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Influence of Diet on Experimental Toxicity of Amphotericin B Deoxycholate

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The effects of pre- and postprandial levels of lipids in serum on the experimental in vivo and in vitro toxicities of amphotericin B deoxycholate (AmB-d) were studied. Normal OF1 mice were tested at baseline, after normal feeding, after 3 h of fasting, or after a sequence of feeding and fasting and vice versa. The 50% lethal dose (LD_{50}) of AmB-d was significantly higher in fed mice than in mice which fasted or at baseline (2.38 ± 0.12 versus 1.53 ± 0.2 and 1.50 ± 0.1 mg/kg of body weight, respectively; $P < 0.05$). When different nutritional regimens were alternated over a short period, the level of in vivo AmB-d toxicity was dictated by the last feeding regimen. Serum triglycerides, but not cholesterol in very-low-density and low-density lipoproteins, correlated significantly ($P < 0.01$) with the LD_{50} of AmB. In vitro experiments showed that the addition of human serum reduced AmB-d-induced toxicity against human erythrocytes, but serum drawn after fasting was less protective than postprandial serum. However, neither serum decreased the in vitro activity of AmB-d against *Candida albicans*. Circular dichroism, a method that enables the amount of free AmB to be measured, showed that both mouse and human total serum lipoproteins bound more AmB-d when serum was isolated postprandially than when it was obtained after fasting. Our results show that AmB-d toxicity is reduced by feeding-induced modifications in serum lipids. The influence of food intake on the clinical toxicity of the drug merits being investigated.

Parenteral amphotericin B (AmB) is the reference treatment for many systemic mycoses. However, its therapeutic use is hampered by a high level of side effects (13, 25). Therefore, many strategies have been developed in an attempt to reduce the toxicity of the commercial formulation for the intravenous route, AmB deoxycholate (AmB-d [Fungizone]). AmB is lipophilic, and its association with liposomes, lipid emulsions, or surfactant leads to a reduction of its toxicity without altering its antifungal activity (2, 3, 7, 14, 16). Some of these formulations are now available for clinical use. They have significant antifungal activity but continue to exhibit some residual toxicity (12, 18) and are very expensive. Since AmB binds to lipoproteins in human serum (5), we hypothesized that the endogenous lipids included in circulating lipoproteins could interfere with AmB-d toxicity. The lipid content of human serum is acutely altered by eating, fasting, or stress (11), and the influences of such variations on AmB-induced toxicity could be significant clinically.

This study was designed to investigate the effects of fasting and feeding on in vivo AmB-d-induced toxicity in mice and to study the effect of variations in the serum lipid content on in vitro AmB-d-induced toxicity against human erythrocytes (RBCs) in the presence of serum.

(This work was presented in part previously [10a]).

MATERIALS AND METHODS

Materials. The commercial formulation of AmB in a micellar dispersion of deoxycholate (AmB-d) was obtained from Squibb (Neuilly-sur-Seine, France). The stock solution was prepared by adding 10 ml of a 5% glucose solution to the lyophilized product; the final concentration of AmB was 5 mg/ml. The stock solution was always prepared on the day of the experiment.

Animals. OF1 male mice, 6 to 7 weeks of age, were obtained from Iffa-Credo (L'Arbresle, France). They were fed a normal diet (the semisynthetic diet contained 1,364.8 J/100 g of food, and its energetic distribution was 67.5% carbohydrates, 10.6% lipids, and 21.9% proteins). Animals were housed in groups of 10 in a temperature-controlled room and were offered food and water ad libitum. The 12-h light and dark cycles started at 7 a.m. and 7 p.m., respectively. Fasting mice had no access to food, but water was available ad libitum.

Determination of serum lipid fractions. (i) Analytic procedures. Blood samples, obtained by cardiac puncture from anesthetized mice and by venipuncture from humans, were allowed to clot for 2 h at room temperature. Sera were prepared by low-speed centrifugation at 4°C. For mouse studies, three to five samples were pooled for each determination. Sodium azide and EDTA (at final concentrations of 0.02 and 0.04% [wt/vol], respectively) were then added. Sera were stored at 4°C, and lipoproteins were isolated on the same day.

Lipoprotein fractions were separated from serum by ultracentrifugation at 15°C. Lipoproteins were defined according to the following density ranges: chylomicrons, <1.006 g/ml; very-low-density lipoproteins (VLDL), 1.006 to 1.019 g/ml; low-density lipoproteins (LDL), 1.019 to 1.063 g/ml; high-density lipoproteins (HDL), 1.063 to 1.215 g/ml. Density adjustments were made with potassium bromide (KBr) (9). Lipoprotein fractions were dialyzed for 12 h at 4°C against phosphate-buffered saline (PBS).

(ii) Chemical analysis. Triacylglycerol, total cholesterol, and phospholipid levels were determined by enzymatic methods using Unipath SA kits 55241, 46601, and 44511, respectively

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(Wako, Dardilly, France). The lipid content of lipoproteins was measured as previously described (8).

Experimental diet protocols. (i) Nycthemeral cycle in mice. Under normal conditions, mice actively feed during the first half of the dark period, and serum lipid levels follow a nycthemeral cycle. Therefore, in further experiments, baseline was defined as the end of the light period (7 p.m.), characterized by a low food intake.

The time of serum sampling for the fed state was derived from preliminary experiments in which serum samples (five samples from three pooled serum specimens each) were obtained every 3 h for 24 h from normally fed animals. These experiments showed that triglyceride concentrations remained constantly low during the light period but increased significantly ($P < 0.05$) during the dark period, peaking at 3 a.m. (2.37 ± 0.23 and 0.90 ± 0.12 mmol/liter at 3 and 9 a.m., respectively). No variation was noted for serum phospholipid levels during the 24-h period of observation. The variations of total cholesterol levels with time were not statistically significant. Given these results, fed mice were defined as mice which received a normal diet and whose serum was sampled at the time of peak triglyceride levels (3 a.m.).

(ii) Controlled food intake experiments in mice. The appropriate fasting duration was also derived from preliminary experiments measuring body weight and serum lipids in samples (five samples from five pooled serum specimens each) obtained every 3 h in mice which were made to fast (fasted mice) from baseline. The weight loss was negligible after 3 h but reached 5% when mice were fasted for longer times. Total cholesterol levels were significantly lower ($P < 0.05$) in 3-h-fasted mice than in mice at baseline and in fed mice (3.16 ± 0.29 versus 3.63 ± 0.21 and 3.91 ± 0.21 mmol/liter, respectively). HDL cholesterol remained stable, and VLDL-LDL cholesterol significantly decreased ($P < 0.05$) from the baseline when mice were fasted for 3 h (0.85 ± 0.21 versus 0.62 ± 0.08 mmol/liter at baseline and after 3 h of fasting, respectively). The decrease observed in triglyceride levels was not significant. Since triglyceride, cholesterol, HDL cholesterol and VLDL-LDL cholesterol levels remained stable when fasting was prolonged over 3 h, fasting was limited to 3 h in further experiments in order to avoid the consequences of weight loss.

In summary, we defined three basic food intake patterns: (i) baseline, at the end of the light period (7 p.m.); (ii) fasting, i.e., 3-h fasting from 7 p.m.; and (iii) fed, coinciding with the peak plasma triglyceride level (3 a.m.). Finally, sequences of feeding followed by fasting and vice versa were also used for studying serum lipid content and AmB toxicity.

(iii) Controlled food intake experiments in humans. The lipid content of normal human serum was determined after 12 to 14 h of fasting and 4 h after a high-calorie, lipid-rich meal which consisted of 736 kcal (ca. 3,000 kJ), for which the composition was as follows: proteins 17.4 g; lipids, 37 g (45% of total energy content); and carbohydrates, 83.8 g. For each regimen, results are expressed as the mean of values measured in nine different serum samples.

In vivo toxicity. Fifty-percent lethal doses (LD_{50}s) and confidence intervals were determined as previously described (22, 23). AmB-d diluted in 5% glucose (0.2 ml) was injected into the tail vein over 15 s. The time of drug administration was chosen according to the food intake pattern studied, as described above. The doses were adjusted according to the mean body weight of the animals in each therapeutic group measured just before the experiment. Toxicity was assessed as mortality over the 7 days following treatment. Groups of 10 mice per dose were used, and each value represents the mean of three different experiments.

Measurements of AmB-induced K^+ leakage and lysis of RBCs and *Candida albicans*. *(i) Toxicity against RBCs.* RBCs were separated from the EDTA-anticoagulated blood of fasting normal donors by centrifugation at 800 × *g* for 10 min at 4°C, washed three times with PBS, and used on the same day. Appropriate amounts of the stock solution of AmB-d (5 mg/ml in 5% glucose) were added to PBS containing 1/10 normal human serum drawn either after feeding or fasting, as defined above, or to PBS alone. The suspensions were immediately mixed by vortexing and incubated for 20 min at 37°C. RBCs were then added at a final concentration of 10^7 cells per ml and the mixtures were incubated at 37°C for 1 h, at which time they were centrifuged at 800 × *g* for 10 min. To measure K^+ and hemoglobin concentrations remaining in RBCs, 0.5 ml of H_2O was added to the pellets of RBCs to cause lysis. The concentration of K^+ was measured by flame emission, and hemoglobin was measured spectrophotometrically at 540 nm. Results are expressed as the means of three different experiments, in which duplicates were run.

(ii) Antifungal activity. *C. albicans* was maintained on Sabouraud dextrose agar. A standard inoculum of the culture was transferred to 10 ml of liquid Sabouraud medium. After 16 to 18 h of incubation at 35°C, 10 ml of fresh medium was added and the mixture was incubated for 1 h. A final concentration of 2×10^7 *C. albicans* organisms per ml in appropriate concentrations of AmB-d was incubated for 1 h at 37°C with shaking. At the end of the incubation, fungal cells were washed three times in PBS. Appropriate dilutions were plated on Sabouraud agar and, after 24 h of incubation, CFU were counted. The remaining cell suspension was boiled for 3 min for K^+ determination. The effect of serum was assessed as described above for RBCs. Results are expressed as the means of three different experiments, in which duplicates were run.

Spectroscopic determination of AmB binding to lipoproteins. For all measurements, we used AmB-d as the AmB source. The protein concentration of lipoproteins was adjusted to 1.2 g/liter, which represents the concentration of total lipoproteins in normal serum (10). AmB-d was diluted to a final concentration of 2 μM. This concentration was selected because it reflects the peak value measured in serum after an AmB-d infusion in patients (1). Since the absorbance spectra of free and bound AmB overlap, there is no wavelength at which only one of the species absorbs and could therefore be quantified by standard spectroscopy. In contrast, AmB circular dichroism enables monitoring of the amount of the drug remaining free in the presence of lipoproteins: AmB bound to lipoproteins does not generate any dichroic signal in the region of 340 nm, in contrast to free AmB, which is responsible for an intense dichroic doublet centered at this wavelength. This doublet is due to the self-associated oligomeric form only, but, since the proportion of monomers is very low at this concentration (2 μM), the size of the doublet can be considered to accurately reflect the amount of the total free AmB fraction present in the medium. Circular dichroism spectra were recorded with a Jobin-Yvon Mark V dichrograph, thermostated at 37°C, as previously described (17), 10 and 30 min after dilution of the AmB-d stock solution in lipoproteins at 37°C, and compared with spectra for AmB-d diluted in 5% glucose.

Statistical analysis. Grouped data are expressed as means ± standard deviations. For more than two groups, multiple means were compared by one-way analysis of variance, and the Scheffe F test was used when allowed by analysis of variance ($P < 0.05$). Student's *t* test was used to compare two groups. The

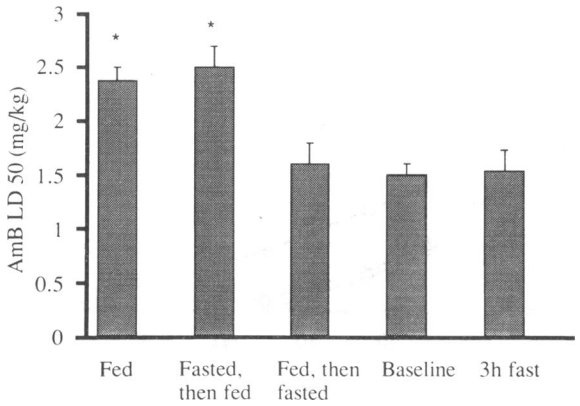

FIG. 1. Influence of feeding pattern on AmB LD_{50}. Each value represents the mean ± standard deviation of three determinations. *, significantly different from the value obtained at baseline; $P < 0.05$. For the definitions of the five experimental diets, see Materials and Methods.

95% confidence intervals were calculated when appropriate. P values of < 0.05 were considered significant. Linear regression analysis was used to correlate the different lipid determinations with LD_{50}s.

RESULTS

Influence of food intake on in vivo AmB-d-induced toxicity and on lipid content in serum in mice. The AmB-d LD_{50} was significantly higher ($P < 0.05$) in fed mice (2.38 ± 0.12 mg/kg of body weight) than in fasted mice (1.53 ± 0.2 mg/kg) or at baseline (1.50 ± 0.1 mg/kg) (Fig. 1). Triglyceride levels were also significantly higher ($P < 0.05$) in fed than in fasted mice or at baseline. The mean VLDL-LDL cholesterol level in fed mice was similar to the value measured at baseline but was higher than that observed in fasted mice ($P < 0.05$) (Table 1).

To further document the effect of diet on the in vivo AmB-d-induced toxicity, two additional experiments were performed: in one, mice were fasted for 3 h and then were fed for 3 h before the LD_{50} was measured; in the other, mice were fed and then fasted before the LD_{50} was measured. The level of AmB-d toxicity was dictated by the last feeding regimen: mice that were fed and then fasted for 3 h before AmB-d administration had low LD_{50}s (1.6 ± 0.2 mg/kg). Conversely, 3 h of food uptake after fasting promptly increased the AmB-d LD_{50} ($P < 0.05$).

A wide range of LD_{50}s was obtained with the five feeding regimens. We studied the correlation between LD_{50} and lipid levels, using the LD_{50} and the mean triglyceride or VLDL-LDL cholesterol value obtained from the same experiment. Triglycerides (in millimoles per liter) correlated with AmB LD_{50}s (milligrams per kilogram) (linear regression: $y = 0.91 \times + 0.108$; $R = 0.71$; $P = 0.0066$) (Fig. 2A). There was no significant correlation between VLDL-LDL cholesterol and the AmB LD_{50} ($P = 0.25$) (Fig. 2B).

TABLE 1. Influence of feeding pattern on triglyceride and VLDL LDL cholesterol levels in normal OF1 mice

Feeding regimen[a]	Serum lipid concn (mmol/liter)[b]	
	Triglycerides	VLDL cholesterol
Baseline	1.13 ± 0.23	0.85 ± 0.21
Feeding alone	2.54 ± 0.15[c]	0.85 ± 0.03
Fast, then feeding	2.65 ± 0.85[c]	0.78 ± 0.23
Feeding, then fast	2.12 ± 0.64	0.67 ± 0.21
3-h fast	0.93 ± 0.45	0.62 ± 0.08[d]

[a] For definitions of the five experimental diets, see Materials and Methods.
[b] Values are means ± standard deviations.
[c] Significantly different from the value at baseline; $P < 0.01$.
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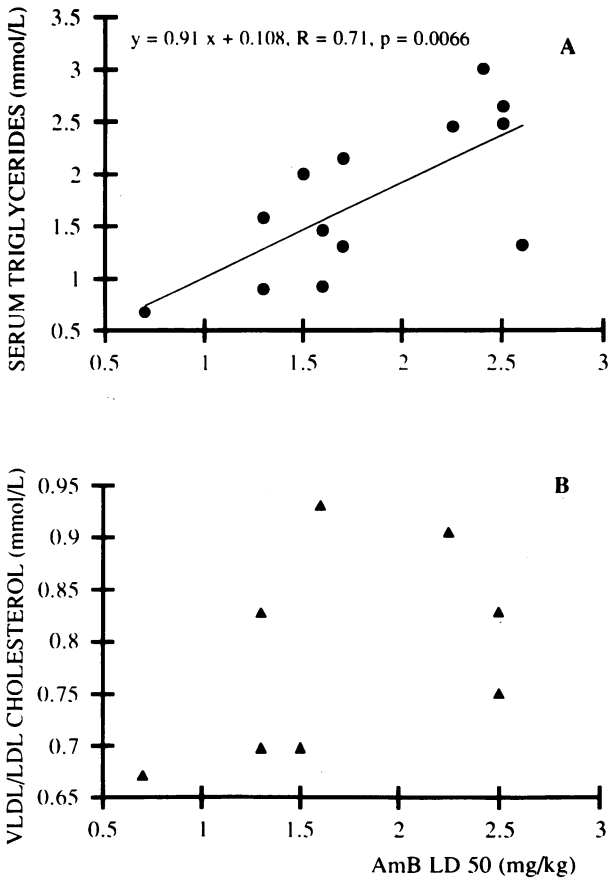
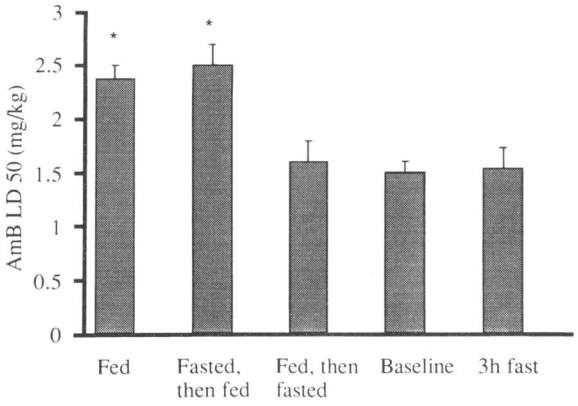


FIG. 2. Relationship between LD_{50} and serum triglyceride values (A) and VLDL-LDL cholesterol values (B). Each point is obtained from the determination of AmB LD_{50} and the mean lipid level obtained in the same experiment.

Influence of serum from fasted or fed volunteers on the in vitro toxicity and activity of AmB-d. In order to investigate whether the above results might be extended to AmB toxicity in patients, the protective role of human pre- or postprandial serum against in vitro AmB toxicity was measured. Normal human serum was obtained either after 12 to 14 h of fasting (preprandial serum) or 4 h after a meal (postprandial serum), and human RBCs were exposed to AmB-d in the absence or presence of serum. AmB-d was less toxic against human RBCs in the presence of serum, but postprandial serum was more protective than preprandial serum against RBC hemolysis induced by 25 μM AmB-d ($P < 0.05$) (Fig. 3A) and against K^+ release induced by 5 μM AmB-d ($P < 0.05$) (Fig. 3B). However, human serum drawn either before or after eating had no influence on the AmB-d-induced K^+ release by and viability of *C. albicans* (Fig. 3C and D). Comparison of lipid concentrations before and after eating showed that total cholesterol levels did not differ, whereas eating increased



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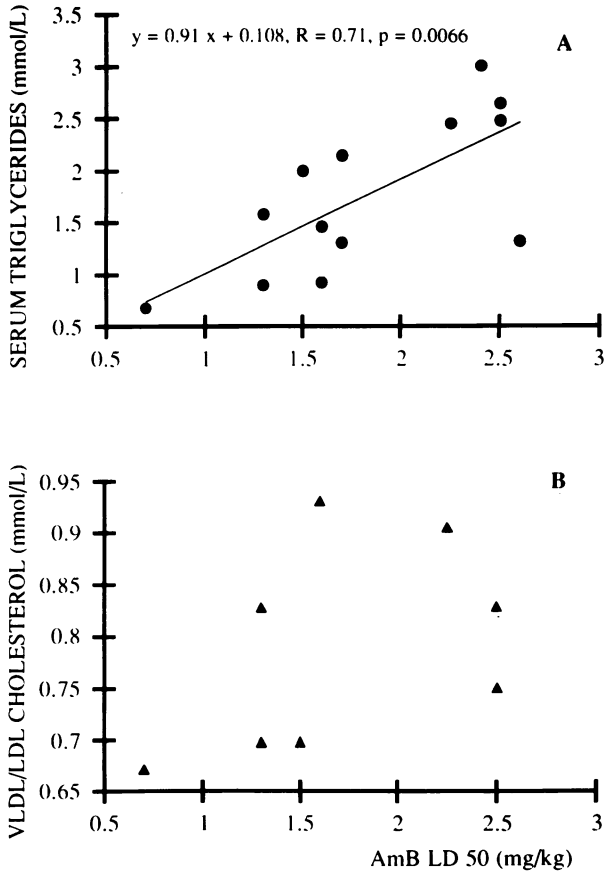

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DISCUSSION

Our results demonstrated mainly that the acute in vivo toxicity of AmB in normal mice was closely related to the feeding pattern. The consequences of food intake on AmB toxicity were rapid and reversible, as shown by the predominant influence of the most recent feeding regimen over a short period of time (less than 3 h).

By means of in vivo and in vitro experiments, we investigated whether these modifications in AmB toxicity were associated with alterations in serum lipids resulting from the different diets imposed. Few in vivo investigations have been performed in this field, and contradictory results have been reported. Koldin et al. (19) failed to demonstrate both the protective effect of high serum cholesterol levels against AmB toxicity in rabbits and the reduced in vivo toxicity of AmB complexed in vitro to lipoproteins. By contrast, Wasan et al. (27) showed that experimentally diabetic rats exhibited an increased tolerance to AmB and a marked increase in serum cholesterol and triglyceride levels. In both studies, serum lipid values were abnormal, due to diabetes or administration of a special diet, leading to experimental conditions that were far from the physiologic situation. To correlate the in vivo toxicity of AmB with changes in the serum lipoprotein content, we chose the mouse model for the following reasons. First, acute lethality after intravenous AmB administration is a simple and reproducible way to investigate AmB toxicity, although the mechanism of death is not known. Second, mouse lipoproteins are well described and easy to isolate from serum (9, 10). We chose to study the consequences on AmB toxicity of spontaneous lipid variations in normal animals, since these variations over a 24-h period, with a normal nutrition and after fasting, paralleled the variations in lipids seen in human serum after a meal or after fasting. The decrease in VLDL-LDL cholesterol during fasting was expected in mice, since, unlike other animal species, mice lack cholesteryl ester transfer activity (15), responsible for transferring cholesteryl ester from HDL back to LDL. We selected a relatively short (3-h) fasting duration in order to avoid the influence of weight loss on our results.

Our data suggest that modifications in serum lipids related to normal feeding were associated with a decrease in AmB toxicity. LD_{50}s correlated with serum triglyceride values but not with VLDL-LDL cholesterol values. This association between an increase in triglycerides and a reduction in AmB-d toxicity suggested that the triglycerides, or their main vehicle in serum, i.e., chylomicrons, LDL, and VLDL, were involved in the protective effect of feeding against AmB-d toxicity. Recently, Souza et al. (24) showed that a triglyceride-rich emulsion that behaves in vivo as chylomicrons was able to reduce the in vitro and in vivo toxicity of AmB. However, we cannot exclude that the protective effect of feeding was related to other, unexplored mechanisms, independent from triglycerides; triglyceride modifications would then be associated with diet alteration but not causally related to AmB-d toxicity.

The spectroscopic investigations shed some light on the possible mechanisms of protection afforded by serum lipid alterations. Free (protein-unbound) AmB is present in aqueous medium in three distinct physical states: soluble monomers, soluble oligomers, and insoluble aggregates. The concentration of the soluble oligomeric form of free AmB, thought to be the most toxic form (2, 4, 20), was reduced in the presence of lipoproteins and was lower when the lipoproteins were isolated from post- rather than preprandial serum. The changes in AmB binding to isolated fractions with a density below 1.063, either from mice or from humans, were similar to those obtained with total lipoproteins, suggesting that diet influences, at least in part, the amount of free toxic drug by modifying its interactions with the VLDL-LDL fraction in mice or the VLDL-LDL fraction and chylomicrons in humans. Since this low-density fraction represents the triglyceride-rich lipoproteins, our binding experiments agree with the correlation between triglycerides and LD_{50} observed in vivo. VLDL and LDL have previously been reported to be more efficient than HDL in reducing the amount of free AmB (5), and this was attributed to the higher cholesterol-to-phospholipid ratio in VLDL or LDL than in HDL. However, it has been shown recently that more than 50% of AmB was recovered in the HDL fraction (26). We cannot exclude that AmB binding to HDL cholesterol was affected by eating or fasting, but, since HDL cholesterol remained unchanged in mouse and human sera in our experiments, we did not investigate this point.

We also indirectly evaluated the protective effect of eating against AmB toxicity in humans by ex vivo experiments using RBCs. Although anemia under AmB therapy results from bone marrow suppression, more than from hemolysis, the interaction of polyene with RBCs has been widely studied since these cells are readily available and their membrane properties are well known. Serum sampled after the volunteers had eaten was more protective than serum drawn after fasting, in agreement with the reduction of free AmB soluble oligomers observed spectroscopically. The K^+ release reflected alterations in membrane permeability, the first step of AmB cellular toxicity. We also investigated AmB-induced hemolysis and demonstrated that protection was not limited to changes of membrane permeability. The high concentrations of AmB tested against RBCs were not representative of the therapeutic concentrations but were selected because lower values were not toxic against RBCs under our experimental conditions because of the short incubation time. Our in vitro results confirmed in vivo data obtained for mice and suggest that they might bear some clinical relevance. Interestingly, antifungal activity remained stable in the presence of serum, drawn either pre- or postprandially. This retained antifungal activity agrees with previous reported data (5, 6, 24) and confirms the fact that the threshold of free AmB needed for interaction with yeast ergosterol-containing membranes is lower than that needed for interaction with cholesterol-containing mammalian cell membranes. This difference supports the selectivity of AmB against yeasts (4).

The in vivo experiments were performed after a single administration of AmB. It has been suggested that AmB binding to lipoproteins may lead to increased internalization of the drug in cells bearing lipoprotein receptors (21) and thereby enhance the intracellular toxicity of the drug in the case of repeated administrations. It therefore remains to be investigated whether an increased binding of AmB to lipoproteins observed under conditions of chronic administration and normal feeding would have long-term deleterious effects.

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