Neutrophil Toxicity of Amphotericin B

C. JOHN CHUNN,* P. R. STARR, AND DAVID N. GILBERT
Leslie Gilbert Infectious Diseases Laboratory, Providence Medical Center,* and the University of Oregon Health Sciences Center, Portland, Oregon 97213

Received for publication 23 February 1977

The toxicity of amphotericin B (AmB) for neutrophils and the protective effect of serum cholesterol were investigated. Neutrophils were exposed in vitro to varying concentrations of AmB. As judged by trypan blue exclusion, neutrophil viability decreased by 40% (P < 0.001) within 30 min of incubation in sterol-free buffer containing 5 μg of AmB per ml. In the presence of 4 mg of cholesterol per 100 ml in buffer, the AmB concentration could be increased to 50 μg/ml before significant (P < 0.01) neutrophil toxicity occurred. Hexose monophosphate shunt activity of neutrophils incubated in serum or cholesterol-containing buffer with 10 μg of AmB per ml was normal. These results suggest that serum contains a protective factor, probably cholesterol, which protects neutrophils in vitro from the toxic effects of AmB.

Amphotericin B (AmB) has remained the cornerstone of systemic antifungal therapy for more than two decades. Its use is not without complication, however, and AmB administration commonly results in fever, anemia, infusion site pain, and renal impairment (1, 4). As do other members of the polyene antimicrobial group, AmB is thought to bind to sterol components of fungal cell membrane, inducing configurational changes of those membranes. The result is altered membrane permeability that promotes ion leakage and, eventually, cell death (5–8, 10, 18).

Since human cell membranes contain sterols (specifically cholesterol), we speculated that AmB might be toxic to neutrophils (16, 19). Therefore, we exposed neutrophils to AmB and assessed their viability. We also considered that serum might protect neutrophils from AmB toxicity because of the presence of cholesterol and investigated this possibility.

MATERIALS AND METHODS

Antibiotics. AmB was graciously supplied by E.R. Squibb & Sons, Princeton, N.J. It was in powder form and free of dispersing agents (i.e., sodium deoxycholate) and contaminants. AmB was dissolved in Me2SO (Sigma Chemical Co., St. Louis, Mo.) at both 1 mg/ml and 10 mg/ml. Portions were frozen at −70°C; these were thawed and diluted in Hanks balanced salt solution (pH 7.4) modified so as to not contain calcium, magnesium, or glucose (MHBSS). Dilutions were stored in a refrigerator for up to 5 days and then discarded, conditions for which chemical stability has been previously shown (P.D. Hoeprich and A.C. Haston, Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 15th, Washington, D.C., Abstr. no. 104, 1975).

Neutrophils. On each day of experimentation, fresh whole blood was obtained from fasting, healthy donors. Dextran sedimentation and hypotonic lysis, by a standard method, resulted in a cell preparation that was consistently 95% neutrophils, the remainder being other granulocytes or lymphocytes (3). Final suspensions were in MHBSS at 12.5 × 10⁶ cells/ml. In all experiments, final cell concentration was 10⁶ cells/ml. Me2SO was 1% by volume in each circumstance. During periods of incubation, cells were kept in a CO₂ incubator at 37°C. Under these conditions, the pH of the buffering solution or serum remained constant.

Soluble cholesterol preparation. Cholesterol-containing low-density-weight beta-lipoprotein (LDL) was prepared by ultracentrifugation of normal human plasma. Briefly, 200 ml of fresh, heparinized (10 U/ml) whole blood was centrifuged to yield 85 ml of plasma with a density of 1.006. This was diluted 2:1 with a sodium chloride solution (density, 1.063) to yield a density of 1.024. The resultant solution was spun at 40,000 rpm in a Beckman ultracentrifuge (model L5-40), with head-type 60 Ti, for 22 h.

The top layer (very low-density-weight beta-lipoprotein) was aspirated and discarded, leaving 93 ml of plasma with a density of 1.024. To this was added 8.2 ml of saline solution with a density of 1.346, raising the plasma density to 1.050. After repeat centrifugation for 22 h, 20 ml of LDL was obtained as the supernatant.

This crude LDL was dialyzed in a cellulose-acceptate bag against frequently changed large volumes of MHBSS over 48 h. The resultant LDL was stored at 4°C. Final characteristics included: pH 7.5, osmolality of 273 mosmol/liter and cholesterol concentration of 250 mg/100 ml.

Periodic cholesterol determinations demonstrated that the LDL maintained a constant cholesterol level over 8 weeks. Cholesterol concentrations were determined by the Lieberman-Burchard method (17).
Neutrophil viability. AmB was added to neutrophil suspensions at specific concentrations. Portions were taken over timed intervals to assess neutrophil viability, as judged by the ability of the neutrophil to exclude a vital dye: trypan blue. To 50 μl of cell suspension was added 5 μl of 0.4% aqueous trypan blue prepared by a standard technique (14). The components were mixed on a microscope slide and covered with a cover slip. After 2 min, 200 cells were counted with a microscope, ×400 magnification. The percentage of viable neutrophils, i.e., those unstained, was noted.

Trypan blue is not a functional stain in the presence of serum, probably because of its greater affinity for albumin than for nonviable cells (13). To assess the efficacy of trypan blue in LDL-cholesterol (as prepared above), previously killed neutrophils were suspended in both MHBSS and LDL-cholesterol. There was complete agreement between trypan blue counts of the two suspensions, indicating trypan blue to be a functional stain in LDL.

Hexose monophosphate shunt activity. The neutrophil's ability to metabolize glucose when exposed to a phagocytic stimulus via the hexose monophosphate shunt was measured by a standard technique (15). Briefly, neutrophils were incubated with AmB at a particular concentration for 30 min and then centrifuged, washed, and suspended in MHBSS. Latex particles (Dow Diagnostics, Indianapolis, Ind.) with a diameter of 0.8 μm were used as a phagocytic stimulus. Glucose, labeled at the C1 position with 14C (New England Nuclear Corp., Boston, Mass.) was used. The specific activity of the final reaction mixture was 1 μCi/0.5 mM glucose. The components were mixed in a sealed Warburg flask on a shaking water bath at 37°C for 30 min. Released 14CO2 was collected in hyamine hydroxide (Sigma) and counted in a liquid scintillation spectrometer (Packard Tri-Carb no. 2425), using Aquasol (New England Nuclear Corp.) as a scintillation cocktail. Results are expressed as counts per minute per 106 cells per 30 min of stimulation.

RESULTS

Effect of AmB on viability in cholesterol-free buffer. Figure 1 illustrates the effect of AmB in varying concentrations on neutrophil viability in cholesterol-free buffer, in this case, MHBSS. Within 30 min, AmB at 5 μg/ml resulted in significant cell death (P < 0.001) when compared with controls. After 2 h of exposure to AmB at 5 and 10 μg/ml, only 22% and 12%, respectively, of the neutrophils examined were viable. Although AmB at 1 μg/ml appeared to decrease neutrophil viability to some degree, the difference was not statistically significant.

Effect of AmB on hexose monophosphate activity in serum. Neutrophils from normal donors were incubated for 30 min in autologous serum that contained 10 μg of AmB per ml. Subsequently, these cells were washed in buffer and then tested for the response of their hexose monophosphate shunt to a phagocytic stimulus. Although there was a fair amount of day-to-day variation in the counts achieved, there was no significant differences between AmB-treated neutrophils and controls within each experiment. The ratio of the counts achieved from the AmB-treated cells to those from the control cells in five separate experiments ranged from 0.91 to 1.34. Thus, serum protected neutrophils after exposure to AmB at a concentration of 10 μg/ml.

Effect of AmB in cholesterol-containing buffer. In previous experiments, cytotoxicity was seen with AmB at a concentration of 10 μg/ml in cholesterol-free buffer. Neutrophils were exposed to AmB, 10 μg/ml, in buffer containing varying concentrations of LDL-cholesterol (Fig.

![Fig. 1. Effect of AmB on neutrophil viability in cholesterol-free buffer.](http://aac.asm.org/)
2). With LDL diluted to a cholesterol concentration of only 4 mg/100 ml, no statistically significant decrease in neutrophil viability occurred. However, after 1 h at a cholesterol concentration of 0.4 mg/100 ml, only 22% of neutrophils examined were viable \((P < 0.001)\).

To further characterize the protective effect of LDL-cholesterol, the cholesterol concentration was held constant at 4.0 mg/100 ml and the AmB concentration was increased (Fig. 3). Significant cell death occurred when the AmB concentration was increased beyond 25 µg/ml. At an AmB concentration of 50 µg/ml, only 57% of the counted neutrophils remained viable \((P < 0.01)\); at 100 µg/ml, only 31% of the counted neutrophils remained viable at 1 h of incubation \((P < 0.001)\). Although increased killing appeared to occur at 25 µg of AmB per ml, the difference was not statistically significant.

Neutrophils were incubated in LDL with a cholesterol concentration of 4.0 mg/100 ml and an AmB concentration of 10 µg/ml for 30 min. Subsequently, the cells were exposed to latex particles and the hexose monophosphate shunt activity was measured. As was seen with serum-incubated cells, AmB-treated neutrophils responded as well as, if not better than, control cells. With one exception, the ratio of the counts achieved from AmB-treated neutrophils...
to those from control cells ranged from 1.07 to 1.19.

Therefore, LDL with a cholesterol concentration of 4.0 mg/100 ml protected neutrophils from overt death due to AmB concentrated to 10 μg/ml and preserved the cells’ hexose monophosphate shunt activity.

**DISCUSSION**

That AmB is capable of killing neutrophils is not surprising. Sterol-polyene interaction on the cell membrane of fungi is the accepted tenet for the fungicidal activity of AmB (7, 10, 18). Human cells, like fungi, contain sterols in their cell membranes. Whereas the fungal cell membrane sterol is ergosterol, human cell membranes incorporate cholesterol (16, 19).

Kinsky et al. reported, in 1963, that AmB could cause lysis of erythrocytes (9). Butler and Cotlove demonstrated increased permeability and potassium leakage in human erythrocytes exposed to AmB and postulated that this interaction may, in part, account for the hemolytic anemia seen with systemic AmB therapy (4). There have been few studies to date on AmB and phagocytic cells. Lin et al. recently reported that AmB enhanced the phagocytic activity of mouse peritoneal macrophages, but this was apparently only when AmB was given in the presence of lymphocytes (11). It is of interest that absolute neutrophil counts began to increase several hours after AmB administration. To our knowledge, only one study has been reported regarding the effect of AmB on human neutrophils. Recently, Bjorksten et al. showed that AmB, in concentrations as low as 2 μg/ml, inhibited chemotaxis of human neutrophils (2). Also, inhibition of neutrophil chemiluminescence was shown at an AmB concentration of 5 μg/ml or greater. Judging from our data, at an AmB concentration of 5 μg/ml, most neutrophils should be nonviable in a cholesterol-free medium (Fig. 1).

Mean serum levels of patients receiving systemic AmB therapy are approximately 1 μg/ml; peak serum levels might certainly be greater (12). AmB at a 1 μg/ml concentration did not kill neutrophils to a statistically significant degree. Such low AmB serum levels may not be important in vivo since our studies show that serum or cholesterol-containing buffer protected neutrophils completely from AmB toxicity. Although the trypan blue viability test was invalid in the presence of serum, the normal post-phagocytic burst of hexose monophosphate shunt activity documents cell viability. The use of purified cholesterol-containing LDL supports the theory that cholesterol is protecting neutrophil viability. We cannot rule out the possibility that lipoprotein alone confers protection.

AmB may have a higher binding affinity for the ergosterol of fungal cell membranes than for the cholesterol in human serum or human cell membrane. This postulate would be in accord with the clinical success in treating acute fungal infections with AmB. There is some experimental evidence to support this hypothesis. Zygmunt and Tavormina demonstrated that exogenous ergosterol inhibited the fungicidal activity of AmB, whereas cholesterol, as well as 24 other steroids, did not (20). However, only 10 mg of cholesterol per 100 ml was used, a concentration far less than the amount found in normal human serum. Also, Cass et al. constructed thin lipid membranes with either ergosterol or cholesterol and exposed them to the polyene nystatin, and the increase in membrane permeability was measured (5). Membranes constructed with ergosterol leaked at a much lower concentration of nystatin than membranes constructed with cholesterol.

In summary, our studies demonstrate that AmB is toxic to normal human neutrophils in a cholesterol-free medium. However, serum or cholesterol-containing LDL protects neutrophils from AmB.

**ACKNOWLEDGMENTS**

Grateful appreciation is extended to Ken Solen, Don Lehman, and William Connor for their role in the preparation of LDL and to Linda Brockway for her technical assistance.

This work was supported by a grant from The Medical Research Foundation of Oregon.

**LITERATURE CITED**


