Heat-Induced Superaggregation of Amphotericin B Modifies Its Interaction with Serum Proteins and Lipoproteins and Stimulation of TNF-α

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ABSTRACT: The purpose of the present study was to examine the influence of heat-induced superaggregation of Amphotericin B (AmB) in the Fungizone® (FZ) formulation on its interaction with human serum components and relate this to reduced toxicity. Whole serum distribution studies showed that a significantly lower percentage of AmB from HFZ was recovered in the high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglyceride-rich lipoprotein (TRL) fractions and a greater percentage recovered in the lipoprotein-deficient plasma (LPDP), though the majority of both preparations were recovered in LPDP. Circular dichroism (CD) and difference absorption spectroscopy were used to determine the stability of FZ and heat-treated FZ (HFZ) in the presence of HDL, LDL, serum, and albumin. The CD studies indicate that the "core" aggregate of HFZ is more stable in the presence of HDL and LDL, whereas the FZ is less stable and more dynamic with the core aggregate dissociating to a greater extent in the presence of either purified lipoprotein. Absorption studies with whole serum and purified albumin suggest that FZ aggregates are far less stable in the presence of albumin than HFZ and that interaction with serum albumin is a dominant feature for both drug preparations. HFZ also has a different effect on the cytokine response *in vitro*. Studies using THP-1 human monocytes show that HFZ provokes a smaller release of tumor necrosis factor (TNF)-α than FZ. This cytokine may be associated with the unpleasant side effects of AmB. These findings suggest that heat-induced superaggregation of AmB alters its interaction with HDL, LDL, serum proteins, and monocytes, and these findings may be important in explaining the reduced toxicity of the superaggregated form of AmB. J Pharm Sci 90:124–133, 2001

Keywords: polyene; antifungal; liposome; drug delivery

INTRODUCTION

Amphotericin B (AmB) is a polyene macrolide antibiotic used to combat systemic fungal infections. The usual formulation of AmB, a preparation consisting of AmB: deoxycholate in a mole ratio of ~1:2 (Fungizone®), is highly toxic to patients, often causing decreased renal function, anaphylaxis, chills, high fever, nausea, phlebitis, anorexia, and a host of other unpleasant effects. This array of untoward effects coupled with long therapeutic regimes nearly negates its usefulness in all but the most life-threatening systemic fungal infections (see review by Hartsel and Bolard[1]). The antifungal mechanism of action of AmB centers on its membrane activity.

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It produces membrane disruptions in the target cells, and the subsequent leakage of ions and small molecules can kill or damage these cells.[2] The reason for its fungal selectivity seems to lie in the greater affinity of AmB for the ergosterol-containing membranes of fungi versus the cholesterol-rich mammalian cell membranes.[1,2] The acute and chronic toxicity toward humans, however, is ample evidence that this selectivity is not absolute. Newer liposomal and lipid-associated drug delivery mixtures have proven very successful at reducing the toxicity of this drug.[3] Their general incorporation into the therapeutic arsenal has been slowed by their great expense, and so the toxic, yet effective, micellar AmB preparation, Fungizone® (FZ), continues to dominate the treatment of serious fungal diseases.

The three lipid-associated pharmaceutical preparations of AmB that are currently on the market are AmBisome (Fujisawa Healthcare, Inc., Deerfield, IL), Amphotec (Alza Corp., Palo Alto, CA), and Abelcet (Liposome Company, Princeton, NJ). They are fundamentally different physically: AmBisome is a true closed liposomal bilayer preparation, Amphotec is a micellar solution with cholesteryl sulfate, and Abelcet is an interdigitated lipid dispersion. Despite these differences, they all possess a far superior therapeutic index to FZ (Bristol Myers-Squibb, Princeton, NJ). As a potential simple and inexpensive alternative, simple AmB or FZ solutions may be treated with moderate heat (70°C for 20 min) to produce a new self-associated state of AmB, the "superaggregate," which we will refer to as heat-treated FZ (HFZ).[4,5] This new species is spectroscopically distinct from FZ, with a blue-shifted absorption maximum, characteristic circular dichroism (CD) spectrum, and pleiomorphic cobweb-like ultrastructure.[6] In model murine infections, HFZ has recently been shown to have a superior therapeutic index.[7,8] This superaggregate species is fascinating both for the possible practical therapeutic benefits and for its potential for explaining the root mechanisms for toxicity reduction in the simplest possible improved AmB drug delivery system. For this reason, the HFZ model system has been chosen for the work described here.

We propose that there are essentially three factors that influence the toxicity and efficacy of AmB preparations: (1) direct membrane toxicity via ion channel formation, (2) differences in distribution and delivery to tissues due to differences in serum lipoprotein and protein binding and stability, and (3) initiation of an inflammatory cytokine response. Previous studies in this laboratory have addressed the first point and have shown that HFZ is intrinsically less active against model cholesterol-containing membranes compared with ergosterol-containing membranes.[9] This result suggests an inherently lower direct toxicity against mammalian cells. However, there are other factors, especially AmB disposition in the serum, that may be just as important in determining the therapeutic index of AmB.[10–14] In this study, we investigate how structural stability, distribution in serum, and cytokine induction differ between FZ and HFZ in an attempt to rationalize the improved therapeutic index of HFZ.

MATERIALS AND METHODS

Chemicals

Purified Amphotericin B (AmB) was a generous gift from Bristol-Myers Squibb Pharmaceuticals (Princeton, NJ). AmB as Fungizone® (FZ; Bristol-Myers Squibb Pharmaceuticals) was purchased from a commercial supplier. Whole human serum was diluted 1:5 in phosphate-buffered saline (PBS) because of the high absorbance of the serum between 300 and 400 nm.

Heat-treated FZ (HFZ) was made by heating a 1.0×10^{-4} M (in AmB) solution of FZ dissolved in PBS (155 mM NaCl, 7 mM Na_2HPO_4, 3 mM KH_2PO_4, pH 7.4) to 70°C in water bath for 20 min. Stock solutions of FZ were removed from the same preparation before heating. Both preparations were purged with argon and kept in darkness to prevent oxidation.

Lipoprotein Distribution Studies

Plasma samples with FZ or HFZ (20 μg/mL of human plasma) were incubated at 37°C for 60 min. The plasma samples were separated into their high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride-rich lipoprotein (TRL), and lipoprotein-deficient plasma (LPDP) fractions by density gradient ultracentrifugation as previously described.[15] Each lipoprotein plasma and LPDP sample was assayed for AmB by a previously developed high-performance liquid chromatography (HPLC) method.[16]

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA; GraphPad Instat). Critical differences were assessed by Tukey–Kramer post hoc test. Differences were considered significant at $p < 0.05$. All data are expressed as means ± standard deviation (SD).

Circular Dichroism and Absorption Studies

Kinetic photodiode array spectral series were taken using a micro-volume stopped-flow reaction analyzer (Applied Photophysics Ltd., UK, and SX.18MV). Spectral series were measured at 37°C from 300 to 450 nm with a diode array detector with an integration time of 10.080 ms. The mixing chamber had a 1.0- or 0.20-cm pathlength, and a monochrometer slit width was generally fixed at 0.5 mm entry and 0.2 mm exit. Oversampling was employed with a 200-s log time base. All samples were protected from light to prevent photooxidation of AmB. Singular value decomposition (SVD) and global analysis software from Applied Photophysics were used for data analysis. SVD analysis was used to reduce spectral noise and suggest a minimum number of species contributing to a spectral family. For difference spectra, reconstituted spectra using the minimal number of species suggested by SVD analysis (number of basis spectra, $n = 2$ in each case here) at $t = 10$ ms and $t = 200$ s were subtracted in DeltaGraph (SPSS, Inc.).

Steady-state CD spectra were taken using the Applied Photophysics CD attachment. FZ, HFZ, or AmB/dimethyl sulfoxide (DMSO) were incubated with HDLs or LDLs in PBS buffer at 37°C for >15 min, and scanned using an Applied Photophysics CD attachment. The human serum was diluted 1:5 with PBS (except where noted) before addition of FZ and HFZ because of heavy absorbance from proteins and bile pigments. Similarly, AmB CD spectra below 300 nm could not be collected in the presence of serum and serum components because of their high absorbance.

Isolation and Purification of HDLs and LDLs

HDL and LDL fractions were isolated from the total lipoprotein precipitate by centrifugation as previously described and further purified by dialyzing against PBS solution (4 L) for 18 h at 4°C.[17] The molecular weight (MW) cutoff of the dialysis tubing used was 1000 Daltons. Following dialysis these lipoprotein fractions were filtered through a 0.2-μm filter and purged under N_2 gas to minimize oxidation.

Tumor Necrosis Factor-Alpha Assay

A THP-1 (ATCC TIB 202) human monocyte cells were grown in RPMI 1640 medium containing 10% low pyrogen fetal calf serum, 100 U penicillin, 100 μg/mL streptomycin and 1 μg/mL gentamycin. The cells were a high tumor necrosis factor (TNF)-producing strain cultured continuously in 1988. The cells were cultured in suspension at 37°C and 5% CO_2. The cells required 6–8 days to reach their terminal density. Cells were centrifuged and placed in fresh media at the outset of the experimental exposure to FZ and HFZ; 10^6 cells/well were used in this assay. The enzyme-linked immunosorbent assay (ELISA) for TNF-α was a sandwich-type assay with alkaline phosphatase/p-nitrophenyl phosphate colorimetric detection from a kit from Immunotech (purchased from Sigma Chemical Company, St. Louis, MO).

RESULTS AND DISCUSSION

Static Distribution of HFZ versus FZ in Whole Serum

We carried out studies of the equilibrium distribution of 2.0×10^{-5} M AmB from HFZ and FZ into human serum components. The results are shown in Table 1. Although 20 μM is a higher concentration than would ever be reached in plasma at equilibrium *in vivo*, it is nonetheless a good representation of the higher local concentration present during infusion of the drug. The data indicate that AmB from FZ binds to all serum lipoproteins to a greater extent than that from HFZ, which remains largely associated with lipoprotein-depleted serum. Even so, the majority of AmB from both forms remains associated with the LPDP. Previous techniques (affinity chromatography) looking at FZ lipoprotein distribution did not adequately separate the LPDP and HDL fraction[11] and thus the present results may seem inconsistent. However, our recent work using a density-gradient ultracentrifugation technique[18] shows that the majority of FZ AmB is, in fact, recovered in the LPDP fraction and not the HDL fraction. Wasan and colleagues have previously shown that incorporation of AmB into one of the

Table 1. Distribution of FZ and HFZ[a]

Treatment Group	Percent Recovery of AmB (%)			
	TRL Fraction	LDL Fraction	HDL Fraction	LPDP Fraction
FZ	5.99 ± 0.50	4.96 ± 0.39	3.56 ± 0.70	75.84 ± 8.93
HFZ	2.16 ± 0.33^b (n = 3)	2.00 ± 0.23^b	0.77 ± 0.12^b	92.61 ± 5.12^b

[a]Determined at AmB concentration in human plasma of 20 μg/mL, with incubation for 60 min at 37°C.

[b]Significantly different than FZ ($p < 0.05$).

Plasma samples were assayed by HPLC at a wavelength of 405 nm for AmB in each of the lipoprotein and lipoprotein-deficient plasma fractions.

Plasma was separated into its lipoproteins and lipoprotein-deficient fractions by affinity chromatography and centrifugation. Total recovery was 90–97% of the original AmB concentration incubated.

Abbreviations: AmB, amphotericin B; LPDP, lipoprotein-deficient plasma that contains albumin and alpha-1-glycoprotein; HDL, high-density lipoproteins; LDL, low-density lipoproteins; TRL, triglyceride-rich lipoproteins that includes very low-density lipoproteins and chylomicrons.

successful liposomal AmB preparations results in AmB bound more avidly to HDL than LDL than compared with FZ. This result suggests that some AmB-induced toxicity may be mediated by cellular uptake via LDL receptors or lack of uptake in critical tissues when bound to HDL.[11,15,16,19] The serum distribution of HFZ is quite different than that of these liposomal preparations, but is nonetheless consistent with this hypothesis because there is significantly less bound to LDL.

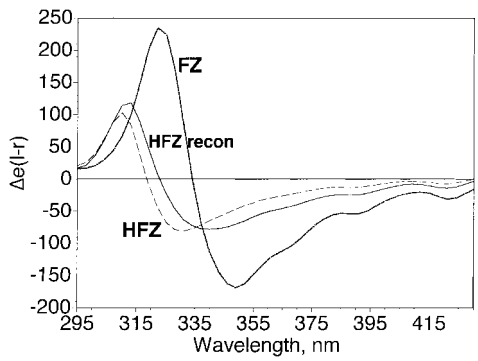

Figure 1. HFZ and FZ CD spectra before and after lyophilization (temperature: 20.1°C; incubated for > 15 min in PBS; pH 7.4; CD units are molar circular dichroism, L mol^{-1} cm^{-1}).

CD Studies of HFZ versus Fungizone

Self-association of AmB molecules can be observed by absorption and CD spectroscopy. Upon self-association, the characteristic polyene vibronic structure of the AmB monomer collapses to an intense blue-shifted band characteristic of some type of stacked array of interacting absorbers. For FZ, for example, the principle transition shifts from 409 nm (in water) to ~332 nm when self-associated. Optical activity of these oligomers, measured by CD spectroscopy, is one of the most sensitive methods for monitoring subtle changes in the supramolecular structure of strongly absorbing molecules.[20] The chromophore of the monomer of AmB is rather symmetric and so has very weak optical activity compared with the oligomer. Hence, we have used CD to demonstrate the conversion of FZ to HFZ and evaluate its stability and permanence, which has not been assessed in previous studies[4–6] (Figure 1). There is a change in the CD intensity on heating of FZ, with a blue shift in the CD crossover point from ~332 to ~318 nm. This shift corresponds to the shift in absorption maximum and agrees well with previous data.[4,5] It should be noted that both absorption spectroscopy in methanol and reversed-phase HPLC indicate that no chemical change in AmB occurs during this treatment; hence, all the changes in structure and activity can be attributed to supramolecular structure change.[9] Figure 1 shows the result of the lyophilization and reconstitution of HFZ from PBS. Note the nearly identical intensities and small shift in the crossover (to 322 nm) band position of the CD spectrum. This small shift is probably due to the re-emergence of minor FZ-like component after reconstitution. These experiments suggest that the supramolecular structure associated with heating has significant "perma-

nence" because lyophilized preparations retain much of the spectral blue shift and characteristic CD on reconstitution. Sugars are often used to stabilize pharmaceutic preparations during lyophilization. CD spectra from reconstitution of HFZ from a 5% dextrose solution show an identical crossover and nearly identical intensity for both, although the crossover is slightly shifted (324 nm) from PBS preparations. For reasons of convenience and shelf-life in a pharmaceutical preparation, this observation has great significance.

The bilobed CD band observed in Figure 1 almost certainly arises from excitonic coupling of the transition moments of a regular (or at least dominant) array of AmB molecules.[21–24] According to exciton chirality theory, an arrangement of AmB monomers showing positive chirality (right-hand screw sense) would give rise to a bilobed CD spectrum with a short wavelength (high energy) band of negative sign, whereas the arrangement of AmB monomers with negative chirality (left-hand screw sense) would give rise to a bilobed CD spectrum with a short wavelength (high energy) band of positive sign.[20] Thus the spectra of HFZ suggest a retention of the negative "chirality" of AmB monomer assembly but point to a change in geometry and/or distance between monomers.

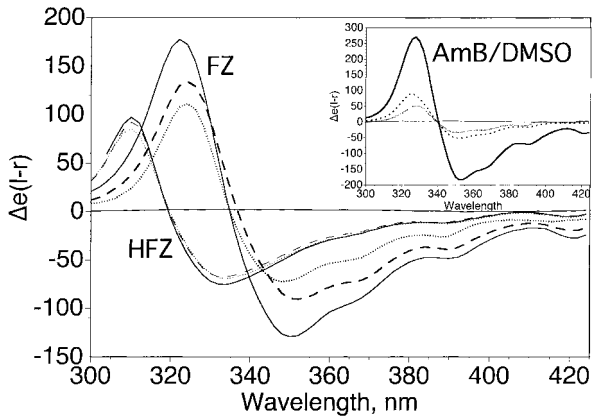

Figure 2. CD spectra of 10 μM FZ, HFZ, and AmB (from 1.0 mM DMSO stock) in PBS buffer alone (———) or with HDLs at 0.85 mg/mL of protein (- - - - - -) or LDLs at 0.4 mg/mL of protein (· · · · · · · · ·). the temperature was set at 37 °C and the solutions were incubated for > 15 min. Inset: AmB delivered from a 1 mM DMSO stock solution. Note that HFZ spectra retain their crossovers at ~320 nm and the intensity shows almost no change. CD units are molar circular dichroism ($L\ mol^{-1}\ cm^{-1}$).

CD Studies of the Interactions between AmB from FZ and HFZ and Human Serum Lipoproteins

The methods used to collect the serum distribution data in Table 1 can only show how much AmB is bound to the different fractions, not what aggregation state this AmB is in when bound. Because stability and self-association state have been previously shown to have a major effect on the membrane activity of AmB, we performed CD studies of FZ and HFZ in the presence of different lipoprotein fractions. Figure 2 shows the CD spectra of FZ, HFZ, and AmB from a DMSO stock in the presence of purified human serum lipoprotein fractions after > 15 min equilibration at 37°C. Because the monomer is essentially optically inactive compared with the self-associated states, the peak-to-trough CD intensity may be linearly related to the relative loss of the self-associated state.[24] We have used the loss of CD intensity to quantitate additional aggregate dissociation. The resulting dissociated AmB may be membrane-adsorbed sterol or protein bound depending on the environment. This result suggests that a larger fraction of AmB from FZ (~35%) dissociates/redistributes into the LDL compared with aqueous dissociation, whereas proportionally less HFZ (~0%) redistributes. In HDL, much the same scenario occurs; that is, ~25% FZ dissociates/redistributes in the presence of HDL with no dissociation of HFZ. AmB from a 1 mM DMSO stock dissociates more extensively than either FZ or HFZ into a monomeric state. These data indicate that AmB in FZ is in a less persistent aggregate structure and can interact with and dissociate into serum lipoproteins, whereas HFZ seems to have a very stable aggregate "core" supramolecular structure that either binds to the lipoproteins without dissociating or interacts very little with these lipoproteins. In light of the whole plasma distribution data in Table 1, it seems that the latter is most probable. The inset of Figure 2 shows that AmB from a 1 mM DMSO stock is even unstable in the presence of lipoproteins.

Distribution of HFZ versus FZ in Serum Lipoproteins, Whole Serum, and Serum Albumin

One feature characteristic of effective AmB drug delivery systems seems to be thermodynamic and/or kinetic stability of the drug complexes; that is, AmB is released slowly and has an effectively lower chemical potential than with FZ.[25] Gaboriau and co-workers[5] have shown that the mono-

mer/aggregate equilibrium is indeed shifted slightly toward its aggregate for HFZ compared with FZ. The concentration where the aggregate is half-dissociated is ~1.0 μM for FZ and ~0.5 μM for HFZ in buffer. However, serum is a complex mixture with many more potential sites for interaction. Difference absorbance spectra can show which serum component has the most significant effect on AmB redistribution from different preparations. We have compared the dissociation of FZ and HFZ in HDL, LDL, whole human serum (containing lipoproteins), and purified human serum albumin (Figure 3a–d). FZ and HFZ were rapidly mixed with the different systems and simultaneously diluted from 1.0×10^{-4} to 9.1×10^{-6} M in AmB, and the spectrum at 10 ms was subtracted from the spectrum at 200 s (near equilibrium9). Consistent with the CD results, FZ dissociates to a greater extent than HFZ in the presence of purified HDL and LDL (Figure 3a,b). The two lowest energy bands (monomer-like) at 409 and 387 nm are similar to monomeric AmB in water or surface adsorbed, but the band shapes and intensities in the presence of LDL indicate at least some interaction with sterols.[9,26] At least part of the additional AmB release into aqueous or adsorbed monomers from FZ may be due to the extraction and binding of deoxycholate from the FZ micelles to the serum lipoproteins.[27] In whole serum and serum albumin, the amplitude differential was much greater for FZ, indicating that there is greater kinetic instability in serum compared with that in HFZ (Figure 3c and d). In addition, the two low energy transitions of the dissociated AmB (at 415 and 391 nm) in whole serum are more similar to the spectrum of AmB in pure human serum albumin (417 and 393 nm, Figure 3d) than to the spectra with lipoproteins (Figure 3a, b). The serum albumin is profoundly destabilizing for the FZ micellar preparation; an estimated 55% of the preparation is albumin-bound (and monomeric) after 200 s. At 30 mg/mL, the serum albumin concentrations in Figure 3d are in the realistic

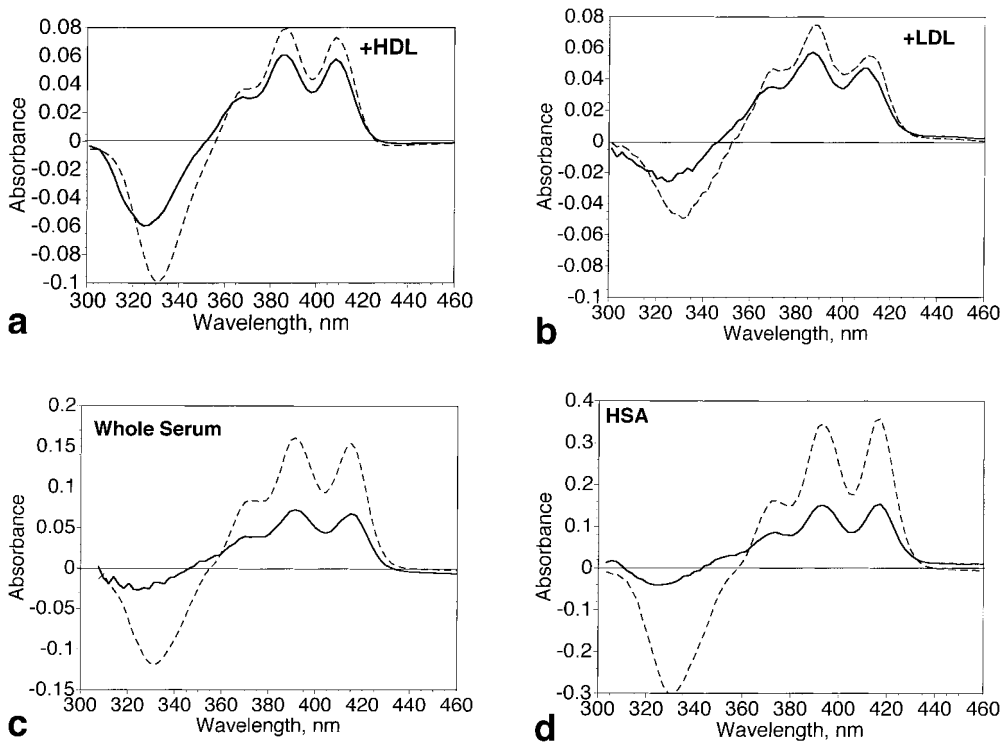

Figure 3. Difference spectra of 9.1×10^{-6} M AmB from FZ (- - - - - -) or HFZ (_____) with (a) 0.85 mg/mL (protein) HDL, (b) 0.4 mg/mL (protein) LDL, (c) human serum diluted 1:5 in PBS (6 mg/mL final total protein), and (d) fatty acid free human serum albumin (30 mg/mL). The AmB from both systems was rapidly diluted from 1.0×10^{-4} M with variable ratio syringes (11:1, serum or lipoprotein: FZ or HFZ solution).

range for whole serum. The CD spectra of FZ and HFZ show a similar trend. In human serum albumin at 15 mg/mL and 37°C after a 15-min incubation, the dichroic doublet characteristic of aggregation is reduced by 23% in the case of HFZ but 82% in the case of FZ (data not shown). With whole human serum (diluted 1:1 in PBS because of high absorbance), the dichroic doublet is reduced by 31% in the case of HFZ but 93% in the case of FZ (data not shown). Note that aggregate instability has previously been correlated with increased toxicity.[25] The related drug Nystatin also binds to albumin and this binding has been shown to increase the Nystatin critical aggregation concentration.[28] In turn, this increase renders Nystatin less hemolytic. However, in our case, the HFZ aggregate has been shown to be nonhemolytic.[4]

It is clear from these data that serum albumin dominates the interaction of FZ and HFZ with whole serum. Because FZ is a micellar AmB–deoxycholate mixture, it is possible that binding of deoxycholate and AmB by albumin hastens the destabilization of the FZ complex and subsequent binding of AmB to albumin.[7] It is unknown as to whether deoxycholate remains as part of the complex after heat treatment, but the same HFZ spectral species can be produced from AmB solutions without deoxycholate.[5] The CD spectra are quite distinct from those of commercial liposomal preparations, suggesting a fundamentally different structure.[29,30] The size distribution of the HFZ particles is not known, but light scattering indicates a larger size for HFZ,[5] which alone could account for the slower redistribution of monomers. It is noteworthy, however that bath sonication for >30 min causes no change in the CD spectra of HFZ, suggesting that the spectral changes are associated with a new, local, supramolecular species rather than merely large undifferentiated aggregates (unpublished results). The larger remaining HFZ aggregate and/or its extra stability in serum may "buy time" for the HFZ allowing its larger aggregates to be taken up into reticuloendothelial system; another characteristic of less toxic AmB formulations.[31] Thus, a common feature for the reduction of toxicity of AmB in any delivery system may be as simple as formulate a large serum-stable aggregate that will be quickly taken out of circulation into macrophage/monocyte reservoirs. Conversely, the FZ distributes rapidly into the serum proteins and lipoproteins, maintaining higher potentially toxic levels of AmB in the serum.

Induction of TNF-α in Human Monocyte Culture by HFZ Versus Fungizone

The third major influence on AmB toxicity after direct membrane effects and serum and body distribution is induction of an inflammatory cytokine response. It has been shown that exposure to FZ (>1 μM) increases expression of cytokine genes as indicated by higher levels of interleukin (IL)-1β and TNF-α mRNA levels in human and murine monocyte cell lines.[32] With this in mind, we have carried out studies of the induction of a bellwether inflammatory cytokine, TNF-α. Using a standard THP-1 human monocyte cell line, we used an ELISA assay to compare concentration-dependent TNF-α production. These results are shown in Figure 4. Both preparations induced TNF-α secretion at 5×10^{-6} M, but the HFZ induced only ~30% of the levels induced by FZ. Little response for either was observed at <1 μM. This result is significant for cytokine researchers who routinely incorporate FZ into their media at ~0.25 μM as a fungistatic agent. The ultimate implication here is that HFZ may show less acute toxicity in animal models because of a reduction in cytokine levels, although cytokine levels have not been tested *in vivo*.

Many of the unpleasant side effects of AmB are acute rather than chronic, so it has been frequently suggested that AmB may trigger a cytokine cascade *in vivo* much the same as bacterial lipopolysaccharide (LPS). Experimen-

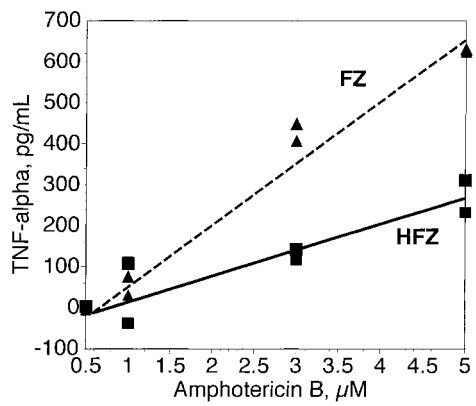

Figure 4. TNF-α production (in pg/mL of cell supernatant) by the THP-1 human monocyte cell line. The ELISA assay was a sandwich-type assay with alkaline phosphatase/pNPP colorimetric detection. The cells were exposed to AmB as FZ or HFZ in various concentrations for 2 h before assay. Cells were grown in 24-well plates in RPMI 1640 medium, and studies at each concentration were performed in duplicate.

tally, this suggestion of a cascade effect has been supported.[33–37] TNF-α, IL-1 and other monokines of the monocyte/macrophage system can have a cascade effect on other cell types. Included in these monokines released are chemokines, proinflammatory cytokines, other interleukins, proteases, growth factors, etc. It is becoming increasing obvious that many of these monokines are pleotrophic and can have multiple activities depending on the cell target. Thus, the monocyte activation can have far-reaching effects on homeostasis functions, including body temperature and others. In fact, AmB has recently been shown to down regulate production of anti-inflammatory cytokines as well as stimulating proinflammatory cytokines. FZ and AmBisome have been compared directly *in vivo* in humans and it was shown that less TNF-α and IL-1 was found in the plasma of the patients receiving AmBisome.[36] Clinical symptoms seemed to be most strongly correlated with increased IL-1 production.

It is not known exactly how AmB enhances cytokine expression but there has been some intriguing recent evidence that it may actually be direct induction of membrane permeability to Ca^{2+} ion that is responsible for AmB stimulation of IL-1β secretion.[38] That the range of concentration of AmB [AmB] associated with channel formation in cholesterol-containing membranes is also the range of TNF-α secretion stimulation; that is, concentrations that do not promote ion channels seem not to stimulate cytokine production. Our previous studies of the membrane activity of HFZ[9] coupled with the present results on TNF-α production support this correlation between ion channel activity and cytokine induction.

CONCLUSION

Our results show that AmB remains mostly associated with the lipoprotein-depleted serum fraction when delivered as either FZ or the novel HFZ. However, there is a still a significantly larger fraction of AmB from FZ bound to lipoproteins. In addition, our experiments suggest that a very different fate befalls the remaining AmB from FZ or HFZ in the presence of serum proteins. Lipoprotein depleted serum, specifically the albumin component, causes a rapid destabilization of AmB from FZ possibly because AmB and deoxycholate bind to at least two sites on albumin.[27] The self-associated state of HFZ, on the other hand, is more robust in the presence of serum albumin. Because this self-associated state is less membrane active in model as well as (RBC) systems,[4,9] AmB is contained in a less toxic state. In addition, the larger remaining aggregate and/or the extra stability may allow the HFZ to remain inert until it is taken up into macrophage reservoirs where AmB can be slowly released. A muted TNF-α response shows that this supramolecular form is much less stimulatory for potentially harmful cytokines as well. It is intriguing to speculate that there may be a direct connection between greater AmB ion channel formation in monocytes and the release of TNF-α. The additional importance of a reduced TNF-α response is highlighted in a recent report that showed that TNF stimulation by an AmB derivative (MS-8209) in turn stimulated human immunodeficiency virus (HIV) replication in macrophages.[39] This important observation points out that reducing inflammatory cytokine response to an AmB preparation may have much greater impact than simply abating acute side effects.

In summary, our studies show that a new self-associated species of AmB obtained by mild heating of FZ may owe its improvement in therapeutic index to all three potential mechanisms mentioned at the outset by showing: (1) reduced model mammalian membrane activity but similar model fungal membrane activity, (2) greater supramolecular stability and less binding/interaction to serum lipoproteins, and (3) reduced stimulation of TNF-α production.

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