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Toxicity and Induction of Resistance to *Listeria monocytogenes* Infection by Amphotericin B in Inbred Strains of Mice

JANINA BRAJTBURG,[1]* SVETLANA ELBERG,[1] GEORGE S. KOBAYASHI,[2,3] AND GERALD MEDOFF[1,3]

Division of Infectious Diseases[1] and Division of Laboratory Medicine,[2] Department of Medicine and Department of Microbiology and Immunology,[3] Washington University School of Medicine, St. Louis, Missouri 63110

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Amphotericin B (AmB) treatment before infection with the bacterium *Listeria monocytogenes* prolonged survival of AKR mice but shortened survival of C57BL/6 mice compared with survival of untreated infected controls. C57BL/6 mice were also more sensitive to the acute toxic effects of AmB than AKR mice, as were (C57BL/6 × AKR)F_1 hybrid mice. Spleen cells and erythrocytes (RBCs) from the C57BL/6 and the F_1 hybrid mice were both more sensitive to the lytic and lethal effects of AmB than corresponding cells from AKR mice. Biochemical analysis indicated that catalase levels in RBCs from C57BL/6 and F_1 hybrid mice were about 60% of those found in RBCs from AKR mice. The lysis by AmB of RBCs from all these strains of mice was inhibited by catalase or incubation in a low-oxygen environment. These findings suggest that (i) the low catalase levels in C57BL/6 and F_1 hybrid mice may limit the protection of cells from the oxidant damage involved in AmB action, and (ii) the toxicity which occurs at low concentrations of AmB in the mouse strains with low intracellular catalase levels may interfere with or ablate the AmB-induced increases in mouse resistance to *L. monocytogenes* infection.

Amphotericin B (AmB) is the principal antimicrobial agent used in the treatment of systemic fungal infections. It binds to sterols incorporated into eucaryotic cell membranes, and its clinical utility is thought to depend on its preferential binding to the ergosterol in fungal cell membranes compared with the cholesterol in host cells (15). AmB does, however, have complex effects on the host. At high doses it kills mice, whereas at lower doses it is a potent immunoadjuvant which stimulates both humoral and cell-mediated immune reactions to defined and complex antigens (3, 21) and enhances host resistance to *Listeria monocytogenes* (25), *Schistosoma mansoni* (17), *Staphylococcus aureus* (2), and *Candida albicans* (2) infections.

Little and associates (13) have demonstrated that the immune effects of AmB on mice are strain dependent. They have been able to classify inbred strains of mice into high and low responder groups on the basis of the immunoadjuvant effects of AmB (12). No such data are available on the strain dependency of AmB-induced resistance to infection and toxicity. The present study was carried out on mouse strains representative of high responder (AKR) and a low responder (C57BL/6) and an F_1 hybrid from both parents (C57BL/6 × AKR) to obtain some information about this dependency.

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MATERIALS AND METHODS

Chemicals. AmB (Fugizone; E. R. Squibb & Sons, Princeton, N.J.) was dissolved in water just before use. Sodium deoxycholate alone, in concentrations equal to those in Fungizone, had no effect on cells. Fungal catalase (catalog no. C-3515) and superoxide dismutase from bovine erythrocytes (catalog no. S-8254) were purchased from Sigma Chemical Company, St. Louis. Mo.

Mice. Female C57BL/6 and female and male AKR mice (6 to 8 weeks old) were purchased from Jackson Laboratories, Bar Harbor, Maine. The (C57BL/6 × AKR)F_1 hybrid strain was bred in our laboratories from breeding sets of C57BL/6 female and AKR male mice; the mice were weaned and the sex was determined at 3 weeks of age. Mice were housed five to six to a cage and allowed free access to tap water and food (Rodent Laboratory Chow 5001; Ralston Purina, St. Louis, Mo.). Only female mice were used in the experiments.

Bacteria. *L. monocytogenes* was originally a clinical isolate from a patient in Barnes Hospital, St. Louis, Mo. The culture has been maintained in our laboratory for about 15 years.

In vivo assays. To measure the effects of AmB on survival after infection with *L. monocytogenes*, the AKR or C57BL/6 mice were divided into the following five groups: mice infected with *L. monocytogenes* and not treated daily with AmB; uninfected mice treated with AmB; mice infected and treated daily with 6.2 mg of AmB intraperitoneally per kg, according to the three different regimens described in the legend to Fig. 1. AKR mice received 4×10^5 viable *L. monocytogenes* organisms, and the C57BL/6 mice received 6×10^5 viable *L. monocytogenes* organisms intravenously through a tail vein. Deaths were recorded up to day 14 after injection of the bacteria. The number of organisms was estimated by optical density measurements according to a standard growth curve and subsequently quantitated by colony counts.

To measure AmB-induced lethality, groups of 10 mice from each strain were given intraperitoneal injections of different doses of AmB on 3 successive days. The doses were adjusted according to the average weight of the animals measured just before the start of the experiments. The dose which resulted in deaths of 50% of the mice (LD_{50}) was

* Corresponding author.

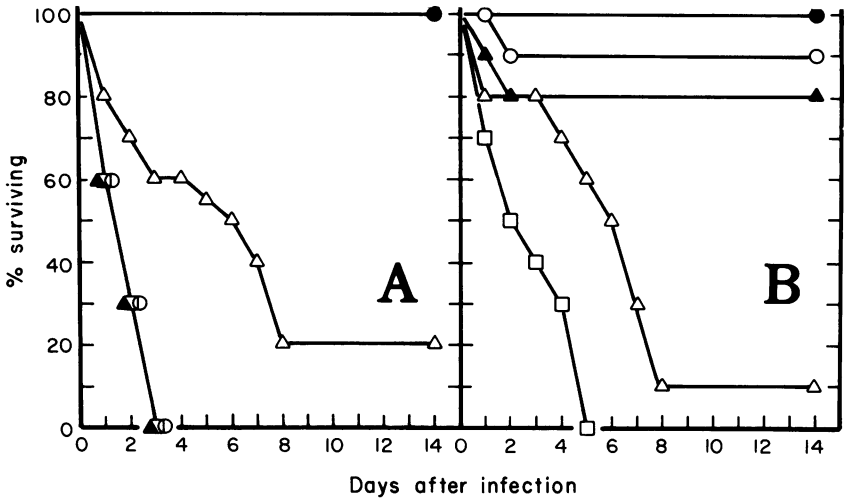

FIG. 1. Effects of AmB on mortality of C57BL/6 (A) and AKR (B) mouse strains uninfected or infected with *L. monocytogenes* on day 0. Symbols: ●, AmB was administered on days −5, −4, −3, and −2, and mice were not infected; ○, AmB was administered on days −5, −4, −3, and −2, and mice were infected; ▲, AmB was administered on days, −4, −3, −2, and −1, and mice were infected; △, AmB-untreated mice were infected; □, AmB was administered to infected mice on days 1, 2, 3, and 4.

calculated by the log dose probit-response curve which was constructed by fitting survival data to a logistic dose-response model (1).

In vitro assays. Spleens were removed from three mice, minced, combined, and placed in RPMI 1650 medium supplemented with 5% fetal calf serum. Fractions containing 4 × 10^6 cells per ml of medium were prepared (16) and incubated with various concentrations of AmB for 1 h at 37°C. The cells were then harvested by centrifugation at 800 × *g* at 4°C. Protein concentrations in pellets were measured by the method of Lowry et al. (14). The fraction of viable cells (those that excluded trypan blue) was estimated by counting the spleen cells in a hemacytometer after they were treated with 0.04% trypan blue.

Blood was obtained from the mice by orbital bleeding into heparinized capillary tubes, and the erythrocytes (RBCs) were separated by centrifugation. The AmB effect on retention of hemoglobin was determined as described previously (4). Briefly, RBCs were incubated for 1 h with AmB at 37°C, centrifuged, and lysed; and the concentration of hemoglobin was measured in a spectrophotometer at a wave length of 550 nm. The progression of lysis over time, oxygen dependence, and the effect of catalase on the progression of AmB-induced hemolysis were estimated at 24°C from the changes in transmittance of RBC dispersions (5) at a wavelength of 650 nm.

Biochemical determinations. Lipids were extracted from RBCs with isopropanol-chloroform (18), rinsed (7), and analyzed for lipid phosphorus (24) and for cholesterol by an enzymatic technique (reagents and procedure were provided by Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Hemoglobin was determined with Drabkin reagent (Sigma) (the procedure was described in Technical Bulletin 525; Sigma). Catalase (22) and superoxide dismutase (26) were estimated by known procedures. Levels of AmB in serum were determined by high-pressure liquid chromatography (10).

Statistical analysis. Data were analyzed with a statistical analysis system (20). When three groups were compared, analysis of variances with Bonfferroni corrections was used. Two groups were compared by Student's *t* test. The differences were considered to be statistically significant at $P < 0.05$.

RESULTS

AmB effects on the survival of mice infected with *L. monocytogenes*. Mice were injected on day 0 through a tail vein with various numbers of *L. monocytogenes* organisms to determine the inocula that would kill 50 to 90% of the animals in about 5 to 8 days. These inocula were 4 × 10^5 organisms per mouse in AKR mice and about 6 × 10^5 organisms per mouse in C57BL/6 mice. The results shown in Fig. 1 were obtained with five groups of 10 mice each. None of the uninfected mice treated with AmB died. In the groups of infected mice not treated with AmB, eight C57BL/6 mice (Fig. 1A) and nine AKR mice (Fig. 1B) died by day 8. The two groups of AKR mice treated with AmB (from days −5 to −2 or from days −4 to −1) before infection with *L. monocytogenes* showed increased survival compared with the untreated infected group. In contrast, AmB given after infection was detrimental, and the median survival time of mice in that group was shorter than in the group not treated with AmB (2 versus 6 days). In the group of infected C57BL/6 mice, AmB given before or following infection with *L. monocytogenes* accelerated death (1 to 2 versus 6 days).

Toxicity of AmB for uninfected mice. Because of the markedly different responses of the two strains of mice infected with *L. monocytogenes* to AmB, we compared the toxic effects of AmB in both strains. The LD_{50} ± confidence limits of an intraperitoneally injected dose of AmB was 42.4 ± 4.5 mg/kg for AKR mice and 17.9 ± 2.8 mg/kg for C57BL/6 mice. The LD_{50} for (C57BL/6 × AKR)F_1 hybrid mice was 21.3 ± 2.4 mg/kg, which was close to that of the C57BL/6 parent. Differences in sensitivity to the lethal effects of AmB were not related to differences in peak concentrations of AmB in serum because levels of AmB in serum in all of the strains were similar. For example, when 25 mg/kg of AmB was injected intraperitoneally into any of the strains of mice and the mice were bled 1 h later, levels of AmB in blood were approximately 2.8 μg/ml.

Toxicity of AmB for spleen cells and RBCs. Little and associates (13) have shown a good correlation between the stimulatory effects of AmB in spleen cells in vitro and the in vivo immunostimulatory effects; that is, AmB is mitogenic to spleen cells from high responder strains and not to spleen cells from low responder strains. We compared the toxic effects of AmB on spleen cells from both strains. We incubated spleen cells with AmB, harvested them by centrifugation, and measured the levels of remaining protein in the cell pellets. In samples incubated with 7.0 μg of AmB per ml, the protein retention decreased to 87.8 ± 3.2% in AKR, 71.6 ± 4.3% in C57BL/6, and 71 ± 3.4% in (C57BL/6 × AKR)F_1 (means ± standard errors [SE] from four experiments). The statistical analysis showed that the AKR group differed significantly from the other two groups. This result of incubation with AmB was due to disintegration of some cells and loss of protein from intact cells. The higher levels of retention of protein in AKR samples indicate that AKR spleen cells were less sensitive to the effects of AmB than spleen cells from the other strains. Other portions of the cell pellets were dispersed in phosphate-buffered saline and treated with trypan blue. The fraction of cells in culture which remained viable after treatment with AmB was estimated by counting the total number of cells and the number of cells able to exclude trypan blue. In Fig. 2 it is shown that in cultures treated with 5.0, 7.0, or 10.0 μg of AmB per ml, the fractions of viable cells remaining were similar for C57BL/6 and (C57BL/6 × AKR)F_1 strains, and both were lower than the corresponding fractions of cells from the AKR strain.

We then questioned whether differences in toxicity of AmB were also present in cells which were not from the immune system. RBCs were used because of our previous experience with this cell type and also because of our previous results that AmB-induced hemolysis is linked to oxidative events (5). The lytic effects of AmB on RBCs were measured as a function of concentrations of AmB or progression of lysis over time. Lysis of 50% of the RBCs, measured as a dose-dependent loss of hemoglobin (Fig. 3),

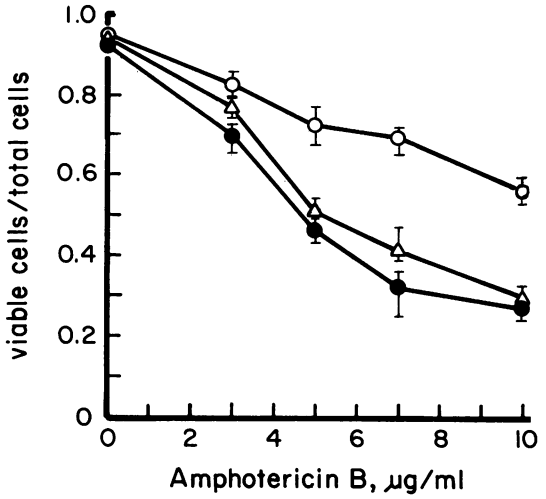

FIG. 2. Effects of AmB on loss of viability (ability to exclude trypan blue) by spleen cells harvested from C57BL/6 (●), AKR (○), or (C57BL/6 × AKR)F_1 (△) mice. Results are means ± SE from four experiments. Results for the AKR group differed significantly from those of C57BL/6 and (C57BL/6 × AKR)F_1 groups at AmB concentrations 5.0, 7.0, and 10.0 μg/ml. Groups C57BL/6 and (C57BL/6 × AKR)F_1 did not differ significantly.

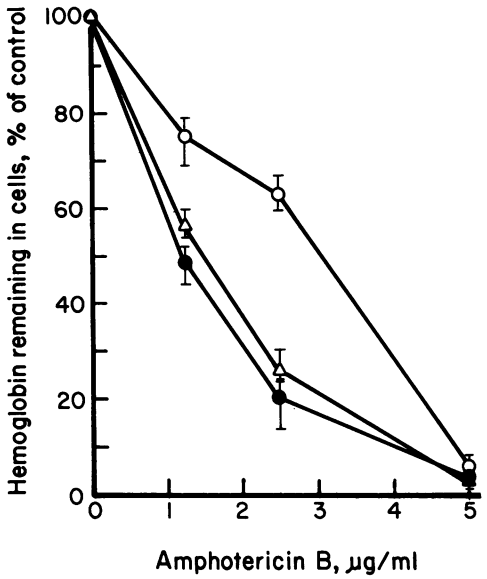

FIG. 3. Effects of AmB on retention of hemoglobin in RBCs from C57BL/6 (●), AKR (○), and (C57BL/6 × AKR)F_1 (△) mice. Results are means ± SE from four experiments for parent strains and from three experiments for the F_1 hybrid. Results for the AKR group were significantly different from those for C57BL/6 and (C57BL/6 × AKR groups)F_1 at AmB concentrations 1.2 and 2.5 μg/ml. Results between the C57BL/6 and (C57BL/6 × AKR)F_1 groups did not differ significantly.

was achieved with 1.3 μg of AmB per ml in cells from C57BL/6 mice, 1.5 μg/ml in cells from (C57BL/6 × AKR)F_1 mice, and 3.2 μg/ml in AKR cells. Thus, the sensitivity of RBCs from the F_1 hybrid closely resembles that of cells from the C57BL/6 parent, and cells from both of these strains were more sensitive to AmB-induced lysis than cells from AKR mice.

When hemolysis was followed over time, RBCs from C57BL/6 mice lysed more rapidly than cells from AKR mice. Hemolysis of RBCs from either strain took longer to occur when the oxygen tension in the incubation mixtures was lowered to 20 mm Hg or when catalase (100 U/ml) was added to the incubation mixtures (Fig. 4). These results are in line with our previous finding with human RBCs (5) and *C. albicans* cells (23) which led to the conclusion that an oxidative mechanism is involved in AmB-induced lysis.

Biochemical analysis. We investigated the probable basis for the differences in sensitivity to AmB of the cells from the different mouse strains. Because the level of sterol in membranes and the molar ratio of sterol to phospholipid are regarded as determinants of AmB-cell interactions (15), we measured cholesterol and lipid phosphorous levels in RBCs from C57BL/6 and AKR mice. No significant strain differences were found. Because our previous results (5, 23) and the data from this study indicate that oxidative damage is involved in AmB toxicity to cells, we measured the levels of cellular enzymes which would likely be involved in cellular defenses against oxidative injury. In agreement with previous results (9), we found that catalase activity in RBCs from C57BL/6 mice was about 60% of that in RBCs from AKR mice: the values (in millimoles ± SE of H_2O_2 decomposed during 10 s/g of hemoglobin) were 90 ± 7 in C57BL/6 cells and 159 ± 29 in AKR cells (means ± SE from five experiments). The catalase activity in RBCs from the (C57BL/6 × AKR)F_1 strain was 101 ± 9 (mean from three experiments),

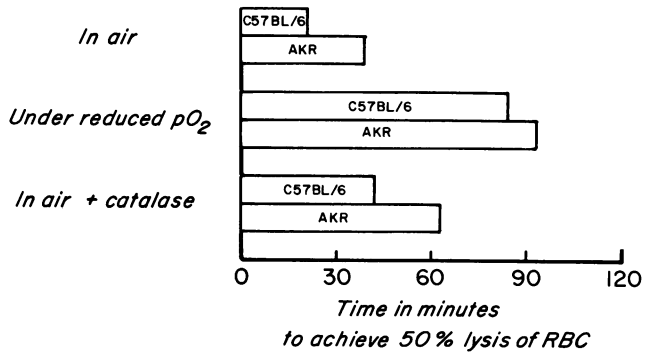

FIG. 4. Effects of low oxygen tension or catalase on time required to achieve 50% hemolysis of RBCs from C57BL/6 or AKR mice. RBCs were treated at time zero with 5.0 μg of AmB per ml. Results are from one representative experiment. Similar results were obtained in two other experiments.

a value comparable to that found in RBCs from the C57BL/6 parent. Statistical analysis showed that catalase levels in RBCs from AKR mice differed significantly from catalase levels in RBCs from the other two strains.

We did not find any significant differences in superoxide dismutase levels in RBCs from C57BL/6 and AKR strains (data not shown).

DISCUSSION

AmB treatment prolonged the survival of AKR mice and shortened the survival of C57BL/6 mice infected with *L. monocytogenes* when the administration of the polyene preceded infection. Survival time was shortened when the drug was given to mice of any strain after infection. Because AmB has no direct antibacterial properties, it is likely that both effects were caused by actions on the host. This notion agrees with results of our previous studies with bacterial infection (25) and is consistent with the results of other investigators who determined AmB effects in several different models of infection in experimental animals (2, 17).

A genetically determined difference in resistance to *L. monocytogenes* infection exists among various strains of mice, and C57BL/6 has been characterized as an innately resistant strain (6, 19). To induce comparable death rates the C57BL/6 mice had to be injected with an inoculum of *L. monocytogenes* which was 50% greater than that injected into AKR mice. This indicates that in comparison with C57BL/6 mice, AKR mice should be regarded as innately sensitive. It is interesting that the responses of the two strains to AmB were the reverse of their responses to *L. monocytogenes* infection. Treatment with AmB augmented the resistance of the AKR mice but not the C57BL/6 mice.

The AmB-induced increased resistance of AKR mice to *L. monocytogenes* infection could be the result of immune stimulation or of a direct effect of AmB on the bactericidal properties of macrophages. Because the lethal effects of *L. monocytogenes* infection were observed very early (1 to 4 days after bacterial challenge), it seems more likely that the enhanced resistance in the AKR mice treated with AmB was due to a very prompt effect of this antibiotic on the host macrophages rather than to the conventional mechanisms of cell-mediated immunity, which require T-cell activation and proliferation as an antecedent to macrophage activation. In support of the second possible explanation is the observation (J. R. Little, personal communication) that H_2O_2 production is stimulated by AmB in AKR macrophages but not in C57BL/6 macrophages.

Like the effects on resistance of mice to infection, the toxic effects of AmB also appear to be strain dependent. The inability of C57BL/6 mice to respond to the immunostimulatory action of AmB has previously been characterized as a dominant genetic trait (11), and it appears that higher sensitivity of this strain to the toxic action of AmB is the same.

The parallelism between the phenotype of whole animal responses and responses of cells harvested from the animal was demonstrated previously by showing that AmB in vitro is a potent mitogen for B lymphocytes taken from mouse strains classified as high responders to the immunostimulation by AmB, but AmB does not activate B lymphocytes from low responder strains (13). In this study we demonstrated that spleen cells and RBCs from C57BL/6 and F_1 hybrid strains are more sensitive to AmB-induced killing or lysis than corresponding cell populations from AKR strains. Because we did not find any differences in sterol or lipid phosphorous content in cells from the different mouse strains, we questioned the basis of the differential cell sensitivity. Recently, using human RBCs (5) and *C. albicans* cells (23), it was demonstrated that oxidative damage was involved in AmB-induced cell lysis and killing. We have shown here that the lysis of murine RBCs is influenced by the level of oxygen and the presence of catalase in the incubation mixture, and we assume that oxidative damage is also involved in AmB-induced lysis of murine RBCs.

The addition of superoxide dismutase to the medium did not influence cellular effects of AmB, and we did not find any strain differences in levels of this enzyme in RBCs. The analysis of spleen cells (J. R. Little, personal communication) and RBCs from low and high responding animals (results of this study) shows that cells harvested from C57BL/6 and hybrid F_1 mice have catalase levels estimated as the ability of the cell to consume H_2O_2 at about 60% of the consumption in cells harvested from the high responder strain. Therefore, it seems probable that the low level of the catalase contributed to the impairment of cellular defense mechanism against oxidative damage involved in the lytic and killing effect of AmB, although the possibility of a group of linked changes in sensitivity to oxygen toxicity cannot be excluded.

It was also observed (J. R. Little, personal communication) that oxidative events are involved in the in vitro mitogenic properties of AmB. If the same mechanism of action of AmB can result both in cell stimulation and toxicity, then the experimental conditions and characteristics of the cell would determine the type of response. Therefore, we interpret our data as suggesting that cellular toxicity of AmB in C57BL/6 mice prevents immunostimulation. Perhaps the detrimental effects of AmB given after infection of AKR mice by *L. monocytogenes* also occur because defense against oxidant damage has been compromised by the infection. The finding that *L. monocytogenes* organisms are capable of generating large quantities of superoxide and hydrogen peroxide (8) suggests that the resistance of the host could be overwhelmed by additive oxidative stress of AmB and bacteria.

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