Journal of Antimicrobial Chemotherapy (1994) **33**, 571–583

Effect of elimination of phagocytic cells by liposomal dichloromethylene diphosphonate on aspergillosis virulence and toxicity of liposomal amphotericin B in mice

Mona Moonis, Imran Ahmad† and Bimal Kumar Bachhawat*

Department of Biochemistry, Liposome Research Centre, University of Delhi, South Campus, New Delhi-110021, India

The role of macrophages in the toxicity and efficacy of liposomal amphotericin B (L-Amp B) was studied in a murine aspergillosis model infection. Macrophages and polymorphonuclear phagocytes (PMN cells) were depleted in the liver and spleen of mice by the administration of liposome encapsulated dichloromethylene diphosphonate. Macrophage depletion had no effect on the lethality of Fungizone, a commercial deoxycholate preparation of Amp B, but significantly increased the lethality of L-Amp B ($P < 0.01$). Macrophage depletion led to an increase in the fungal loads in the lung, liver and kidney ($P < 0.05$) and to an increase in the virulence of aspergillosis ($P < 0.05$). Tissue distribution analysis of L-Amp B revealed that in macrophage/PMN-depleted mice there was a decrease in the concentration of Amp B in the liver, with concomitant increases in the circulation, spleen and lung, both in the uninfected and in the infected conditions. The results clearly demonstrate that depletion of macrophage/PMN cells increases the virulence of aspergillosis, as well as the toxicity of L-Amp B. Moreover, L-Amp B treatment does not improve the survival rate of macrophage/PMN-depleted mice subjected to aspergillosis challenge.

Introduction

Aspergillosis, a common systemic fungal infection, is the major cause of mortality in immunocompromised hosts (Diamond, Huber & Haudenschild, 1983). Macrophages play an important role in the defence against fungal pathogens by an effective elimination of the spores in their conidial stage (Schaffner, Douglas & Braude, 1982). Studies have shown that *Aspergillus fumigatus* spores phagocytosed by macrophages *in vitro*, as well as *in vivo* after intravenous administration are rapidly removed by the reticuloendothelial system (Schaffner *et al.*, 1982).

Amphotericin B (Amp B) is the drug of choice for the treatment of systemic fungal infections such as aspergillosis, but its use is restricted due to severe toxicity. Liposomal intercalation of the drug offers an effective means both to reduce its toxicity and improve the therapeutic index when given to control systemic fungal infections (Taylor *et al.*, 1982; Lopez-Berestein, 1987; Ahmad, Sarkar & Bachhawat, 1989*a*). We and others have shown that most of the intravenously injected liposomal Amp B (L-Amp B) is taken up by the macrophage-rich organs such as the liver, spleen and

*Corresponding author.
†Present address: The Liposome Company Inc., One Research Way, Princeton-Forrestal Center, Princeton, New Jersey, USA.

0305–7453/94/030571+13 $08.00/0

lung (Lopez-Berestein, Rosenblum & Mehta, 1984; Ahmad, Sarkar & Bachhawat, 1990). Studies reported Lopez-Berestein *et al.* (1984) further suggested that the concentration of Amp B was higher in lung and liver from infected animals *vis à vis* the healthy controls.

Considering the importance of macrophages as primary defence cells in the control of systemic fungal infection, we have studied the role of macrophages and polymorphonuclear phagocytes (PMN cells) in controlling aspergillosis virulence and in reducing the toxicity of L-Amp B. We have determined the effect of eliminating tissue macrophage/PMN cells from the liver and spleen by intravenous administration of liposomal dichloromethylene diphosphonate (L-DMDP). Phagocytic uptake of the liposomes *in vivo* by macrophages causes release of DMDP into the interior of the cell resulting in cell death (van Rooijen, 1992). Intravenous administration of DMDP encapsulated in liposome tends to eliminate, selectively and reversibly, macrophages from the spleen and the liver without affecting the alveolar and other macrophages (Classen & van Rooijen, 1984; van Rooijen, 1992).

The present communication compares the infectivity, toxicity, therapeutic efficacy and tissue distribution of L-Amp B under in-vivo conditions in uninfected and in diseased animals, with and without macrophage depletion.

Materials and methods

Chemicals

DMDP was a gift from Proctor Eaton Pharmaceuticals Inc. USA; Amp B and cholesterol were purchased from Sigma Chemical Co., St Louis, USA; soya phosphatidylcholine (SPC) was from Dianorm, Munich, Germany; Fungizone, the commercial deoxycholate preparation of Amp B, was obtained from Sarabhai Chemicals, Baroda, India and was reconstituted in 5% dextrose before use. All other reagents used were of analytical grade.

Animals

Male BALB/c mice (body wt 20–25 g) were obtained from the laboratory animal facility of the Central Drug Research Institute, Lucknow, India and were maintained in our animal house.

Animal model for aspergillosis in BALB/c mice

The animal model for aspergillosis in BALB/c mice was established as described by Ahmad, Sarkar & Bachhawat (1989*b*). In brief, $1{\cdot}8 \times 10^7$ *Aspergillus fumigatus* spores suspended in sterile saline were injected intravenously into BALB/c mice via the tail vein. In the case of macrophage depleted mice the spores were injected after 1 h of L-DMDP treatment. Animals were also infected with a lower dose of fungal spores ($1{\cdot}8 \times 10^6$), both with and without macrophage depletion, to study the roles of PMN and tissue macrophages in controlling aspergillosis virulence after infection with a sublethal dose of *A. fumigatus*. Infection was confirmed by the presence of colony forming units (cfu) in lung, liver, spleen and kidney tissues.

Preparation of liposomes

DMDP-encapsulated liposomes were prepared as described by Claassen & van Rooijen (1986) with the slight modification of using SPC instead of egg phosphatidylcholine. In brief, 75 mg SPC and 19 mg cholesterol were dissolved in chloroform/methanol (2:1 v/v). The organic solvent was removed under reduced pressure in a rotary evaporator and the resulting film was dessicated overnight. The thin dry film was hydrated in 10 mL of 10 mM phosphate buffered saline (pH 7·4) containing 1·89 g DMDP. The preparation was kept for 2 h at room temperature under nitrogen and then sonicated for 3 min at 20°C in a water bath sonicator. It was kept at room temperature for another 2 h. The liposome suspension was centrifuged twice at 100,000 g for 30 min to remove free, non-entrapped material and liposomes were finally resuspended in 4 mL phosphate buffered saline.

Liposome-intercalated Amp B (1 mg Amp B for 45 mg total lipid), having the molar lipid composition, SPC/cholesterol (7:3) was prepared and sonicated for 45 min as described by Ahmad *et al.* (1989*b*).

Depletion of phagocytic cells by liposomal dichloromethylene diphosphonate as studied by transmission electron microscope

Male BALB/c mice were injected intravenously with liposome-encapsulated dichloromethylene diphosphonate. Twenty-four hours after the L-DMDP injection three mice were killed and their lungs, livers and spleens were removed in cold, physiological saline. The tissue samples were immediately immersed in fixative comprising 1% paraformaldehyde, 2·5% glutaraldehyde in 0·1 M cacodylate buffer, pH 7·3. The tissues were fixed for at least 4 h at 4°C, post-fixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol solutions and then embedded in plastic resin. Thin sections were stained with uranyl acetate (1%) and lead citrate (0·3%) and observed under CM 10 Philips transmission electron microscope at 80 Kv.

Determination of LD_{50} of Amp B

L-DMDP was administered via the tail vein into 20 BALB/c mice. Twenty-four hours later free Amp B (Fungizone, commercially available form, 0·5–4·0 mg/kg) was administered to half of the animals and liposome-intercolated Amp B (1·0–8·0 mg/kg) to the other half. Each set of 10 animals was split into four groups of BALB/c mice (weight 20–25 g, five animals in each group). The logarithm of the successive dosage levels differed by a constant d, such that the geometric factor (R) is related to d by the relation $d = \log R$. Survival was checked over a period of 4 days. Median lethal doses (LD_{50}) were calculated by the formula $\log (LD_{50}) = \log$ dose (minimum) $+ \log$ ratio $(f+1)$, as described by Weil (1952). The confidence level of the assay was 5%. For LD_{50} determination of liposome-intercalated Amp B, without macrophage depletion, mice were injected with 2·5–20·0 mg/kg body weight. An approximate test of the significance of difference between the two LD_{50} values was made by using the test criteria:

$$\frac{Z_o = m_1 - m_2}{\text{S.E.}(m_1 - m_2)}$$

where m_1 and m_2 are the LD_{50} values with and without macrophage depletion and S.E.$(m_1 - m_2)$ is the standard error of their difference. This is obtained as S.E.$(m_1 - m_2)$ =

Var. m_1 + Var. m_2 where Variance $m_1 = m_1^2$ Var. (f_1), d_1^2 where $d_1 = \log_{10} R$ (common ratio of the doses) and f_1 is a function of the mortality number in a given sequence as available in the tables of Weil (1952). Similarly, for set 2 the suffixes 2 are used in the corresponding quantities to obtain Var. (m_2). The difference is deemed significant at the 5% level if Z is greater than 1·96 (normal distribution) and at 1% level if Z is greater than 2·58.

Treatment of infected mice with and without macrophage depletion

BALB/c mice were injected via the tail vein with 0·2 mL of L-DMDP, followed 1 h later by the administration of $1{\cdot}8 \times 10^7$ *A. fumigatus* spores. Animals were divided randomly into two groups of 20 animals each. Twenty-four hours after infection animals in one group were injected with L-Amp B (0·5 mg/kg); the other group was left untreated. For survival experiments, ten animals were used in each experiment. The same protocol described above was followed. Survival was also checked in groups of animals which received only L-DMDP and in groups of animals to which were administered L-DMDP and L-Amp B (0·5 mg/kg).

The effect of L-Amp B was evaluated from the survival of animals, together with the number of cfus in lung, liver, spleen and kidney tissues. Survival was checked for up to 7 days after therapy with L-Amp B. To study the fungal load and numbers of cfus in lung, liver, spleen and kidney were determined as follows: three animals from each group were killed on alternate days after therapy and their lungs, livers, spleens and kidneys were excised aseptically, weighed and homogenized in 10 mL sterile 0·15 M NaCl. A 25-fold dilution was made and aliquots were plated on Sabouraud Dextrose medium. Colonies were counted after 48 h incubation at 37°C.

Statistical analysis of cfu and survival counts

Statistical analysis of the cfu values was done by using the student *t*-test to compare two mean values with equal frequencies as given by Snedecor & Cochran (1968). The survival data were analysed by using the χ^2 test of 2×2 table by Yate's correction of the Chi-Square, as well as by the Fisher's exact test.

Determination of amphotericin B in liposomes and in various organs

L-Amp B was determined both spectrophotometrically at 405 nm and by high performance liquid chromatography (HPLC) at 345 nm. The intercalation efficiency was found to be greater than 90%. HPLC analysis of Amp B in various organs and serum was done according to the method of Nilsson-Ehle *et al.* (1977), modified as reported earlier (Ahmad *et al.*, 1989*b*). The recovery of Amp B from various tissues ranged from 60–75%. The recovery of Amp B from various tissues ranged from 60–75%. The recovery from serum was more than 95%. Recovery of Amp B from serum was assessed as follows: blood samples drawn from the hearts of five mice were pooled and the mixture was allowed to clot. It was centrifuged to obtain serum. Pooled serum (0·5 mL) was homogenized with 1 mL of methanol containing 10–20 μg of Amp B and centrifuged at 12,000 **g** for 15 min. Samples (20 μL) of the supernatant were injected into a C_{18} reverse phase column for HPLC analysis.

Amp B levels were determined in normal and infected animals, killed 1 and 24 h, respectively, after iv administration of L-Amp B (2 mg/kg). L-DMDP was adminis-

tered 1 h before administration of fungal spores and L-Amp B was injected 24 h after administration of fungal spores. For tissue distribution analysis, groups of six mice were killed; blood and organs (lung, liver, spleen and kidney) were removed, pooled and frozen until required for analysis. The amount of Amp B in various organs was determined. Final values were calculated by adjusting the measured value to take account of the recovery factors.

Results

Depletion of phagocytic cells by liposomal dichloromethylene diphosphonate as studied by transmission electron microscope

Electron microscope studies show that 24 h after L-DMDP treatment the macrophage/PMN cell populations in the liver and the spleen were almost completely depleted and the few remaining cells were undergoing degenerative changes, as indicated by the appearance of a pycnotic nucleus, as well as a highly vacuolated cytoplasm (Figures 1 and 2(a)). The multilobed nuclei of PMN cells from the liver and spleen were also seen to be undergoing degenerative changes and the cells showed the presence of large cytoplasmic inclusions (Figures 1 and 2(b)). The alveolar macrophage/PMN cells (Figure 3(a), (b)) however, showed no degenerative changes 24 h after L-DMDP administration and displayed well defined nuclei and dense phagocytic granules.

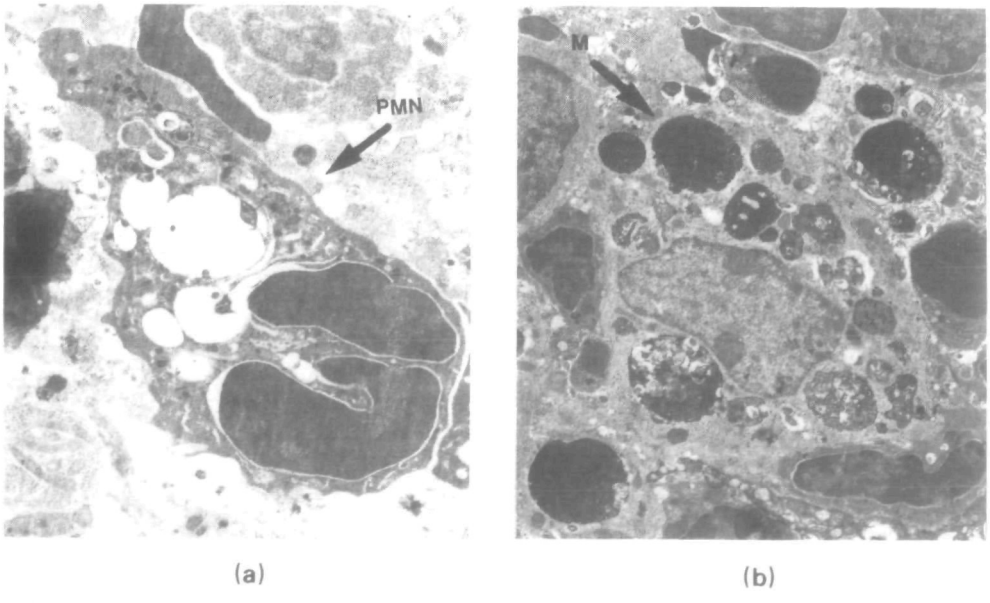

Figure 1. Electron micrographs of (a) a degenerating macrophage from liver showing cytoplasmic vacuolization indicating degenerative process (× 8900). 24 h after treatment with L-DMDP. (b) A degenerating PMN and macrophage (M) from liver after 24 h of L-DMDP treatment. The multi-lobed nucleus of the PMN displays degenerative changes and cytoplasmic vacuolization Macrophage (M) nucleus appears pycnotic (× 6600).

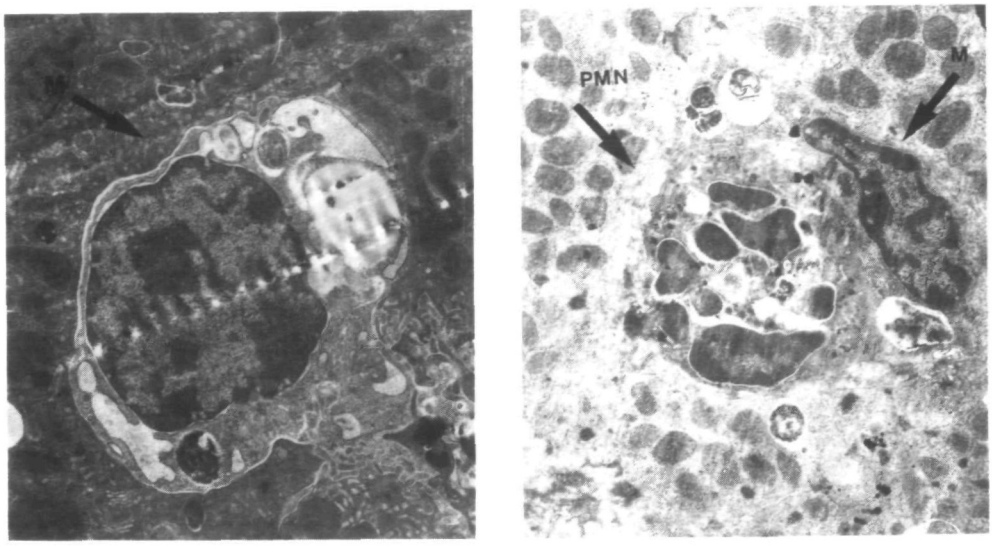

Figure 2. Electron micrographs of (a) a degenerating PMN from spleen, 24 h after L-DMDP treatment Note pycnotic nucleus and vacuolated cytoplasm (× 8900) (b) A degenerating PMN from spleen, 24 h after treatment with L-DMDP showing remnants of a necrotic cell (× 5200).

Infectivity

Animals which received a lower dose ($1{\cdot}8 \times 10^6$) of fungal spores showed similar fungal loads in various organs irrespective of macrophage depletion. In macrophage-depleted animals, the lungs and kidneys were more infected while in animals with normal macrophage populations these organs showed lower fungal counts (Table I). The fungal load was seen to decrease to negligible values in all organs after 6 days in the case of animals without macrophage depletion (100% survival after the sixth day of infection). In contrast, macrophage-depleted mice showed 100% mortality 3 days after fungal spore challenge (Table I). In the case of animals infected with $1{\cdot}8 \times 10^7$ *A. fumigatus* spores disseminated fungal infection was observed in both control and macrophage-depleted mice (Table II). Since disseminated fungal infection was observed

Table I. Fungal load[a] (cfu/organ) in various organs of BALB/c mice infected with *A. fumigatus*

Expt. No.	L-DMDP treatment	Days after infection	Lung	Liver	Spleen	Kidney
1a	–	2	4000 ± 700^b	$40{,}000 \pm 2800$	$200{,}000 \pm 28{,}000$	4000 ± 1400^b
1b	–	6	$25 \pm 5{\cdot}4$	200 ± 45	25 ± 6	50 ± 11
2a	+	2	8000 ± 1400^b	$35{,}000 \pm 2400$	$150{,}000 \pm 14{,}000$	9000 ± 1000^b
2b	+		100% mortality 3 days after fungal challenge			

[a]Values are expressed as mean error ± S.E. of means of cfu counts from duplicate readings from three animals. i.e. six observations.

[b]Significant differences at the 5% level between (1a) and (2a).

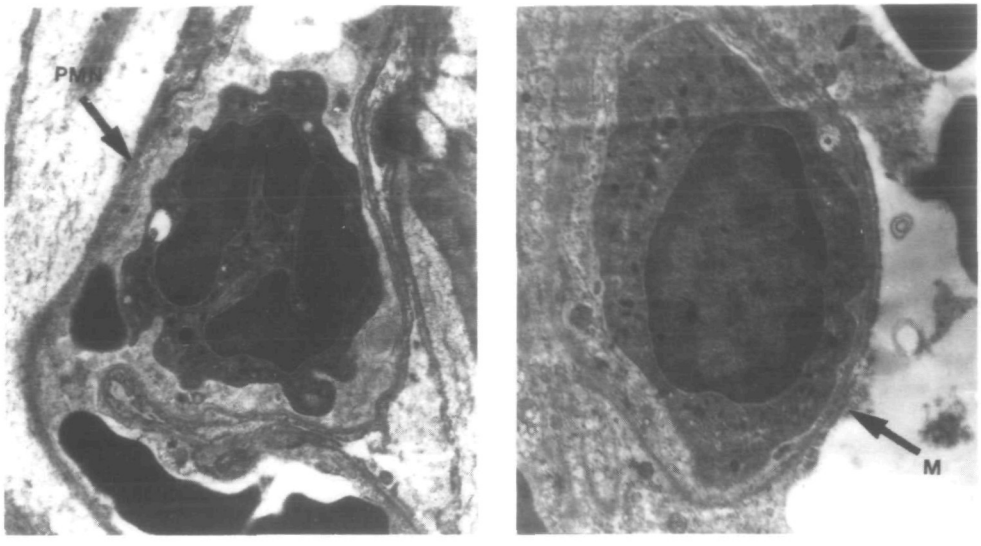

Figure 3. Electron micrographs of (a) a normal PMN cell from the lung after 24 h of L-DMDP treatment. Note the well defined multilobed nucleus and presence of phagocytic granules (×8900) (b) A normal macrophage from lung 24 h after L-DMDP treatment (×11,500).

both with and without macrophage depletion after inoculation with $1{\cdot}8 \times 10^7$ fungal spores, this dose was used in all subsequent experiments.

LD_{50} of L-Amp B

The LD_{50} values of various liposomal formulations are given in Table III. As evident from Table III there was a 2·6-fold increase in the toxicity of L-Amp B with L-DMDP

Table II. Fungal load (cfu/organ) in various organs of *A. fumigatus* infected BALB/c mice given liposomal amphotericin B therapy

Group[a] No.	Days after infection	Days after L-Amp B therapy	L-DMDP treatment	cfu (×10³) lung	liver	spleen	kidney
1	2	no L-Amp B therapy	–	12·0 ± 4	388 ± 62	62·0 ± 9·0	7·20 ± 0·4
2	2	1	–	3·80 ± 1·7	30 ± 3·8	19·0 ± 8·3	3·70 ± 1·8
	4	3		1·20 ± 0	8·2 ± 3·8	2·50 ± 1·7	0·70 ± 0·1
	6	5		0·20 ± 0·1	1·4 ± 1·0	0·10 ± 0·03	0
	8	7		0·17 ± 0	0	0·05 ± 0·03	0
3	2	no L-Amp B therapy	+	35·0 ± 21	273 ± 139	32·0 ± 14	20·0 ± 7·0
4	2	1	+	120 ± 35	690 ± 15	22·5 ± 7	12·0 ± 3·5

[a]L-DMDP was administered to groups 3 and 4 only.

Table III. LD_{50} of various formulations of Amp B, with and without administration of L-DMDP, for BALB/c mice

Drug formulation	L-DMDP	LD_{50} (mg/kg)
Fungizone	−	1·2[a]
Fungizone	+	1·4[b]
L-Amp B	−	9·5[a,c]
L-Amp B	+	3·7[b,c]

[a,b,c]Significant differences (1% level).

treatment, as compared with the untreated animals. On the other hand, macrophage depletion had no effect on the toxicity of Fungizone.

Survival of infected animals after L-Amp B therapy with and without macrophage depletion

The survial patterns of various groups of animals are shown in Figure 4. All uninfected animals were alive after the seventh day of administration of either L-DMDP or L-DMDP followed by L-Amp B (0·5 mg/kg) (not shown in Figure 4). In the absence of L-Amp B therapy, infected animals (with no macrophage depletion) survived for up to 2 days of infection (100% survival), while the macrophage-depleted animals showed 70% mortality during the same period. In the case of infected animals given L-Amp B

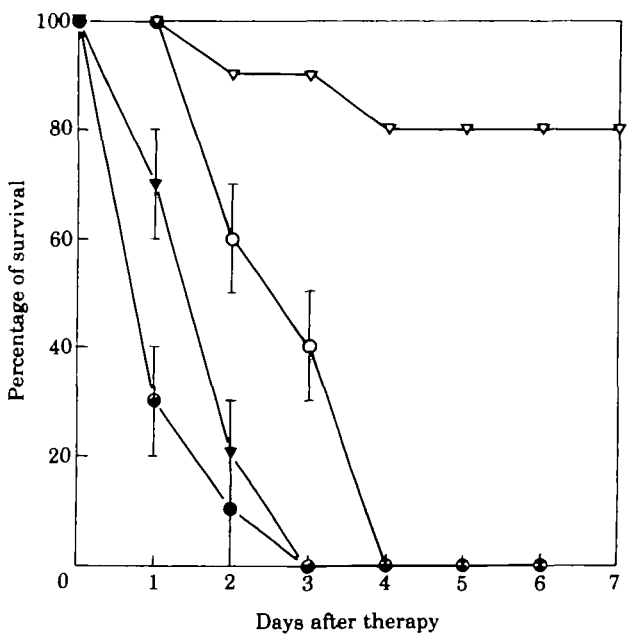

Figure 4. Survival of infected BALB/c mice with and without L-DMDP administration: ○, untreated controls; ●, macrophage depleted, untreated; ▽, infected mice, L-Amp B therapy; ▼, infected and macrophage depleted mice, L-Amp B therapy. Values are expressed as mean of % survival of animals of two separate expeciments and the vertical bars represent standard error of means at each time point.

therapy, the survival of animals with and without macrophage depletion was found to be 70% and 100%, respectively, one day after L-Amp B therapy. While 80% of the infected animals not subject to macrophage depletion and given L-Amp B treatment survived for at least 7 days, those mice with macrophage depletion had a 100% mortality rate during the same time period, even with L-Amp B therapy. The chi-squared test revealed that the survival ratios were heterogenous, as indicated by the significant (1% level) values for each. The Yates' correction of the chi-squared and Fischer's exact test were found to be significant after the fourth day (5% level) within the groups.

Cfu in various organs of infected BALB/c mice after liposomal therapy with and without macrophage depletion

Table II shows the effect of L-Amp B therapy in reducing the fungal load in the lungs, livers, spleens and kidneys of infected mice. Disseminated fungal infection was observed both with and without macrophage depletion after 2 days of fungal infection. In infected animals without macrophage depletion and which were given L-Amp B therapy there were marked reductions in fungal counts in all organs after 1 day of therapy. However, in macrophage-depleted mice, which were given L-Amp B therapy, the cfu count was consistantly high in all organs after 1 day of therapeutic treatment.

Tissue distribution pattern of L-Amp B in normal and infected mice with and without macrophage depletion

The organ concentrations of Amp B, following the administration of L-Amp B, in uninfected mice at 1 h and 24 h, with and without macrophage depletion, are shown in Table IV. Tissue distribution studies revealed marked increases in the concentrations of Amp B in the lungs, spleens, kidneys and sera of macrophage-depleted animals; in contrast, there was a decrease in the liver. The most significant increase (6·6-fold) in the concentration of Amp B in mice with macrophage depletion was observed in the spleen. Twenty-four hours after the administration of L-Amp B there were increases in the concentration of Amp B in the lungs (3·3-fold), spleens (3·2-fold) and kidneys (1·4-fold) of macrophage-depleted mice as compared with control values, i.e. in organs of mice not subject to macrophage depletion.

Table IV. Organ concentration of Amp B following intravenous administration of L-Amp B to normal mice, with and without administration of L-DMDP

	Organ (μg Amp B/g tissue) and serum (mg Amp B/L) concentration of Amp B			
	without L-DMDP		with L-DMDP	
	1 h	24 h	1 h	24 h
Lung	4·5 ± 0·7	3·2 ± 0·28	9·1 ± 0·28	10·7 ± 1·4
Liver	7·6 ± 1·4	14·7 ± 0·7	6·2 ± 0·28	7·7 ± 1·4
Spleen	3·5 ± 0·7	4·0 ± 0·28	23·3 ± 1·4	12·7 ± 1·4
Kidney	1·3 ± 0·42	4·3 ± 0·28	3·7 ± 0·98	6·2 ± 0·56
Serum	0·8 ± 0·14	0·6 ± 0·14	3·5 ± 0·42	N.D.

N.D., Not detectable.

Table V. Organ concentration of Amp B (mean ± S.D.) following intravenous administration of L-Amp B to infected mice, with and without administration of L-DMDP

	Organ (μg Amp B/g tissue) and serum (mg Amp B/L) concentration of Amp B			
	without L-DMDP		with L-DMDP	
	1 h	24 h	1 h	24 h
Lung	7·7 ± 0·28	1·2 ± 0·28	11·0 ± 0·10	6·6 ± 0·56
Liver	52·5 ± 2·1	31·2 ± 1·4	5·7 ± 0·98	7·9 ± 0·28
Spleen	8·4 ± 1·4	2·1 ± 0·35	22·7 ± 1·4	16·3 ± 1·4
Kidney	0·3 ± 0·07	3·1 ± 0·42	5·5 ± 0·42	3·9 ± 1·4
Serum	0·6 ± 0·14	0·5 ± 0·14	8·6 ± 0·56	N.D.

N.D., Not detectable.

It is evident from Table V that there was a marked alteration in the tissue distribution of Amp B during infection at 1 h and 24 h after drug administration to macrophage-depleted animals. In spleen (2·7-fold), kidney (18·0-fold) and serum (14·0-fold) the concentrations of Amp B were significantly enhanced by macrophage depletion, whereas there was a significant decrease in the concentration of Amp B in the liver (9·2-fold), in comparison with animals without macrophage depletion. Tissue distribution of L-Amp B in infected animals after 24 h followed a similar pattern to that observed in uninfected mice with macrophage depletion. Increases in the concentration of Amp B were observed in the lungs (5·5-fold), spleens (7·8-fold), kidneys (1·2-fold) while a decrease was noted in the liver (3·9-fold), as compared with mice without macrophage depletion.

Discussion

The results presented in this paper demonstrate that depletion of PMN and resident macrophages from liver and spleen accentuates both the virulence of aspergillosis and the toxicity of liposomal Amp B. The therapeutic index of L-Amp B is also greatly reduced, as inferred from the poor survival of macrophage/PMN-depleted mice after aspergillosis challenge.

van Rooijen and co-workers have shown histochemically depletion of macrophages in both the liver and the spleen 24 h after iv administration of L-DMDP (van Rooijen & Nieuwmegen, 1984); this was also confirmed ultrastructurally in the spleen (van Rooijen, Nieuwmegen & Kamperdijk, 1985). Our electron microscope studies are in agreement with the ultrastructural studies of van Rooijen *et al.* (1985) on the elimination of macrophages from the spleen and confirm the depletion of macrophages in the liver. Our ultrastructural studies also show depletion of PMN cells from the liver and the spleen 24 h after administration of intravenous L-DMDP. Furthermore, in agreement with the findings of van Rooijen (1992) macrophages in the lung were not affected by L-DMDP treatment and neither were PMN cells.

The increased virulence of *A. fumigatus* in macrophage/PMN-depleted animals is evident from the fact that when a sublethal dose of fungal spores was injected into these animals they did not survive for more than 2 days after infection. In contrast, in similarly challenged animals without macrophage depletion the fungal count after 6 days showed a marked decrease and all the animals survived. It is well known that

macrophages phagocytize fungal cells during systemic infection. Studies have shown that tissue macrophages play a key role in the control of infectivity of intracellularly growing pathogens, e.g. *Listeria monocytogenes* (Pinto *et al.*, 1991). The results in this study show that tissue macrophages and PMN cells play an important role in the control of aspergillosis virulence, although *A. fumigatus* is not an intracellular pathogen.

Liposomal Amp B is primarily known to be taken up by the reticuloendothelial system and the drug accumulates in lung, liver and spleen tissues of infected animals (Lopez-Berestein, *et al.*, 1984). The importance of PMN cells and resident macrophages in the control of fungal disease is evident from the fact that there was an 80% survival after 7 days of infected animals which had been given L-Amp B therapy, while for macrophage-depleted animals there was 100% mortality of infected animals after one day of L-Amp B therapy. The importance of macrophages/PMN cells is further supported by our data on fungal load in animals which were given L-Amp B therapy. In infected animals, subsequently given L-Amp therapy, there were marked reductions in fungal counts in all organs. However, in the case of macrophage-depleted mice treated with L-Amp B, the fungal counts did not decline after therapy and, as a consequence the drug therapy was ineffective.

Comparative toxicity studies with L-Amp B and Fungizone indicated that, in contrast to free Amp B, there was a marked increase in the toxicity of L-Amp B in macrophage/PMN-depleted mice. The increase in circulation time of L-Amp B may give greater opportunity for the drug to interact with potentially sensitive cells resulting in enhanced toxicity (Szoka, Milholland & Barza, 1987). It is interesting to note that the concentration of Amp B in the kidneys of macrophage-depleted animals was found to be comparable to that observed for Fungizone in a previous study (Ahmad *et al.*, 1989*b*). This increase could also be one of the factors responsible for the enhanced toxicity, as well as for the poor therapeutic efficacy, of L-Amp B.

Tissue distribution of L-Amp B in uninfected mice, with and without macrophage depletion, followed essentially the same pattern as that reported by Claassen & van Rooijen (1984). However, there was a dramatic change in the tissue distribution of L-Amp B in infected mice, with and without macrophage depletion. As expected a marked increase in the levels of Amp B was observed in all the infected organs, except liver. These findings support our contention that PMN cells and resident macrophages have major roles to play in the capture of the drug in the diseased tissues, enhancing thereby its effectiveness against the fungus. In the present study, we observed an increase in the concentration of Amp B in the spleen, even though L-DMDP treatment results in the depletion of splenic macrophages. This could possibly be due to the phagocytic activity of other cells, such as the sinus lining cells of the spleen, which have been shown to assume part of the phagocytic function (Claassen & van Rooijen, 1984). This functional capability has not been observed in liver. The increase in the concentration of Amp B in circulation in macrophage-depleted mice could also be due, indirectly, to the depletion of resident macrophages and PMN cells from the liver and the spleen.

In conclusion, our observations on in-vivo toxicity, therapeutic efficacy and tissue distribution profile of L-Amp B in BALB/c mice, with and without macrophage depletion, suggest that resident macrophages and PMN cells have important rôles in the containment of aspergillosis virulence, although *A. fumigatus* is not an intracellular pathogen. Also, resident macrophages/PMN cells play a pivotal rôle in the uptake of

liposomal Amp B from serum, which may account for the decreased toxicity of the drug.

Acknowledgements

Mona Moonis is a Senior Research Fellow and Bimal Kumar Bachhawat is a Bhatnagar Fellow of the Council for Scientific and Industrial Research, India. We thank Dr Shakti N. Upadhyay of the National Institute of Immunology, New Delhi for his help in performing the TEM studies. We thank Dr Daljeet Singh for statistical analysis and Professor U. N. Singh for critically evaluating the manuscript. This project has been supported by a grant from the Department of Biotechnology, Government of India.

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(*Received 16 February 1993; revised version accepted 29 October 1993*)