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In Vitro Tumor Necrosis Factor Induction Assay for Analysis of Febrile Toxicity Associated with Amphotericin B Preparations

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Amphotericin B can stimulate macrophages to produce tumor necrosis factor alpha (TNF_{alpha}), one of the inflammatory mediators that may be responsible for the febrile toxicity associated with drug administration. The purpose of this study was to compare the in vitro TNF-inducing activity of one recalled lot of an amphotericin B preparation which was associated with more frequent febrile reactions in patients with a preparation not associated with a greater incidence of febrile reaction. We found that the former preparation induced significantly more TNF than the latter preparation, and the in vitro data correlated with the results of rabbit pyrogen testing. The in vitro TNF induction assay may serve as a screening tool for the selection of the least pyrogenic lots of amphotericin B preparation.

Intravenous administration of amphotericin B (AMB) is frequently associated with a severe febrile reaction. Compounding the problem is the variability among patients and among drug lots in the severity of these febrile reactions. Recently, a higher incidence of febrile reactions was reported in patients receiving a generic preparation of AMB, prompting a voluntary recall of all lots in February 1988 (4).

Federal regulations (5) require that AMB in a preparation of AMB exceed or equal 75%, and that the concentration of amphotericin A (AMA) not exceed 5%. All lots must also pass a pyrogenicity test in rabbits (6). Apparently, these requirements for purity and nonpyrogenicity are not sufficient to detect lots with greater pyrogenicity for humans. *Limulus* amoebocyte lysate (LAL) gelation assay of the commercial formulation cannot be done without substantial dilution because of the presence of Desoxycholate, which interferes with gelation and causes false-negative results (1). In addition, a negative LAL test would not indicate the absence of any pyrogenic substances other than endotoxin.

Discovery of an assay which would predict the pyrogenicity of AMB for humans would allow more effective screening of manufactured lots. This search would be aided if the cause of the febrile reactions was known. It has been speculated that one of two amphotericin molecules other than AMB, AMA or AMX, or another unknown impurity in the preparation causes the reaction. Supporting this possibility is the fact that a highly purified lot of AMB was found to be less pyrogenic (10). Since AMB is a fermentative product that requires multiple purification steps, there is always a possibility of contaminating traces of endotoxin. Another possibility is that AMB is inherently pyrogenic. We and others recently reported that AMB induces in vitro production of tumor necrosis factor alpha (TNF_{alpha}) by murine and human macrophages and that the inducing effect is not due to contaminating endotoxin (2; J. A. Gelfand, K. Kimball, J. F. Burke, and C. A. Dinarello, Clin. Res. 36:456A, 1988). In the present study, we compared one of the AMB lots recalled because of frequent febrile reactions with a lot not associated with any greater incidence of adverse drug reactions. These two preparations were compared with respect to pyrogenicity in rabbits and TNF release from murine macrophages.

AMB (Fungizone; lot 6F86883) was purchased from a local pharmacy. Lyphomed AMB (LAMB; lot 233723) was provided by the manufacturer (Lyphomed, Inc., Rosemont, Ill.). The two AMB 50-mg preparation vials contained the same quantity of AMB, by weight, sodium Desoxycholate, and sodium phosphate, and the AMB solutions were reconstituted in pyrogen-free distilled water to a stock AMB concentration of 5 mg/ml. The relative percentage of AMB and congeners in each preparation was determined by high-pressure liquid chromatography with an Altex C-18 reverse-phase column (Waters Associates, Inc., Milford, Mass.) using a standard procedure (7, 8) with some minor modifications. The mobile phase consisted of acetonitrile, methanol, and 0.5 M sodium acetate with 3 mM EDTA (pH 5) at a ratio of 28:36:36. AMB and AMX were detected at a wavelength of 405 nm, and the two different peaks were separable by their different retention times. AMA was detected at 302 nm with nystatin as the standard. The intrarun and interrun variations were less than 7 and 5%, respectively. The stock solution was diluted to a 1- to 5-μg/ml concentration for the high-pressure liquid chromatography analysis. Endotoxin contents of serial dilutions of AMB preparations were determined by the LAL gelation assay, employing the procedure provided by the manufacturer (Cape Cod Associates, Woods Hole, Mass.). The reported sensitivity of 3 pg/ml for endotoxin detection was verified by endpoint dilution of an *Escherichia coli* endotoxin standard.

Various concentrations of AMB were incubated with 10^6 murine RAW264.7 macrophages in Dulbecco modified essential medium supplemented with 10% fetal calf serum (Hyclone, Hogan, Utah), glutamine, penicillin, and streptomycin for 2 to 18 h, and the TNF bioactivity of culture supernatants was determined with a murine L929 fibroblast cytotoxicity assay as previously described (9). LAMB, at concentrations of 1.5 and 3 μg/ml, induced significantly more TNF production at 6 h poststimulation than AMB did (Fig. 1A). Higher concentrations of AMB were cytotoxic. Two other experiments produced essentially identical results.

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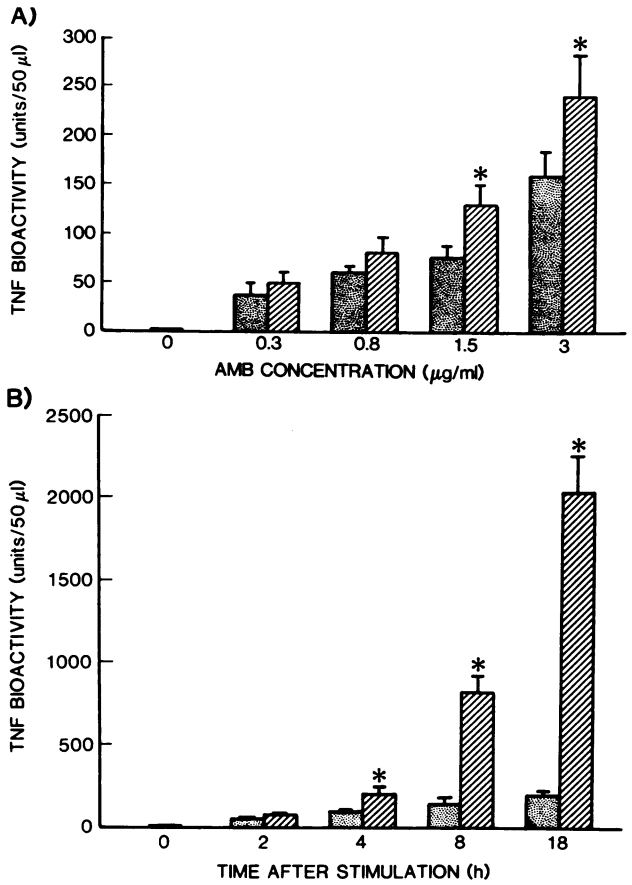

FIG. 1. Effect of two different AMB preparations on TNF production by murine RAW264.7 macrophages. (A) Concentration-dependent response. RAW264.7 macrophages (10^6) were stimulated with various concentrations of LAMB (hatched bars) and AMB (dotted bars) for 6 h, and the TNF bioactivity of macrophage supernatants was determined as cytolytic activity on dactinomycin-sensitized L929 murine fibroblasts. The results are expressed as the means ± the standard errors of the means of triplicate determinations. *, Difference between the two preparations was statistically significant ($P < 0.05$). (B) Time-dependent response. Murine macrophages (10^6) were stimulated with 3 μg of LAMB or AMB per ml for various incubation periods, and the TNF bioactivity was determined as described above.

The TNF responses of macrophages to a maximal, nontoxic concentration of AMB over a longer incubation period were evaluated. Interestingly, whereas the TNF bioactivity induced by 3 μg of AMB per ml remained relatively constant for 18 h after stimulation, LAMB-stimulated macrophages produced dramatically higher TNF bioactivity over time, suggesting a more sustained synthesis and release of bioactive TNF (Fig. 1B). The difference in TNF levels induced by the two AMB preparations over time was not due to a difference in cell viability, which was determined by trypan blue exclusion. The cytolytic activity of macrophage supernatants was also completely neutralized to less than 4 U/50 μl by a rabbit polyclonal anti-murine TNF_{alpha} antibody at a dilution of 1:200 (Genzyme, Boston, Mass.).

The relationship between the relative magnitude of the in vitro TNF responses and the respective febrile responses to the different preparations in rabbits was examined. Six paired female New Zealand White rabbits were randomized and tested with the two AMB preparations, and six additional rabbits were injected with pyrogen-free normal saline as controls. After temperature stabilization, each rabbit of the treatment group received 0.25 mg of AMB per kg of body weight in 5 ml of pyrogen-free 5% glucose water via the marginal ear vein by slow infusion over 5 min, and the rectal temperature was monitored over 4 h. No animals were excluded for data analysis. The control rabbits had no temperature elevation (<0.1 ± 0.1°C) in response to pyrogen-free saline. LAMB induced a significantly faster and higher pyrogenic response in these nonprimed rabbits than AMB did, although the maximal increases in temperature at 200 and 240 min following infusion were not statistically different for the two preparations (Fig. 2). The temperature response to the dose of AMB used in this study was comparable to the results reported by Arroyo (1).

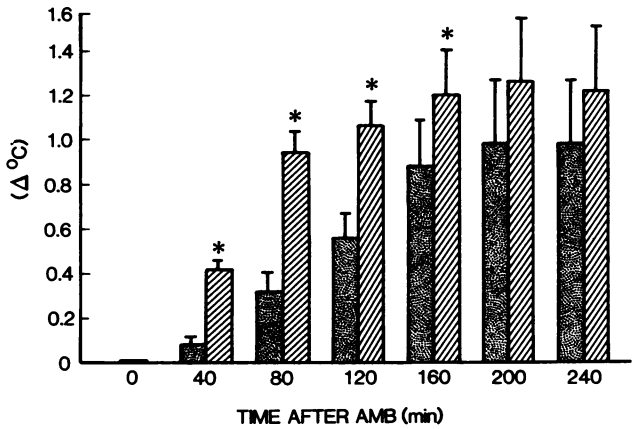

FIG. 2. Rabbit pyrogenic response to different AMB preparations. Six pairs of female New Zealand White rabbits (2 kg each) were injected with 0.5 mg of AMB (dotted bars) or LAMB (hatched bars) via the marginal ear vein over a period of 5 min, and the temperature response was monitored over the next 4 h. The results are expressed as the mean changes in temperature ± the standard errors of the means for six animals. *, Difference between the two groups is statistically significant ($P < 0.01$).

The concentrations of contaminating AMA and AMX in the AMB preparation were 2 and 4%, respectively, versus <1 and <3% in LAMB. Direct testing of AMB and LAMB stock solutions with the LAL assay had negative results, even when endotoxin was added to the tested specimens. A 40- to 100-fold dilution was required to prevent the effects of sodium desoxycholate. The endotoxin concentrations in LAMB were barely detectable but were more than those in AMB (>2.4 but <12 and <2.4 endotoxin units per mg of AMB, respectively).

These data indicate that the TNF-inducing activity of different AMB preparations may vary. The LAMB preparation, which was reportedly associated with a higher incidence of febrile reactions, induced more TNF production in vitro and a significantly faster pyrogenic response in rabbits than did a standard AMB preparation. The concentrations of AMA and AMX in the two preparations were inversely related to the TNF response in vitro and the pyrogenic response in rabbits, which made it unlikely that these congeners were responsible for the febrile responses.

Whereas the difference between TNF responses to the two AMB preparations was relatively minor at 6 h following stimulation, the difference at 18 h was dramatic. This observation suggests that the LAMB preparation is a much more potent and persistent inducer of TNF bioactivity. The endotoxin level in the LAMB preparation was between 2.4 and 12

endotoxin units per mg (24 to 120 pg/mg of AMB), which is at or slightly above the Food and Drug Administration-proposed limit of 2.5 endotoxin units per mg for this product. At the concentration found in the LAMB preparation, endotoxin alone would not induce significant TNF production by the RAW264.7 macrophages (J. Chia, unpublished observation) or be pyrogenic in rabbits (3), although it is possible that trace quantities of endotoxin act in synergy with AMB to induce TNF production. Therefore, the trace quantity of endotoxin in the LAMB preparation may be responsible for the difference in pyrogenic activity and the greater TNF induction with prolonged stimulation of the macrophages.

The febrile response in rabbits to AMB can be quite variable and is dependent on the rate of infusion (1). If the maximal temperature elevation had been used as the only criterion for excluding more-pyrogenic lots, the difference between the two AMB preparations could have been missed in this study. These results suggest that the in vitro TNF induction assay may be a more sensitive indicator of human febrile response than the rabbit pyrogen test, and they raise questions about the efficacy and consistency of the rabbit pyrogen test for preclinical screening of different AMB preparations.

The use of an in vitro pyrogen induction test for detecting contaminating endotoxin in biological materials has been previously described in Dinarello et al. (3). Our study evaluated the relative potency of two different AMB preparations for the in vitro induction of TNF production by a murine macrophage cell line. The RAW264.7 macrophages produce detectable TNF bioactivity in response to subnanogram amounts of endotoxin. Although the LAL assay is more sensitive (detecting amounts of endotoxin as low as 3 pg/ml), it is not infallible, particularly when it comes to endotoxin detection in the AMB-sodium Desoxycholate colloidal suspension (1). The routine use of a simple, reproducible, in vitro TNF induction assay may complement the LAL assay for quality control of the commercial preparations and thereby minimize the variability in febrile reactions seen with different lots of AMB.

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ADDENDUM

Lyphomed has developed a new process to rectify the endotoxin problem in its AMB preparation. New AMB lots screened in our laboratory are free of detectable endotoxin and have low TNF-inducing activity in vitro.

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