage in Erythrocyte Lysis Induced by Amphotericin B

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Lysis of human erythrocytes induced by amphotericin B was retarded when the oxygen tension of the incubation mixture was reduced or when the antioxidant catalase was added; lysis was accelerated when cells were preincubated with the prooxidant ascorbate. In the atmosphere of reduced oxygen tension, the erythrocytes containing carboxyhemoglobin lysed at a slower rate than did the cells containing oxyhemoglobin. Consistent with a role for oxidative damage in lysis, the mixture of erythrocytes and amphotericin B showed an increase in malonyldialdehyde, the product of peroxidation, which paralleled the progression of hemolysis. In contrast, the permeabilizing effect of amphotericin B, measured as a decrease in intracellular K^+, was not affected by changes in oxygen tension, catalase, or ascorbate treatment. These results imply that oxidant damage is involved in the lytic, but not in the permeabilizing, action of amphotericin B.

Amphotericin B (AmB), a heptaene macrolide antibiotic used clinically in the treatment of systemic mycoses, binds to sterols and is toxic to both fungal and mammalian cells (10). At low levels AmB has a reversible permeabilizing effect on erythrocytes (RBC); at higher levels is causes lysis. The connection between these two cellular actions (permeabilizing and lytic) of the antibiotic has been extensively studied. Some investigators have proposed that lysis is an extension or direct consequence of the permeabilizing effect (6). Others have suggested that increased permeability can be separated from the more destructive effects of the polyenes (3, 5).

An aspect of the chemistry of polyene antibiotics that has interested us is their susceptibility to autooxidation (1, 4, 8). Since compounds that autooxidize may give rise to several forms of active oxygen that are toxic to cells (see reviews in references 7 and 15), we wondered whether oxidant damage might be involved in the anticellular effects and particularly in the lytic action of AmB.

The experiments that we describe here study the influence of oxygen tension, catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6), or ascorbate, a known stimulator of oxidative hemolysis (9), on the permeabilizing and lytic effects of AmB on RBC. The results imply that oxidant damage is involved in the lytic, but not the permeabilizing, action of AmB.

MATERIALS AND METHODS

Chemicals. AmB (Fungizone; E. R. Squibb & Sons, Inc., Princeton, N.J.) was dissolved in water just before use. Sodium deoxycholate alone, in concentrations equal to those in AmB, had no effect on cells or the assays. Catalase (C-3515) and ascorbic acid were purchased from Sigma Chemical Co., St. Louis, Mo.

RBC. Venous blood obtained from healthy volunteers was collected in heparinized tubes and centrifuged at 1,500 × *g* for 15 min, and the plasma and buffy coat were removed. The cells were washed with 0.15 M NaCl solution buffered with 10 mM sodium phosphate to pH 7.4 (PBS) and used the same day they were obtained.

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Functional assays. RBC were dispersed at a concentration of 0.5% (vol/vol) in PBS. All experiments were done on 1-ml samples in duplicate or triplicate at 24 to 26°C.

The time course of AmB-induced hemolysis was measured as an increase in transmittance at 700 nm on a Coleman Junior II spectrophotometer. The results are presented as percent hemolysis. Time 0 is the time of AmB addition.

Intracellular K^+ loss from RBC was estimated by comparison of K^+ retention in AmB-treated and untreated cells (2). Measurements of cell-associated K^+ were done in a Corning Flame Photometer 430, and the results are expressed as the percentage of values found in untreated cells.

To test the oxygen dependence of AmB effects, RBC were dispersed in 2-ml portions of PBS in 7-ml vacutainer tubes (Becton-Dickinson and Co., Paramus, N.J.). Nitrogen or air was flushed into the tubes for 90 min through a spinal needle inserted through the cap to a point just above the liquid level; gas exited the tubes through a small vent needle. The AmB was then added, the dispersion was flushed with nitrogen or air for an additional 30 min, and both needles were removed. Oxygen pressure (pO_2) in the RBC dispersions in the tubes flushed with nitrogen (measured in a pH-Blood Gas Analyzer, model 813; Instrumentation Laboratory, Lexington, Mass.) was 15 to 20 mmHg (ca. 2.0 to 2.6 kPa) and remained stable in samples stored in the capped tubes for at least 30 h. The progress of lysis was monitored in parallel in the samples under air and nitrogen. Since deoxygenation of hemoglobin itself caused a 7% decrease in transmittance at 700 nm, the results were correspondingly adjusted.

RBC containing carboxyhemoglobin (HbCO) were prepared by bubbling carbon monoxide through 0.5% suspensions of RBC in PBS; 98% conversion of hemoglobin to HbCO was achieved after 4 min. The percentage of HbCO in the RBC was still 91% after storage in air for 3 h.

To test the effect of catalase, the enzyme was added first to PBS at concentrations of 50 to 300 U/ml. Then RBC were added, followed 5 min later by AmB. To test the effect of ascorbate, RBC were incubated at 37°C with 2 mM ascorbate for 2 h unless otherwise stated. The cells were har-

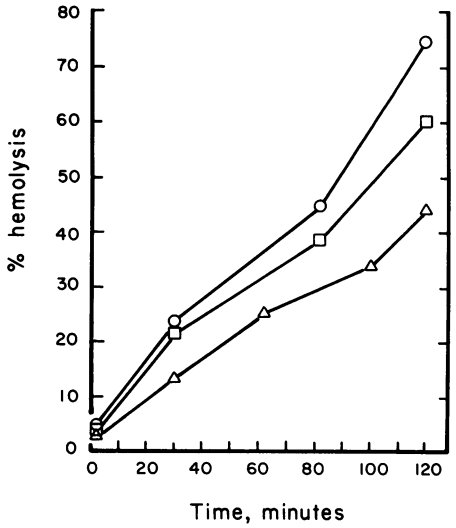

FIG. 1. Lysis of RBC over time, as induced by 5.0 (△), 10.0 (□), or 15.0 (○) μg of AmB per ml. These results were from one representative experiment; similar results were obtained in five other experiments.

vested by centrifugation (not washed) for 10 min at 1,500 × *g*, dispersed in fresh PBS, and exposed to AmB.

To test the effect of light on AmB-induced hemolysis, solutions of AmB in PBS with or without RBC were illuminated with a fluorescent lamp (General Electric Co., Schenectady, N.Y.; F15T8-CW 15-W cool white) at a distance of 10 cm.

Measurement of malonyldialdehyde formation. Malonyldialdehyde was assayed by the procedure of Trotta et al. (14). RBC suspensions (19%, vol/vol) were incubated with AmB for 2 h at 37°C with constant shaking. Malonyldialdehyde was measured by the colorometric procedure involving reaction with tiobarbituric acid. The differences in absorbancy at 532 nm and that at 600 nm were calculated and compared with those of controls without AmB. For these calculations, standard curves were prepared with malonyldialdehyde prepared from 1,1,3,3-tetramethoxypropan (Aldrich Chemical Co., Milwaukee, Wis.). Absorption was measured in a Gilford 260 spectrophotometer.

RESULTS

Hemolytic and permeabilizing effects. Incubation of RBC for 0.5 h with 1.2 μg of AmB per ml was sufficient to cause depletion of at least 90% of the intracellular K^+ ions. However, the K^+ depletion was not sufficient to lead to lysis, and the RBC did not lyse even after 20 h. RBC incubated with 2.5 μg of AmB per ml did not lyse for 3 h (data not shown). RBC that were incubated for 3 min with 5 to 15 μg of AmB per ml retained only 3 to 5% of their initial intracellular K^+. The lysis of these cells with identical levels of remaining K^+ occurred at different rates depending on the AmB concentrations used (Fig. 1). AmB at 5 μg/ml lysed 42% of the cells after 120 min of incubation, AmB at 10.0 μg/ml required 100 min to lyse 50% of the cells, and AmB at 15.0 μg/ml required 82 min.

The continuous presence of AmB in the medium was not required for the progression of hemolysis. When RBC which had been incubated with 5.0 μg of AmB per ml for 10 min were harvested by centrifugation (without washing) and suspended in PBS without AmB, the time course of hemolysis was identical to that of cells incubated continuously in the presence of AmB.

To assess a possible role for oxygen in AmB-induced damage, we tested for AmB-induced lysis and permeability after placing RBC under reduced oxygen tension. When the oxygen tension (pO_2) of the incubation mixture was decreased from 150 mmHg (ca. 20 kPa) to 20 mmHg by flushing with nitrogen for 90 min, the time required for 50% lysis by AmB at 5.0 μg/ml increased from 66 min to 108 min (Fig. 2). Comparable inhibition of lysis by reduction of oxygen tension was also seen when the cells were treated with 10.0 or 15.0 μg of AmB per ml (data not shown).

HbCO-containing RBC were made to determine the role of oxyhemoglobin in AmB-induced lysis. When HbCO-containing and oxyhemoglobin-containing RBC incubated in air were treated with 5.0 μg of AmB per ml, the rate of lysis was identical (Fig. 2). In contrast, when the pO_2 was lowered to 20 mmHg, the RBC containing HbCO lysed at a slower rate than did the cells containing oxyhemoglobin (Fig. 2).

In contrast to the oxygen dependence of lysis, the permeabilizing effect of AmB was unaffected by the pO_2 of the incubation mixture; identical AmB-induced levels of K^+ depletion were obtained when the incubations were carried out under normal or reduced O_2 tension (Fig. 3).

In the next series of experiments, catalase was used as an antioxidant. We found that catalase, in a dose-dependent manner, retarded or completely prevented hemolysis when RBC were continuously incubated with catalase and 10.0 or 15.0 μg of AmB per ml (Fig. 4). Hemolysis was also inhibited when RBC were first incubated with catalase and AmB and then harvested and incubated in AmB-free, catalase-free PBS as well as when RBC were first incubated with AmB and then harvested and incubated in PBS free of AmB, but containing catalase. No protection was seen when cells were first incubated with catalase and then harvested and treated with AmB. In further controls, bovine serum albumin, used at concentrations comparable to that of catalase (40.0 μg/ml), did not affect hemolysis.

To learn whether catalase also inhibited the permeabilizing effect, we measured K^+ loss by RBC exposed to 5 to 15 μg of AmB per ml in the absence or presence of 200 U of

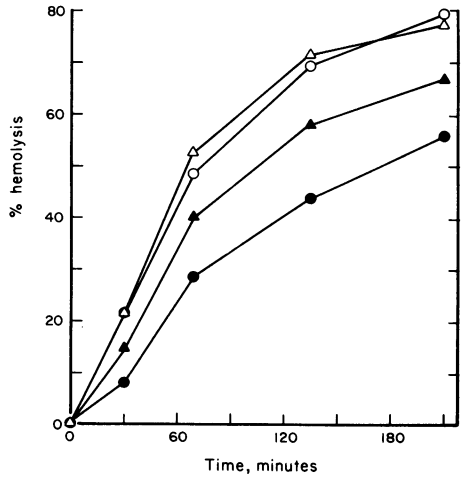

FIG. 2. Effect of decrease in oxygen tension from 150 (○, △) to 20 (●, ▲) mmHg on the time course of hemolysis induced by 5.0 μg of AmB per ml. These results were obtained in one representative experiment with RBC containing oxyhemoglobin (△, ▲) or HbCO (○, ●). Similar results were obtained in two other experiments.

catalase per ml. In both conditions after 5 min of incubation loss of K^+ from cells was 93 to 95% of initial values (data not shown). Since AmB at concentrations as high as 1.0 μg/ml was very potent in permeabilizing cells, a small protective effect of catalase could have been overwhelmed. Therefore, we examined the effect of 200 U of catalase per ml on RBC incubated for 30 min with 0.6 μg of AmB per ml. There was no significant difference in the retention of K^+ by the RBC incubated with or without catalase.

The effects of ascorbate on AmB-induced lysis and permeability were measured by comparing AmB effects on RBC that had been incubated without or with ascorbate at 37°C, harvested, and then exposed to AmB in fresh PBS. Ascorbate by itself did not cause hemolysis. The potentiating effect of ascorbate on hemolysis was seen 2 h after the treatment of RBC with 2.5 μg of AmB per ml (Fig. 5A) and within 15 min of treating RBC with 10.0 μg of AmB per ml (Fig. 5B). This effect of ascorbate on lysis occurred in RBC preincubated with ascorbate and then exposed to AmB either in air or under reduced pO_2 (Fig. 6).

When the effect of ascorbate on AmB-induced permeabilization was measured, we found that RBC preincubated with ascorbate and then exposed to 0.6 μg of AmB per ml for 0.5 h retained the same level of intracellular K^+ as RBC not pretreated with ascorbate.

Therefore, our experiments indicated that oxidative damage was involved in AmB-induced hemolysis; no evidence for a similar involvement in the permeabilizing effects of AmB could be demonstrated.

Effect of light on AmB-induced hemolysis. Polyene antibiotics are light sensitive. Thus it was of interest to test how light influenced the lytic effects of AmB. We compared the effects of light on dispersions in PBS of AmB alone, RBC alone, or AmB together with RBC.

In the first set of experiments, samples of 10.0 μg of AmB per ml were dispersed in 1-ml portions of PBS; half of the samples were illuminated for 0.5, 1, or 2 h by a fluorescent lamp, and the other half were stored in the dark. RBC were then added to the mixtures, and the progression of lysis was monitored spectrophotometrically. In three separate experiments, RBC incubated with AmB that had been stored in the dark lysed more rapidly than did those incubated with AmB that had been illuminated. Moreover, the differences between the illuminated and non-illuminated AmB increased with the length of exposure to light. In one typical experiment, the time to achieve hemolysis of 50% of RBC was 60 min when AmB protected from light was used, 95 min for AmB illuminated for 30 min, and 125 min for AmB illuminated for 1 h. Only 5% of RBC lysed 2.5 h after exposure to AmB that had been illuminated for 2 h. Therefore, the gradual decomposition of AmB exposed to light resulted in a decrease in its lytic effects on RBC.

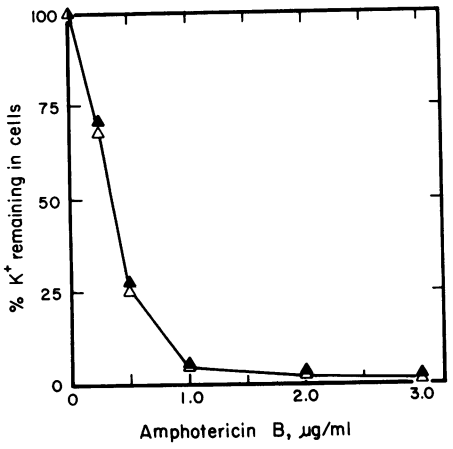

FIG. 3. Loss of K^+ by RBC incubated for 10 min with AmB in air (△) or under pO_2 of 20 mmHg (▲). These results were obtained in one representative experiment; similar results were obtained in two other experiments.

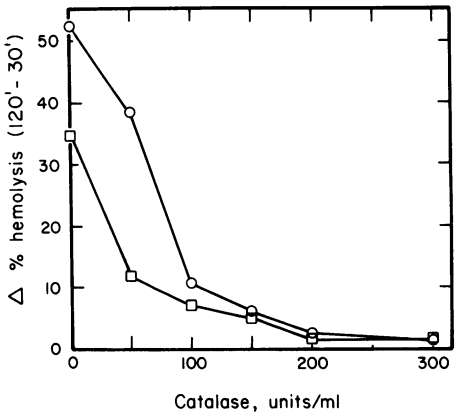

FIG. 4. Inhibitory effects of catalase on progression of lysis (from 30 to 120 min) of RBC incubated with 10.0 (□) or 15.0 (○) μg of AmB per ml. These results were obtained on one representative experiment; similar results were obtained in five other experiments.

In a second set of experiments, RBC were added to PBS; replicate samples were illuminated for 60 min or incubated in the dark. Both samples were then exposed to 5.0 or 10.0 μg of AmB per ml. There was no difference in the rate of hemolysis between RBC that had been illuminated and RBC that had not.

In the third set of experiments, RBC were incubated with 5.0 μg of AmB per ml and were illuminated or stored in the dark for periods up to 2.5 h. The rate of hemolysis in the two groups of cells did not differ. Catalase added with AmB at the onset of the incubation protected against hemolysis equally well in the two groups. These findings and the results obtained in the first set of experiments imply that RBC protected AmB from light-induced decomposition. The observation that the decrease in anticellular potency of AmB induced by exposure to light was inhibited in the presence of RBC is consistent with previous demonstrations of stabilization of AmB biological activity by interaction with the cholesterol in lipoproteins (2).

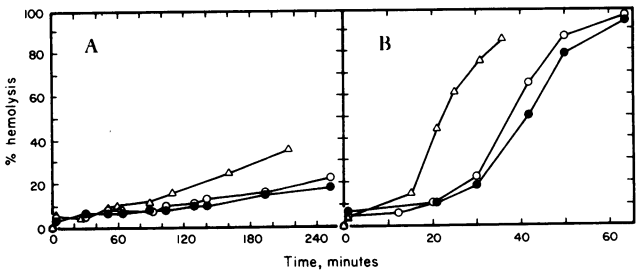

FIG. 5. Effects of preincubation of RBC with ascorbate on hemolysis induced by AmB at 2.5 (A) or 15.0 (B) μg/ml. Cells were preincubated for 2 h without ascorbate (●) or preincubated with ascorbate for 0.5 (○) or 2 (△) h. These results were obtained in one representative experiment; similar results were obtained in three other experiments.

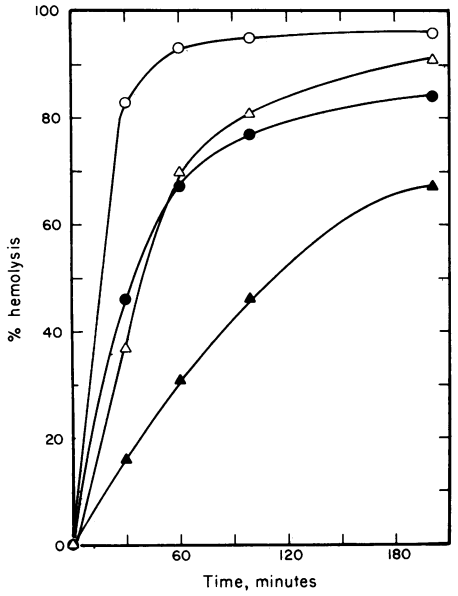

FIG. 6. Effect of ascorbate on lysis of RBC induced by AmB in air or under reduced oxygen tension. Cells were preincubated with (○, ●) or without (△, ▲) ascorbate and then exposed to AmB at 10.0 μg/ml in air (○, △) or in pO_2 of 20 mmHg (●, ▲). These results were obtained on one representative experiment; similar results were obtained in two other experiments.

Production of malonyldialdehyde in RBC-AmB mixtures. Malonyldialdehyde is an end product of lipid peroxidation of polyunsaturated phospholipids (14). It can also be formed by oxidation of the chain of conjugated double bonds in heptaene macrolides (8). RBC were incubated with several concentrations of AmB. A portion of each sample was used for measurements of lysis, and the other half was used for determination of levels of malonyldialdehyde. We found that the increase in the extent of hemolysis in response to increasing AmB concentrations occurred concurrently with an increase in the formation of malonyldialdehyde (Fig. 7).

DISCUSSION

RBC placed in an atmosphere of reduced oxygen tension (pO_2 lowered to 20 mmHg) underwent AmB-induced lysis more slowly than did those incubated in air (pO_2 of 150 mmHg). AmB-induced lysis was also inhibited by catalase and accelerated by ascorbate; moreover, the levels of malonyldialdehyde generated by incubations of AmB with RBC correlated with the extent of hemolysis. Taken together, all of our results convincingly indicate that oxidative damage is involved in AmB-induced hemolysis.

Although HbO_2 can act as an initiator of lipid peroxidation (14), the decreased availability of HbO_2 (caused by exposure of RBC to CO) had a negligible effect on AmB-induced lysis in air, probably because of the abundance of oxygen in the incubation mixture. However, in the atmosphere of reduced oxygen tension, the difference in concentrations of HbO_2 (about 20% in the population of untreated cells, as evaluated from oxygen dissociation curves [13] and 2% in the population of CO treated cells) resulted in retardation of lysis in CO-treated cells.

The possibility that the anticellular effect of another heptaene polyene antibiotic (candicidin) may at least in part be secondary to oxidant damage has been independently considered by Gutteridge and Thomas (8). In addition, at least three other studies and our unpublished finding with Candida albicans have reported results consistent with this hypothesis. Sippel and Levine (12) observed the oxygen dependence of AmB action on fungi and the decrease of the effect in anaerobic condition. Weis and Levine (16) reported inhibitory effects of reducing compounds on hemolytic and antifungal activity of AmB. The potentiation of AmB toxicity against yeast by ascorbate has been reported by Beggs et al. (1). These workers assumed that ascorbate acted by protecting AmB from decomposition during prolonged incubations. Since we observed the potentiation of AmB effects by ascorbate after only 15 min of incubation with AmB, in our experiments ascorbate was probably acting as a prooxidant (9).

We were not able to demonstrate that oxidant damage was involved in AmB-induced permeability of RBC. The loss of K^+ that occurred rapidly at low AmB concentrations was not affected by lowering the oxygen tension, adding catalase to the medium, or preincubating cells with ascorbate. Thus we tentatively conclude that oxidative injury is not necessary for AmB-induced permeability. This notion is consistent with results that have suggested a distinction between the mechanism of a loss of cellular K^+ and lytic effects. For example, the lytic effects of 15.0 μg of AmB per ml were more pronounced than those that occurred with 5.0 μg/ml (Fig. 1), despite equal levels of K^+ depletion in the two cell populations. In the past, we (3) and others (5) have viewed the increase in permeability and the lysis or cell killing as two separate dose-dependent stages with different characteristics. Possibly the development of oxidative effects contributed to the final fate (lysis or death) of cells that had been already made permeable to K^+.

The protection of AmB from light-induced decomposition by RBC likely resulted from binding to cellular cholesterol. It has been shown that incorporation of cholesterol into liposomes reduced the rate of peroxidation of unsaturated phospholipids (11). Although the mechanism of the protection is not clear, it appears that hydrophobic binding of molecules to cholesterol may decrease the vulnerability of molecules to oxidation.

The mechanism(s) of the oxidative effect of AmB is unknown. Perhaps AmB extends the effects of normal peroxidation in cells by providing a better or an additional substrate. If so, AmB may also propagate damaging and lytic reactions generated by other oxidizing agents. Should this

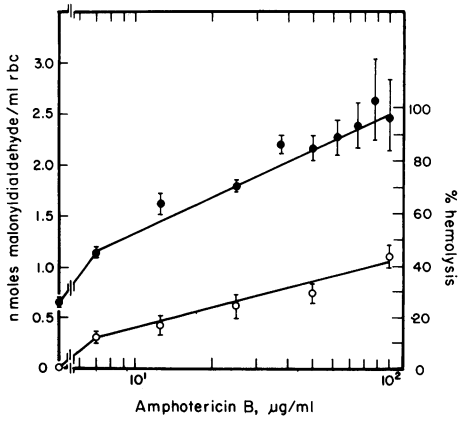

FIG. 7. Formation of malonydialdehyde (●) in AmB-RBC mixture and concurrent hemolysis (○). The results shown were the means ± standard deviations from six experiments.

prediction be valid, new synergistic combinations of AmB and other classes of drugs are suggested that might improve antifungal and antitumor therapy.

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