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Liposomal hamycin in the control of experimental aspergillosis in mice: effect of phosphatidic acid with and without cholesterol

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Hamycin incorporated into liposomes containing phosphatidylcholine (SPC) and phosphatidic acid (PA) had reduced toxicity and an enhanced antifungal activity in experimental aspergillosis in balb/c mice. Incorporation of cholesterol into liposomes led to a dose dependent decrease in the toxicity of hamycin. The LD_{50} (mg/kg) of hamycin contained in SPC/cholesterol/PA (molar ratio 4:5:1) liposomes was 2·8 whereas that in SPC/PA liposomes (molar ratio 9:1) was 0·35. Although the free drug had little or no protective effect on the animals, those administered liposomal hamycin at an equivalent dose (0·1 mg/kg) in the absence of cholesterol (SPC/PA; molar ratio 9:1) showed 90% survival after seven days of therapy. On the other hand the presence of cholesterol in the carrier phosphatidic acid liposomes (SPC/cholesterol/PA; molar ratio 4:5:1) at a similar dose (0·1 mg/kg) led to a 60% survival over the same time period. Hamycin incorporation in phosphatidic acid liposomes both in the presence or absence of cholesterol was found to be effective in reducing the fungal load in lung, liver, spleen and kidney. Studies with distribution of hamycin in various tissues by HPLC showed a significant reduction in the concentration of the liposomal drug in circulation as compared to those seem after administration of free drug.

Introduction

Hamycin, an aromatic heptaene isolated from *Streptomyces pimprina*, has been used topically against a variety of fungal infections (Thirumalachar & Narasimhan, 1987). The interest in the use of aromatic polyenes against fungal infections is because their in-vitro antifungal activity on eukaryotic membranes is two to three orders of magnitude higher than that of their non aromatic counterparts (Hammond, 1977; Cybulska *et al.*, 1983). Hamycin acts by binding to ergosterol in the plasma membrane of the fungal cell leading to the formation of pores which allow metabolites to leak out, resulting in cell death. Parenteral administration of hamycin is associated with acute toxicity (Dave & Kaul, 1964; Williams *et al.*, 1965) which appears to be related to the stability, residence time, and the intrinsic permeability properties of the polyene—sterol complex (Mazerski, Bolard & Borowski, 1983).

Liposomes are being increasingly exploited as a drug delivery system in the treatment of fungal infections (New, Chance & Heath, 1981; Lopez-Berestein, *et al.*, 1987) to reduce the in-vivo toxicity and enhance the antifungal activity of the drug. A number

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of laboratories including ours have reported on the reduced toxicity of liposomal amphotericin B leading to a significant improvement in its therapeutic index in the treatment of systemic fungal infections (Tremblay *et al.*, 1984; Lopez-Berestein, 1986; Ahmad, Sarkar & Bachhawat, 1989). Furthermore our laboratory (Moonis, Ahmad & Bachhawat, 1992) and Mehta *et al.* (1991) have shown that liposomal hamycin is effective *in vivo*.

In the present studies we have used liposomes bearing a strong negative surface charge so as to reduce their circulation time *in vivo* as macrophages are reported to have a high affinity towards negatively charged liposomes (Senior, 1987). We have investigated the effect of phosphatidic acid liposomes on in-vivo toxicity and antifungal activity of hamycin. We also present data on the tissue distribution of liposomal hamycin in the presence and absence of cholesterol in normal mice and compared with that in mice infected with *Aspergillus fumigatus*.

Materials and methods

Drugs, lipids and chemicals

Hamycin was a gift from Dr S. R. Naik of Hindustan Antibiotics Ltd., (Pimpri, India). Soya phosphatidylcholine (SPC) was from Dianorm, (Germany), egg phosphatidic acid (PA) was from Sigma Chemical Co, (St. Louis, USA) and cholesterol was from the Centre for Biochemicals, (New Delhi, India). All other reagents used were of analytical grade.

Animals

Male balb/c mice (body wt, 20–25 g) were obtained from the animal house facility of the National Institute of Nutrition, (Hyderabad, India) and maintained in our animal house.

Animal model for aspergillosis in balb/c mice

A. fumigatus strain V.P. chest 256 isolated from a patient was supplied by Dr Z. U. Khan of V.P. Chest Institute, Delhi. The animal model for aspergillosis in balb/c mice was established as described by Ahmad, Sarkar & Bachhawat (1989*b*). In brief $1{\cdot}8 \times 10^7$ viable *A. fumigatus* spores were suspended in sterile saline and injected into balb/c mice via a tail vein.

Preparation of hamycin suspension

Hamycin suspension was prepared as described by Moonis *et al.* (1992). In brief, an appropriate volume of stock hamycin solution (200 mg/L in methanol) mixed with 0·9% NaCl containing 1% Tween—80. The suspension was sonicated in a water bath sonicator for 30 min under nitrogen to completely disperse the drug.

Liposome preparation

Liposomal hamycin was prepared as described by Moonis *et al.* (1992). The lipid compositions used for the preparation of liposomes were SPC/PA (molar ratio, 9 : 1)

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and SPC/cholesterol/PA (molar ratio, 4:5:1). In brief, the lipids (1 mg hamycin for 45 mg lipids) were dissolved in chloroform/methanol (2:1 v/v) and a solution of hamycin in methanol added. The organic solvents were evaporated under vacuum using rotary evaporator and kept in a dessicator overnight. The thin dry film obtained was resuspended in 0·15 M NaCl and sonicated for 45 min under nitrogen in a bath type sonicator (Model Decon FS-200, Utrasonic Ltd., England). Free hamycin was removed by overnight dialysis at 4°C in 200 volumes of 0·9% NaCl with two changes. The amount of hamycin entrapped in liposomes was determined both spectrophotometrically at 380 nm and by HPLC at 355 nm (Moonis *et al.*, 1992). The intercalation efficiency was between 60–80%.

LD_{50} determination

Free hamycin (0·0625–0·5 mg/kg) and liposomal hamycin (0·125–4·0 mg of hamycin/kg) were injected iv into four groups (five mice per group) of balb/c mice (weight 20–25 g) such that the logarithms of successive dosage levels differed by a constant (d), the geometric factor being denoted by R where $d = \log R$. Survival was checked over a period of four days. LD_{50}s were calculated by the formula $\log (LD_{50}) = \log$ dose (minimum) + log ratio (f + 1) where f is a function of the mortality taken from the tables in Weil (1952). The confidence level of the assay was 5%.

Treatment of infected mice

Seventy male balb/c mice were infected with $1{\cdot}8 \times 10^7$ *A. fumigatus* spores iv via the tail vein. After 24 h, mice were divided into groups of ten. The first and second groups received 0·05 and 0·1 mg/kg hamycin in SPC/PA liposomes respectively whereas the third, fourth and fifth groups received 0·1, 0·25 and 0·5 mg/kg hamycin in SPC/cholesterol/PA liposomes respectively. The sixth group was administered 0·1 mg/kg free hamycin and the last group was the untreated control group.

The in-vivo antifungal activity of liposomal hamycin was evaluated on the basis of survival and the number of fungal cfu in homogenates of the lung, liver, spleen and kidney. Survival was checked for seven days after therapy. The cfu in various organs was determined as follows: two animals from each group was sacrificed on alternate days after therapy and their lung, liver, spleen and kidney were removed aspetically, weighed and homogenized in 0·9% NaCl. Serial dilutions of the homogenate were plated on Sabouraud Dextrose medium. Colonies were counted after 48 h incubation at 37°C.

Statistical analysis of cfu and survival

The cfu data were statistically analysed by the method of analysis of variance of one way classification as described by Snedecor & Cochran (1968). Since the data were in the form of cfu counts and were highly variable (some values being 0) a log transformation was made on the values of the cfu counts + 1. Heterogeneity of means for the respective organs was tested by the F-ratio of treatment variance to the experimental error variance. The latter was used to find the standard error of difference between the pairs of means by calculating the observed t-value and comparing it with the tabular t-value of degree of freedom. The survival data was analysed by using the Chi-squared with Yates correction and by Fisher's exact test.

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HPLC analysis of hamycin

The HPLC system for the analysis of hamycin was as described by Moonis *et al.* (1992). The mobile phase was 0·005 M EDTA-Methanol (2:8 v/v) and the column was Hypersil C_{18} (Shandon Scientific Ltd, Cheshire, UK). Detection of hamycin was by UV absorbance at 355 nm. Peak areas were determined using a PC integrator attached with the HPLC system. Standard curve linearity was determined by injecting 20 μL of the appropriately diluted hamycin from a stock solution (10 mg/L in methanol) in duplicate. The areas under the peak areas were plotted against the amount of drug to obtain standard curve. Correlation coefficient (r^2), for the linear regression of peak areas versus concentration range between 0·012–0·2 μg hamycin was greater than 0·96.

Estimation of liposomal hamycin

Twenty μL of hamycin was injected in duplicate into the HPLC. The peak area obtained after injection of methanol lysed liposome was compared with that of the hamycin standard to calculate the percentage intercalation.

For analysis of hamycin spectrophotometrically, an aliquot of liposome was lysed with methanol and absorbance was read at 380 nm against a methanol blank. The absorbance obtained was compared with the absorbance of standard hamycin solution at 380 nm to calculate percentage intercalation.

Recovery of hamycin from tissue and serum

For recovery of hamycin from various organs and serum, groups of five mice were killed and various organs including blood were removed. Pooled organs (0·5 g) and pooled serum (0·5 mL) were homogenized in 2 mL of methanol containing 10 μg of hamycin. The samples were centrifuged at 9000 *g* for 15 min and 20 μL of the supernatant was injected into the HPLC. The amount of hamycin was estimated as described above.

Tissue distribution of liposomal hamycin

Hamycin was assayed in normal and infected mice killed 1 h following iv administration of liposomal hamycin. Groups of six mice were killed, blood and lung, liver, spleen and kidney were taken out and frozen until analysis. In the case of hamycin incorporated in SPC/cholesterol/PA liposomes, tissue distribution studies were also performed 24 h after liposomal administration as the drug (dose 1·5 mg/kg) could still be measured 24 h after administration. Pooled organs (0·5 g) were homogenized in methanol, centrifuged and the supernatant was taken for HPLC analysis. For serum, 1 mL of methanol was added to 0·5 mL of pooled serum and centrifuged at 9000 **g** for 15 min. The concentration of hamycin assayed in the various organs was multiplied by the recovery factor. In the case of SPC/PA liposomes, at 1 h, because of the low dose administered (0·2 mg/kg), additional hamycin (1 mg/L) was added while homogenizing the tissue samples. The concentration in the sample was estimated by subtracting the additional added amount from the total concentration measured.

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Table I. LD_{50} of various formulations of negatively charged liposomal hamycin

Formulation tested	Molar ratio	LD_{50} (mg hamycin/kg)
Free hamycin		0·17
SPC/PA liposomes	9 : 1	0·35
SPC/cholesterol/PA liposomes	6 : 3 : 1	0·71
SPC/cholesterol/PA liposomes	4 : 5 : 1	2·80

LD_{50} was calculated by the method of Weil (1952). The experiments were repeated twice with no variation in the results. SPC, Soya phosphatidylcholine; PA, phosphatidic acid.

Results

Animal model for infection

Disseminated fungal infection in the lung, liver, spleen and kidney was observed after injection of $1{\cdot}8 \times 10^7$ *A. fumigatus* spores via the tail vein (data not shown). The infected animals survived up to two days which gave sufficient time for administration of hamycin therapy for comparative evaluation.

Effect of phosphatidic acid and cholesterol on the toxicity of liposomal hamycin

The LD_{50} of various formulations of phosphatidic acid liposomes are given in Table I. Addition of cholesterol had a marked effect in reducing the toxicity of liposomal hamycin. A 4·2-fold reduction in the toxicity of hamycin in cholesterol containing liposomes (SPC/cholesterol/PA, molar ratio 6 : 3 : 1) was observed. A further increase in the LD_{50} of hamycin in SPC/cholesterol/PA liposomes was observed when cholesterol was incorporated at a molar ratio of 4 : 5 : 1 showing a 16-fold reduction in the toxicity of hamycin as compared to that due to the free drug. The maximum tolerated dose (MTD) of SPC/PA liposomes in infected animals was 0·22 mg/kg whereas the MTD for SPC/cholesterol/PA liposomes was 1·8 mg/kg.

Survival and fungal load in infected animals with liposomal hamycin therapy

The survival pattern of animals on various days after therapy is shown in Figure 1. The data shows only 40% survival of infected animals after 3 days of infection and 100% mortality after the fourth day. Similar survival pattern (50% survival on the third day; with 100% mortality thereafter) was observed with infected animals which received 0·1 mg/kg of free hamycin. In contrast, the animals which received a single iv dose of 0·05 mg/kg of hamycin in SPC/PA liposomes (molar ratio 9 : 1) showed 20% survival seven days after therapy. Animals which received 0·1 mg/kg of hamycin in SPC/PA liposomes showed 90% survival after seven days of therapy. Administration of 0·25 mg/kg hamycin in SPC/PA liposomes induced high toxicity resulting in the immediate death of the mice. Incorporation of 0·1 mg/kg hamycin in SPC/cholesterol/PA liposomes produced 60% survival of animals after seven days of therapy. Increasing the dose of the drug in SPC/cholesterol/PA liposomes led to an increase in survival rates. Animals which received 0·25 mg/kg or 0·5 mg/kg SPC/cholesterol/PA liposomes showed 80% survival after the 7th day of therapy.

The fungal load in various organs on alternate days after therapy (up to seven days) with various liposomal formulations of hamycin is presented in Table II, data from

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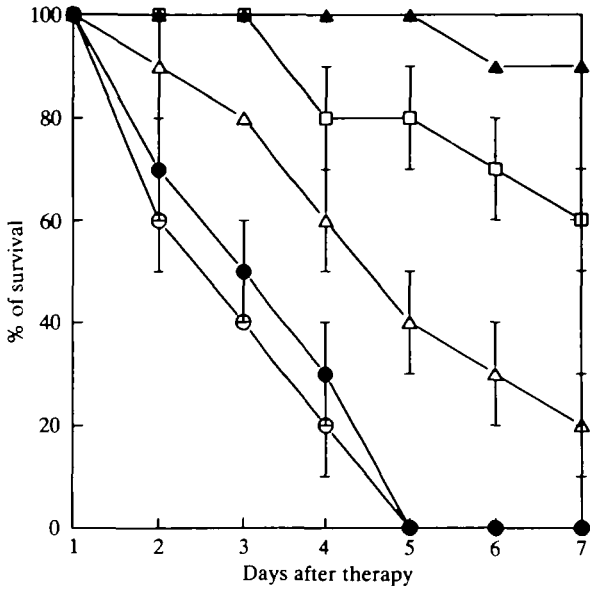

Figure 1. Survival of infected balb/c mice given hamycin therapy in phosphatidic acid liposomes in the presence or absence of cholesterol. Infected animals ($n = 10$) were given liposomal hamycin therapy 24 h after infection with *A. fumigatus* spores. Yates correction of the chi-squared analysis and Fisher's exact test indicated significant differences between the groups ($P < 0.001$, two tailed) on the fifth and seventh days after therapy whereas differences were insignificant on the third day after therapy. ○, untreated controls; ●, free hamycin (0·1 mg/kg); △, hamycin in SPC/PA liposomes at 0·05 mg/kg; ▲, hamycin in SPC/PA liposomes at 0·1 mg/kg (single experiment using 20 mice); □, hamycin in SPC/cholesterol/PA liposomes at 0·1 mg/kg. Values are expressed as mean % survival of animals from two experiments. The vertical error bars represent S.E.

Moonis *et al.* (1992) for free hamycin has been included for comparison. Liposomal hamycin in the presence or absence of cholesterol induced a marked reduction in cfu in all organs as compared to that in untreated animals and in animals treated with free drug. The cfu counts obtained after therapy with SPC/PA liposomes were not significantly different from those observed for SPC/cholesterol/PA liposomes at the same dosage of liposomal hamycin (0·1 mg/kg).

Recovery of hamycin from tissues and serum

Recovery of hamycin from tissues and serum ranged from 64% to 88%. The percentage recovery from lung, liver, spleen, kidney and serum were 77, 73, 64, 88 and 82, respectively.

Tissue distribution of hamycin contained in phosphatidic acid liposomes in normal and infected mice

Tissue distribution of hamycin in normal animals 1 h after the administration of the liposomal drug is shown in Figure 2. Considering the recovery of the drug from tissues (64–88%), a less than two-fold difference in drug concentration in various organs may not be highly significant. However, differences in tissue levels above two-fold could be significant. The concentration of hamycin in the lung with SPC/cholesterol/PA lipo-

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Table II. Fungal load in various organs of infected mice on alternate days after therapy with free hamycin or negatively charged liposomal hamycin

Formulation Tested	Days after therapy	Mean Log cfu (±S.E.) lung	liver	spleen	kidney
Free hamycin (0·1 mg/kg)	1	3·7±0·01**	5·4±0·18*	4·7±0·04	4·1±0·09*
	3	2·7±0·02**	4·6±0·03*	3·6±0·1	3·4±0·34
SPC/PA 9:1 (0·05 mg/kg)	1	3·4±0·15	4·9±0·05	4·4±0·01	4·0±0·1
	3	3·6±0	3·5±0	2·5±0	3·9±0·25
	5	3·0±0·02	3·0±0·16	2·3±0·09	3·5±0·13
	7	0	2·3±0·15	1·7±0·01	3·4±0
SPC/PA 9:1 (0·1 mg/kg)	1	2·7±0·03**	4·6±0·01*	4·6±0·02	3·3±0·19
	3	2·7±0·01**	4·2±0·43*	3·1±0·19	3·2±0*
	5	2·6±0	4·0±0·33	2·2±0·05	2·8±0·08
	7	0	3·5±0·04	0	2·8±0·04
SPC/cholesterol/ PA 4:5:1 (0·1 mg/kg)	1	2·6±0·01**	4·4±0·01*	4·4±0·13	3·6±0
	3	2·4±0·02**	4·0±0·17*	3·4±0·20	3·5±0·10*
	5	2·1±0·03	3·8±0	2·2±0	3·1±0·04
	7	0	3·1±0·22	1·1±0·69	2·6±0·08
SPC/cholesterol/ PA 4:5:1 (0·25 mg/kg)	1	2·4±0·01	4·6±0·10	4·4±0·04	3·4±0·25
	3	2·1±0·02	4·4±0·19	3·5±0·03	3·3±0·01
	5	1·5±0·1	3·5±0·05	3·0±0·1	3·1±0·1
	7	0	3·5±0·1	1·1±0·7	2·7±0·1
SPC/cholesterol/ PA 4:5:1 (0·5 mg/kg)	1	2·9±0	4·2±0·09	4·4±0·02	3·0±0
	3	2·8±0	3·9±0·30	3·5±0·30	2·9±0·02
	5	1·5±0·06	3·6±0·25	2·1±0·2	2·9±0
	7	0	3·2±0·14	1·1±0·69	2·5±0·01

Two animals from each group were killed at each time point for cfu counts. The analysis of variance between free drug, hamycin incorporated in SPC/PA and SPC/cholesterol/PA liposomes (0·1 mg/kg) revealed that the treatment means were heterogeneous on various days after therapy for the respective organs at the 5% level (*) and 1% level (**). The analysis of variance between SPC/PA and SPC/cholesterol/PA at 0·1 mg/kg was not significant except for the lung at the 1% level on third and fifth day after therapy and the liver on the 1st day after therapy (5% level). SPC, Soya phosphatidylcholine; PA, phosphatidic acid. The data for free hamycin are taken from Moonis *et al.* (1992).

somes was 2·9-fold higher than that previously observed by Moonis *et al.* (1992) with free drug. In serum on the other hand a significant decrease in the concentration of hamycin was observed with liposomal preparations containing SPC/PA (4·1-fold) and SPC/cholesterol/PA (3·8-fold) as compared to that previously obtained with the free drug, (Moonis *et al.*, 1992).

The distribution of hamycin in the tissues of infected animals 1 h after the administration of the liposomal drug is shown in Figure 3. In the lung there was a 2·8-fold decrease in the concentration of hamycin with SPC/PA liposomes as compared to that obtained with free drug (Moonis *et al.*, 1992). In spleen on the other hand there was a 3·3-fold increase in the concentration of hamycin SPC/PA liposomes and a 1·9-fold enhancement with SPC/cholesterol/PA liposomes in comparison with the free drug (Moonis *et al.*, 1992). In serum the concentrations of hamycin were reduced by a factor of 4·4 and 4·0 for the liposomal preparations (SPC/PA and SPC/cholesterol/PA) in comparison with the concentrations previously reported due to the free drug (Moonis

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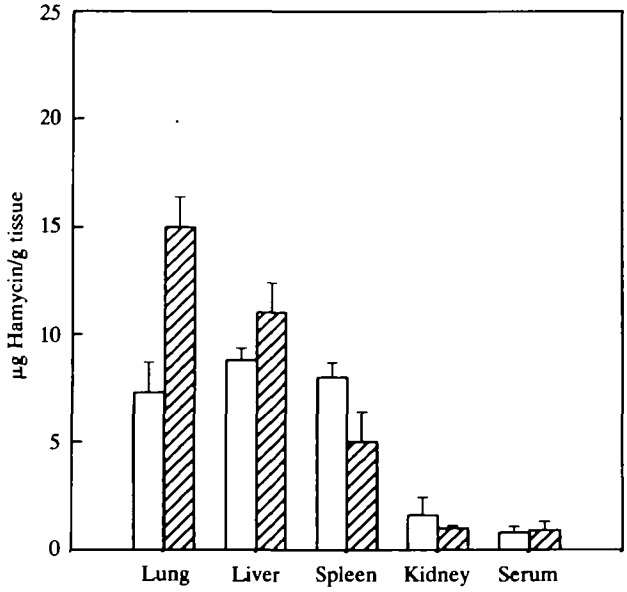

Figure 2. Organ and serum concentration of hamycin 1 h after iv administration to mice in phosphatidic acid liposomes with (■) and without (□) incorporation of cholesterol. Mice were injected 0·2 mg/kg hamycin in SPC/PA liposomes and 1·5 mg/kg hamycin in SPC/cholesterol/PA liposomes. For the purpose of comparison both the doses have been normalized to 1 mg/kg. Bars represent the S.D. of duplicate samples obtained from pooled organs of six mice.

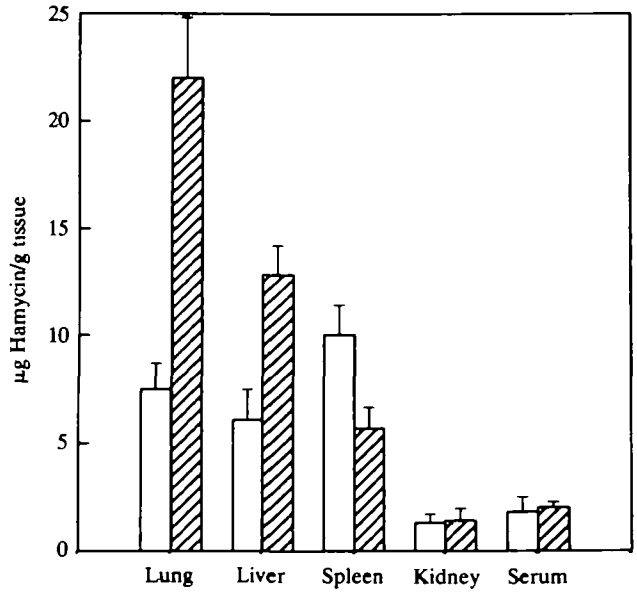

Figure 3. Organ and serum concentration of hamycin 1 h after iv administration to infected mice in phosphatidic acid liposomes with (■) and without (□) incorporation of cholesterol. Mice were injected 0·2 mg/kg hamycin in SPC/PA and 1·5 mg/kg hamycin in SPC/cholesterol/PA liposomes. Other experimental protocols are described in the legend to Figure 2.

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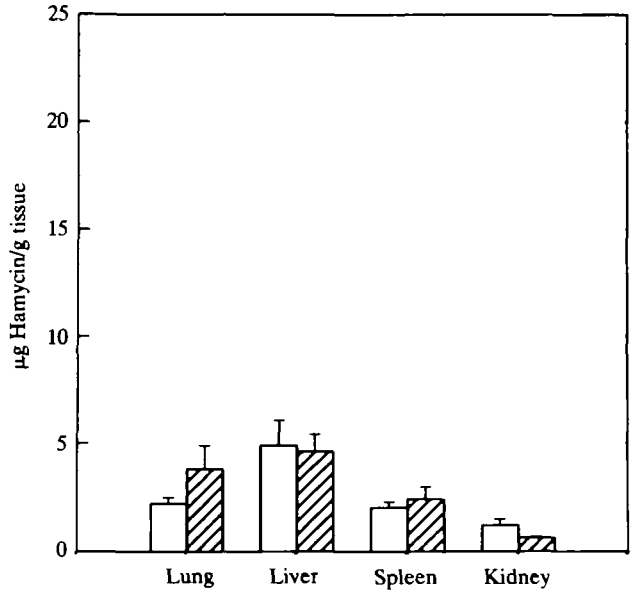

Figure 4. Organ concentration of hamycin 24 h after iv administration in phosphatidic acid liposomes in the presence of cholesterol (SPC/cholesterol/PA) in normal (□) and infected (■) mice. Dose of liposomal hamycin injected was 1·5 mg/kg. For the purpose of comparison dose of hamycin has been normalized to 1 mg/kg. Bars represent the S.D. of duplicate samples obtained from pooled organs of six mice. Hamycin was not detectable in serum.

et al., 1992). Tissue distribution of hamycin in SPC/PA liposomes could not be determined at 24 h as the amount of injected drug with this formulation was too low (0·2 mg/kg) to be accurately estimated, and a higher dose could not be administered due to toxicity. Figure 4 shows the tissue distribution of hamycin administered as a liposomal preparation (SPC/cholesterol/PA; molar ratio 4:5:1), 24 h following administration, in normal and infected animals. In the lung there was a 2·7-fold increase in the concentration of hamycin in infected animals whereas in kidney there was a 1·8-fold decrease. In liver and spleen the hamycin concentrations were comparable in both normal and infected animals.

Discussion

In the present communication we have shown that intercalation of hamycin into phosphatidic acid liposomes significantly reduces the toxicity as well as enhances the in-vivo antifungal activity of hamycin in experimental aspergillosis. The observed reduction in the toxicity of hamycin *in vivo* is determined by the amount of cholesterol incorporated into phosphatidic acid liposomes. This is consistent with our earlier observations (Moonis *et al.*, 1992) and those of other investigators (Mehta *et al.*, 1991). The reason for the reduced toxicity of hamycin in cholesterol-containing phosphatidic acid liposomes may be due to their reduced circulation time which may minimize the deleterious effects arising from the interaction of the drug with the RBC's (Juliano & Stamp, 1975). The toxicity of hamycin in cholesterol-containing phosphatidic acid liposomes was significantly lower than that of the free drug and drug intercalated into neutral liposomes (Moonis *et al.*, 1992).

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We suggested that due to similar binding affinities of aromatic polyenes to fungal as well as host cell sterol, hamycin cannot preferentially bind to ergosterol in the presence of liposomal cholesterol (Moonis *et al.*, 1992). To circumvent this we designed liposomes having strong negative surface charges which are known to be rapidly taken up by the reticuloendothelial system thus accelerating their clearance from the circulation. This could in turn augment the hamycin-ergosterol interaction in diseased organs leading to enhanced antifungal activity *in vivo*. Tissue distribution studies revealed an enhanced uptake of liposomal hamycin at infected sites with a concomitant decrease in the serum hamycin concentration. The enhanced in-vivo antifungal activity of hamycin contained in phosphatidic acid liposomes could be due to the increased interaction of hamycin with ergosterol as a consequence of the enhanced uptake by the macrophages. In the same disease model, hamycin intercalated into neutral liposomes at an equivalent dose (0·1 mg/kg) was not as effective in controlling experimental aspergillosis (Moonis *et al.*, 1992) as seen in the present study with phosphatidic acid liposomes.

In the present work the in-vivo antifungal activity of liposomal hamycin was not enhanced by the addition of cholesterol; an observation in contrast to that observed for liposomal amphotericin B (Ahmad *et al.*, 1990). These observations seem to be validated by physicochemical studies on aromatic polyenes which reveal that in contrast with amphotericin B, aromatic polyenes bind both cholesterol and ergosterol with equal affinity (Witzke & Bittman, 1984). At an equivalent dose (0·1 mg/kg) of hamycin in phosphatidic acid liposomes with and without cholesterol we observed 60% and 90% survival of animals respectively after seven days of therapy. In a recent report Mehta *et al.* (1991) achieved in-vivo therapeutic efficacy of liposomal hamycin in the presence of cholesterol. However, they did not analyse the comparative in-vivo antifungal activity of hamycin in the presence and absence of cholesterol.

The data in the present study indicates that the most effective therapeutic formulation of hamycin for the control of experimental aspergillosis is SPC/cholesterol/PA liposomes (molar ratio; 4:5:1) as it significantly reduces the toxicity of the drug, allowing higher doses to be administered. Although the SPC/PA liposomal preparation (9:1; molar ratio) was also effective at a lower dose but due to its high toxicity could not be used at higher doses.

Our results clearly demonstrate the potential usefulness of liposomes as a carrier in improving the therapeutic index of hamycin in the treatment of fungal infections by reducing its toxicity. This is particularly significant as the latter has previously severely restricted the use of hamycin.

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