Immunological Effects of Amphotericin B and Liposomal Amphotericin B on Splenocytes from Immune-Normal and Immune-Compromised Mice†

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The immunological effects of amphotericin B and liposomal amphotericin B were studied in vitro by measuring B- and T-lymphocyte proliferation on splenocytes from immune-normal, cyclosporine-compromised, and cyclophosphamide-compromised mice. Cellular viability of cells from immune-normal mice was also evaluated. The concentrations used (0, 0.5, 1, 2, 4, 8, and 16 μg/ml) encompassed clinically relevant doses. Amphotericin B consistently reduced the abilities of B cells and T cells to proliferate, especially when administered at higher than clinically relevant doses. Direct cytotoxicity probably played only a minor role, since viability studies showed that, compared with its liposomal analog, amphotericin B reduced the number of viable cells by no more than 10%. Clinically relevant doses of liposomal amphotericin B (A. S. Janoff, L. T. Boni, M. C. Popescu, S. R. Minchey, P. R. Cullis, T. D. Madden, T. Tarash, S. M. Gruner, E. Shyamsunder, M. W. Tate, R. Mendelsohn, and D. Bonner, Proc. Natl. Acad. Sci. USA 85:6122–6126, 1988; R. Mehta, G. Lopez-Berestein, R. Hopfer, K. Mills, and R. L. Juliano, Biochim. Biophys. Acta 770:230–234, 1984) did not inhibit any of the immune parameters examined. Liposomes may, therefore, be a useful means of delivering more drug to a host infected with a fungal organism without further compromising the patient’s already suppressed immune system.

The incidence of systemic fungal infections has steadily increased, especially since the onset of the AIDS epidemic. Although amphotericin B (AmB) remains the drug of choice for these life-threatening mycotic infections, complications due to renal toxicity severely limit its clinical use. AmB has been reported to augment both humoral and cell-mediated immunity (3), including the stimulation of B lymphocytes, T lymphocytes, and macrophages (11). Other reports indicate that AmB’s ability to stimulate the immune system might even have important prophylactic implications against fungal infections (16). Other reports, however, indicate that AmB suppresses both humoral and cell-mediated immunity, as well as the activity of macrophages (13).

A new formulation, L-AmB, is significantly less toxic to the kidneys and has also been implicated as an immune stimulant. Its stimulatory capabilities purportedly exceed those of nonliposomal preparations (10, 11, 12).

The purpose of this study was to explore the specific effects of AmB and L-AmB on mitogen-stimulated B- and T-lymphocyte proliferation as well as the viability of splenocytes exposed to these drugs in vitro as indicators of immune system functions. Immune-normal and immune-compromised BALB/c female mice were euthanized, and spleen cells were harvested and incubated with each of the drugs. The doses (0, 0.5, 1, 2, 4, 8, and 16 μg/ml) bracketed clinically relevant concentrations in plasma. Cyclosporine and cyclophosphamide were chosen as immune suppressants to simulate treatment received by specific, immuno-suppressed patient populations which experience significant morbidity and mortality from fungal opportunists, specifically, organ transplant patients and patients undergoing cancer chemotherapy, respectively.

MATERIALS AND METHODS

Immunosuppression in mice. Splenocytes from 8- to 10-week-old BALB/c females (Simonsen Laboratories, Inc., Gilroy, Calif.) were utilized. Mice were immunosuppressed in vivo by injecting 50 mg of cyclosporine IV (SandImmune, IV; Sandoz Pharmaceuticals Corp., East Hanover, N.J.) per kg of body weight into the tail vein 24 and 48 h prior to euthanasia and splenocyte collection. Mice immunosuppressed with cyclophosphamide (Sigma Chemical Company, St. Louis, Mo.) were given intraperitoneal injections of 100 mg/kg 5 days and 24 h prior to euthanasia and splenocyte collection.

Preparation of splenocytes. Following in vivo administration of immunosuppressive drugs, animals were euthanized by cervical dislocation, and the spleens were removed aseptically and placed into sterile, 15-ml polypropylene centrifuge tubes (Corning Glass Works, Corning, N.Y.) containing 10 ml of prewarmed RPMI-PS consisting of 99% RPMI 1640 (GIBCO-BRL, Grand Island, N.Y.) and 1% penicillin-streptomycin (GIBCO).

The spleens and RPMI-PS from each centrifuge tube were poured into sterile, regular-sized lab bags (Tekmar Company, Cincinnati, Ohio) and homogenized for 1 min by using a stomacher tissue homogenizer (Lab-Blender 80; Tekmar). Any remaining pieces of spleen were carefully pressed out with a rubber print roller.

Ethrocyes were lysed by conventional techniques, and cells were washed and suspended in 5 to 10 ml of media. Media consisted of 1% 5 mM 2-mercaptoprotoanol (Sigma)–1% sodium pyruvate (GIBCO)–1% penicillin-streptomycin—
heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, Utah) added shortly prior to use and RPMI 1640.

Cells from each immune model were pooled into separate sterile glass bottles. The three pooled samples were counted by trypan blue exclusion and were diluted to \(10^7\) cells per ml with media. These served as stock splenocyte suspensions for each immune model.

Viability studies. The viability of splenocytes was ascertained for immune-normal cells in 96-well, round-bottom tissue culture plates (Corning). A single plate contained six replications of each drug concentration (0, 0.5, 1, 2, 4, 8, and 16 \(\mu g/ml\)) for both AmB (Fungizone Intravenous; E. R. Squibb & Sons, Inc., Princeton, N.J.) and L-AmB (AmBisome; Vestar, Inc., San Dimas, Calif.) as well as six control wells containing cells in media but neither drug nor drug vehicle. Prior to incubation, the final concentration of cells in the plates was determined to be \(5 \times 10^6\) cells per ml in a 10% fetal bovine serum solution. The plate was incubated for 24 h at 37°C in 5% CO\(_2\). The concentration of cells in each well was then determined with a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.).

Lymphocyte proliferation studies. Lymphocyte proliferation was determined by using the B-cell mitogen, lipopolysaccharide (LPS) (Sigma), and concanavalin A (ConA) (Sigma), a T-cell mitogen. LPS and ConA were both dissolved in sterile RPMI and used at final well concentrations of 20 and 10 \(\mu g/ml\) (LPS) and 2.5 and 1.25 \(\mu g/ml\) (ConA). One 96-well, flat-bottom tissue culture plate (Corning) contained six replications of each drug dilution for both AmB and L-AmB as well as six control wells containing immune-normal splenocytes exposed to mitogen but to neither drug nor drug vehicle and six background wells containing splenocytes exposed to neither drug, drug vehicle, or mitogen. Final concentrations of AmB and L-AmB were 0, 0.5, 1, 2, 4, 8, and 16 \(\mu g/ml\). The final splenocyte concentration was \(5 \times 10^6\) cells per ml in a 10% fetal bovine serum solution.

Plates were incubated for 24 h at 37°C in 5% CO\(_2\). After 24 h, cells were pulsed with 2.0 \(\mu l\) (0.4 mCi/mmol) of [methyl-\(^3\)H]-thymidine (DuPont NEN Products, Boston, Mass.) and incubated for an additional 24 h under the same conditions. Cells were harvested on standard, self-aligning glass fiber filters (Packard Instrument Company, Meriden, Conn.) with a Packard Microplate 196-well harvester (Packard) by aspirating the cells through the filter and washing the wells four additional times. The filters were then dried for 1 h at 60°C. Filters were read for 10 min with a Matrix 96-well direct beta counter (Packard), and the data were expressed as counts per minute.

Data analysis and statistical methods. Average values from the different experimental replications were graphed for both viability and proliferation studies (see Fig. 1 to 7). For the viability studies, cell counts were converted to a percentage by taking the ratio of the original concentration of cells. For the studies assessing lymphocyte proliferation, the mean value of the immune-normal control wells (illustrated by a single dotted line on each graph) served as a reference in determining the relative amount of stimulation or inhibition of immune function that had occurred secondary to drug exposure.

Minitab Statistical Software (State College, Pa.) was used in the analysis of variance for each immune model and for each mitogen level. This included an analysis between AmB and L-AmB as well as between the different doses of each drug used. The standard error of the mean used in the graphs indicates that values from each experiment received equal weight.

**RESULTS**

Viability studies: immune-normal splenocytes. Viability studies were conducted on splenocytes from immune-normal animals. Although the difference demonstrated between AmB and L-AmB is statistically significant (\(P < 0.001\), demonstrating reduced viability with AmB exposure, the differences were relatively minor and are therefore of questionable biological significance (Fig. 1). At lower drug concentrations, representative of achievable concentrations in plasma for AmB, toxicities associated with the two drugs differed by approximately 5%.

There were significant differences (\(P < 0.001\)) demonstrated between the doses, but there was no meaningful stimulation or inhibition of viability by either drug. This may have implications for the use of L-AmB, since it can be administered at higher doses without the side effects of renal toxicity associated with the free form of the drug.
The decrease in viability to 80% for those cells treated with the liposomal preparation did not differ from the toxicity exhibited by the drug vehicle alone. This implies that the reduction in viability was due either to the drug vehicle or to the harvesting technique employed rather than to L-AmB.

B-cell proliferation studies. (i) Immune-normal splenocytes. The effects of both AmB and L-AmB on B cells at both concentrations of LPS are significant ($P < 0.001$) (Fig. 2). There was also a significant difference ($P < 0.001$) between the different concentrations of each drug.

Over the concentrations tested, AmB inhibited B-cell activity, particularly at concentrations exceeding clinically relevant plasma concentrations of 0.5 to 2 $\mu$g/ml (14). L-AmB neither inhibited nor stimulated B cells at concentrations through 8 $\mu$g/ml. At concentrations exceeding 8 $\mu$g/ml, however, L-AmB stimulated B cells at 20 $\mu$g of LPS ml$^{-1}$ and slightly inhibited B cells at 10 $\mu$g of LPS ml$^{-1}$. L-AmB's lack of marked inhibition at higher levels in serum together with the ability to achieve higher concentrations in plasma without associated renal toxicity may have important clinical implications.

(ii) Cyclosporine-compromised splenocytes. AmB and L-AmB differ significantly ($P < 0.001$ for both mitogen levels) in their effects on cyclosporine-compromised splenocytes (Fig. 3).

As was the case for immune-normal splenocytes, AmB was much more inhibitory at higher drug concentrations than at the lower therapeutic levels between 0.5 and 2 $\mu$g/ml. Most concentrations of L-AmB failed to markedly stimulate or inhibit cyclosporine-compromised cells. At both mitogen levels, however, concentrations that exceeded 8 $\mu$g/ml apparently increased activity, indicative of immune stimulation which could be of value when treating cyclosporine-compromised patients.

(iii) Cyclophosphamide-compromised splenocytes. A significant difference ($P < 0.001$) is demonstrated at 10 $\mu$g of LPS ml$^{-1}$ but, again, only when drug concentrations exceeded the achievable therapeutic levels for AmB. A significant difference was not demonstrated between the two drugs at 10 $\mu$g of LPS ml$^{-1}$. At the higher mitogen level, the drugs did not differ in their effects on cell activity until a dose of 4 $\mu$g/ml, after which AmB demonstrated toxicity while L-AmB's effect remained stable. At both mitogen levels, AmB eventually inhibited the cells, whereas L-AmB neither stimulated or inhibited cells significantly, a factor which could be of clinical importance in the treatment of immunocompromised individuals suffering from fungal infections. At both levels of mitogen, significant differences ($P < 0.001$) are demonstrated between the different drug concentrations (Fig. 4).

T-cell proliferation studies. (i) Immune-normal splenocytes. At both levels of ConA, there are significant differences ($P < 0.001$) between the two drugs as well as between the different concentrations used. AmB inhibited T-cell proliferation when clinically relevant therapeutic levels of 0.5 to 2 $\mu$g/ml were exceeded, most notably following 4 $\mu$g/ml (Fig. 5).

L-AmB did not significantly inhibit T-cell activity. Concentrations exceeding 8 $\mu$g/ml appeared to stimulate these cells at both mitogen concentrations.

(ii) Cyclosporine-compromised splenocytes. As shown in Fig. 6, the overall T-cell activity was depressed by cyclosporine. At both concentrations of ConA, significant differences ($P < 0.001$) exist between the drugs and between different drug doses. Higher than relevant therapeutic concentrations of AmB again inhibited T-cell response. The inhibition was apparent at levels exceeding 2 $\mu$g/ml, compared with levels exceeding 4 $\mu$g/ml in the immune-normal model. AmB slightly decreased activity at 0.5 $\mu$g/ml and severely decreased activity beyond 2 $\mu$g/ml.
L-AmB again did not appreciably affect T-cell response. All values were somewhat lower than for the immune-normal control cells which, as mentioned above, would be expected from cyclosporine's immunosuppressive effects. When concentrations exceeded 8 μg/ml, however, the liposomal drug slightly increased the activity of the T-cells, perhaps because of immune stimulation. Again, this could be beneficial in the treatment of organ transplant patients.

(iii) Cyclophosphamide-compromised splenocytes. As represented in Fig. 7, immunosuppression of splenocytes by cyclophosphamide greatly reduced T-cell activity. With ConA at 2.5 μg/ml, no significant difference is demonstrated between the two drugs. A significant difference (P < 0.001) is demonstrated between the drugs with ConA at 1.25 μg/ml and between concentrations of the drugs at both mitogen concentrations.

When doses exceeded 4 μg/ml, AmB markedly inhibited T-lymphocyte activity, whereas L-AmB levels exceeding 8 μg/ml slightly increased T-lymphocyte activity. Lower concentrations of both drugs may have stimulated the T cells. Once again, however, it appears that higher doses of L-AmB may not have the immunoinhibitory effects associated with AmB.

DISCUSSION

Organ transplant patients receiving cyclosporine, individuals undergoing cancer chemotherapy with drugs such as cyclophosphamide, and those suffering from AIDS are at increased risk for opportunistic fungal diseases. AmB has long been the drug of choice in serious, life-threatening mycoses because, unlike most other antifungal compounds, it is fungicidal rather than fungistatic. Because of renal toxicity, however, it is useful only within a very narrow therapeutic index. Toxicity is minimized by incorporating nanometer-sized particles of the free drug into liposomal vesicles. This is the basis for the formulation of L-AmB.

On the basis of the results of in vivo and in vitro tests, both AmB and L-AmB have been characterized as immunostim-
ulants. The results of our in vitro tests, however, do not indicate that AmB is an immunostimulant. We ascertained the effects of AmB on splenocytes from immune-normal, cyclosporine-compromised, and cyclophosphamide-compromised mice. Splenocytes were exposed either to LPS to stimulate B cells (20 and 10 μg/ml) or to ConA to stimulate T cells (2.5 and 1.25 μg/ml). Our results are not consistent with those of Blanke et al. (3) who concluded that AmB augmented both humoral and cell-mediated immunity in vivo (3). In this study, exposure to levels of AmB that exceeded 2 to 4 μg/ml severely suppressed these two cell populations. (T cells from cyclophosphamide-compromised mice appeared to be weakly stimulated, but the effect is probably not biologically relevant.) We also did not find that AmB activated populations of T cells, as was reported by Mehta et al. (13). The concentrations of AmB that elicited a notable immunoinhibitory response from splenocytes generally exceeded the clinically relevant drug doses of 0.5 to 2 μg/ml, however, which is very similar to the range where other tissues, such as the kidneys, are most prone to drug toxicity. The toxicity of AmB may be similar for all mammalian cells.

L-AmB did not demonstrate the same severe inhibitory effects associated with AmB, particularly at higher concentrations. The activities of mitogen-stimulated cells exposed to different concentrations of this liposomal formulation remained relatively constant, and higher concentrations (greater than 8 μg/ml) appeared to stimulate B and T cells in both immune-normal and immune-compromised splenocytes. The limitations of an in vitro study should be remembered, however, for in vivo effects of other immune cells, such as those of monocyte lineage, could affect both humoral and cell-mediated immunity. The possible effects of empty liposomes on immune parameters have also been studied by Mehta et al. (13). They determined that empty liposomes had no effect on the mitogen-induced proliferation of T cells. Also noted was a depression of antibody production in mice exposed to empty liposomes; however, the proliferative response of B cells was not determined.

Drug interactions between the immune suppressants used in this study and AmB have been reported when both immune-suppressant and antifungal agents are administered concurrently in vivo (2, 4, 8). Since immune suppression in this study occurred in vivo and AmB and L-AmB were later administered to the already immunosuppressed cells in vitro, the likelihood of possible drug interactions was greatly minimized. Cyclophosphamide, by itself, is rather biologically inactive and requires activation by the hepatic cytochrome P-450 enzymes (4). The further generation of active metabolites would not have been possible following euthanasia. Because of a relatively long half-life and the tendency to be sequestered in tissues because of its extreme hydrophobicity, there would be little interaction between cyclosporine and AmB. Cyclosporine has been reported to increase the accumulation and decrease the efflux of such chemotherapeutic agents as doxorubicin and daunomycin in multidrug-resistant cells (6, 15). Whether any residual cyclosporine could have interacted similarly with AmB in the splenocytes in vitro is beyond the scope of this study.

The viability of immune-normal cells exposed to AmB was between 5 and 10% lower than that of cells exposed to L-AmB (Fig. 1). This difference in viabilities cannot explain the severe toxicity to proliferating cells, however, and is therefore more suggestive of inactivation than of direct cell lysis. These results are consistent with the theory that the toxicity of AmB is due to the creation of ion pores (1, 5, 7, 17). The fact that AmB did not reduce splenocyte viability as much as occurred in the mitogen-stimulated proliferation assays suggests that the drug may affect immune responsiveness without actually destroying these cells (e.g., it may inhibit the cellular division of lymphocytes). This is especially suggestive at higher drug concentrations. This could be explained by the creation of ion pores in the cell membrane that consequently disrupt the Na⁺/K⁺ gradients and therefore deplete stored cellular ATP as the ATP-dependent Na⁺–K⁺ pumps attempt to restore and/or maintain normal ion gradients. This would deplete cellular energy reserves and reduce the potential for cell proliferation. The actual mechanism of inhibition to immune cells warrants further investigation.

Even though AmB did not appear to be immunotoxic within clinically relevant concentrations, it was highly inhibitory at higher concentrations. Higher concentrations of the liposomal preparation, however, did not appear to inhibit these immune cells. Because L-AmB has been shown to demonstrate similar antifungal activity at the same concentration of AmB (13) and because it is much less toxic to the kidneys, allowing the use of higher concentrations, the results of these in vitro studies suggest that L-AmB may be promising therapy for use in patients with compromised immune status.

REFERENCES


