Volume 326, number 1,2,3, 56–58 FEBS 12659 July 1993

Tuftsin-bearing liposomes as drug vehicles in the treatment of experimental aspergillosis

Mohd. Owais[a], I. Ahmed[b], B. Krishnakumar[a], R.K. Jain[a,*], B.K. Bachhawat[b] and C.M. Gupta[a]

[a]*Institute of Microbial Technology, Chandigarh-160014, India* and [b]*Liposome Research Centre, Delhi University South Campus, Benito Juarej Road, New Delhi-110021, India*

Received 28 April 1993; revised version received 17 May 1993

Encapsulation of amphotericin B in tuftsin-bearing liposomes greatly increased its efficacy in treatment of human aspergillosis in mice. Also, the drug efficacy was significantly increased by pretreating the animals with drug-free tuftsin-bearing liposomes. These results demonstrate that macrophage activation can considerably enhance the therapeutic efficacy of antifungal drugs, like amphotericin B.

Tuftsin; Macrophage activation; Fungal infection; Liposome; Amphotericin B

1. INTRODUCTION

Tuftsin is a natural killer activator of macrophages/monocytes [1] which can easily be grafted onto the surface of liposomes [2] by attachment of a fatty acyl chain through an ethylenediamine spacer arm (Thr-Lys-Pro-Arg-NH-$(CH_2)_2$-NH-$COC_{15}H_{31}$) to its C-terminus. The tuftsin-bearing liposomes (Tuft-Lip) thus formed have been shown to be quite effective in activating the host's macrophages [3] and also in increasing non-specific resistance against parasitic infections [4,5].

Elimination of fungi from the tissues of normal healthy subjects is often associated with stimulation of cell-mediated immune defences which, for antifungal activity, essentially involve activation of mononuclear phagocytes by sensitized T cells [6]. The main function of these T cells in this case is to release lymphokines which in turn arm the macrophages to exhibit the fungistatic or fungicidal activity [6]. In the case of immune compromised individuals, however, these mechanisms do not operate optimally due to impaired cellular immunity [6]. Since these individuals are the main target of systemic fungal infections, treatment of such patients with antifungal drugs along with some agent which can provoke macrophages/monocytes for their normal function should afford obvious advantages over conventional chemotherapy. Keeping this in view, we considered it of interest to evaluate the usefulness of Tuft-Lip as amphotericin B (Amp B) vehicles in the treatment of experimental aspergillosis, as these liposomes, besides reducing drug toxicity [7–10], are also expected to activate the host's macrophages due to the presence of the tuftsin on their surface [3].

Correspondence address: C.M. Gupta, Institute of Microbial Technology, PO Box 1304, Sector 39-A, Chandigarh 160014, India. Fax: (91) (172) 44252.

**Present address:* Division of Gynecologic Oncology, Roswell Park Cancer Institute, Elm & Carlton Streets, Buffalo, NY 14263, USA.

2. MATERIALS AND METHODS

2.1. *Materials*

All the reagents used in the study were of the highest purity available. Cholesterol was bought from Centron Research Laboratory, Bombay, and used after crystallizing it three times from methanol. Egg phosphatidylcholine (egg PC) was isolated and purified according to the published procedure [11]. Tuftsin modified at the C-terminus was prepared as described earlier [2]. Amp B was purchased from Sigma Chemical Co.

2.2. *Liposomes*

Liposomes were prepared from egg PC (49 μmol) and cholesterol (21 μmol) with or without modified tuftsin (7–8% by PC weight). All the ingredients, along with Amp B (1.0 mg), were dissolved in a round-bottomed flask in a minimum volume of chloroform/methanol (1:1, v/v). The solvents were carefully removed under reduced pressure so that a thin lipid film formed on the wall of the flask. Final traces of the solvent were removed by leaving the flask in vacuo overnight at 4°C. The dried lipid film was hydrated with 2.0 ml of 150 mM sterile saline under vigorous stirring for 1 h in a bath-type sonicator under N_2 atmosphere. The sonicated preparation was dialysed against saline for 24 h at 4°C in dark, and then centrifuged at 10,000 × *g* for 1 h at 4°C to remove traces of undispersed lipids. The supernatant was analysed for both Amp B and tuftsin.

2.3. *Estimation of Amp B and tuftsin*

The amount of liposome-intercalated Amp B was determined by measuring its absorbance at 405 nm. The intercalation efficiencies of Amp B in tuftsin-bearing and tuftsin-free liposomes were about 95% and 85%, respectively.

The amount of tuftsin incorporated into liposomes was estimated as described earlier [2]. The incorporation efficiency was found to be about 98%.

 Published by Elsevier Science Publishers B.V.

2.4. *Animals*

Male Balb/C mice weighing 18 ± 2 g were used in the study. The animals were given a pellet diet (Hindustan Lever Ltd.) and water ad libitum.

2.5. *Infection*

Aspergillus fumigatus (strain VP_{236}) originally isolated from an aspergillosis patient at V.P. Chest Institute, New Delhi, was maintained in vitro and also in animals. The animals were given a known number of spores intravenously 24 h prior to the drug treatment. For prophylactic studies, the infection was given after treating the animals with drug-free Tuft-Lip.

2.6. *Treatment*

Two sets of experiments were carried out under this study. In the first set, the efficacy of the various drug formulations was determined against the *A. fumigatus* infections by measuring both the fungal load (CFU) in various organs and survival time of the animals. In the second set, the effect of tuftsin-mediated macrophage activation on the therapeutic efficacy of Amp B was analysed. In this case, the antifungal activity of Amp B was evaluated in the animals that were treated with Tuft-Lip prior to infection.

2.6.1. First set

The infected animals were divided into 5 groups, and then treated by being given as follows: Group I, normal saline; Group II, drug-free Tuft-Lip; Group III, drug- free, Tuft-free liposomes; Group IV, Tuft-free Lip-Amp B; Group V, Tuft-Lip-Amp B.

2.6.2. Second set

The animals were first divided into two groups. The first group was pretreated for 3 consecutive days with Tuft-Lip (50 μg Tuft/animal/day) on day 1–3 prior to infection, while the second group was left untreated. Each group, after beinig infected with *A. fumigatus* (~ 1.8×10^7 spores/animal) was further divided into 3 groups; thus the animals were divided into a total of six groups. Group I, pretreated animals given no Amp B (PT(+),DT(−)); Group II, pretreated animals given Lip-Amp (PT(+),Lip-Amp B)); Group III, pretreated animals given Tuft-Lip-Amp B (PT(+),Tuft-Lip-Amp B)); Group IV, untreated animals given no drug (PT(−),DT(−)); Group V, untreated animals given Lip-Amp B (PT(−),Lip-Amp B)); Group VI, untreated animals given Tuft-Lip-Amp B (PT(−), Tuft-Lip-Amp B)).

For CFU determination, 3 animals were taken out from each group of all the three sets. The animals were sacrificed and livers, lungs, kidneys and spleens were dissected out aseptically. The organs were minced in normal saline (5 ml) and 200 μl of this suspension was plated on rich media, and the plates were incubated for 48 h at 37°C. The colonies were counted and the fungal load calculated by multiplying with the dilution factor.

Table I

Effect of Lip-Amp B on *A. fumigatus* infections in Balb/c mice

Formulation	Survival on day 7 post-treatment			
	Exp. I	Exp. II	Exp. III	Exp. IV
Saline	0/4	0/5	0/10	0/14
Free drug	–	0/5	0/10	–
Empty Liposome	0/4	–	–	–
Empty Tuft-Lip	0/4	–	–	–
Lip-Amp B	3/8	3/10	9/25	4/11
Tuft-Lip-Amp B	6/8	11/15	19/25	9/13

Drug dose, 0.5 mg/kg (single, i.v.); challenge dose, ~ 1.8×10^7 spores/animal.

3. RESULTS AND DISCUSSION

Balb/c mice infected with *A. fumigatus* (~ 1.8×10^7 spores/animal) were treated with various doses (single, intravenous) of Amp B encapsulated in egg PC/cholesterol liposomes. No significant antifungal effects were observed when the administered dose of Amp B was < 0.5 mg/kg, but at a dose of 0.5 mg/kg, at least 35% of the infected animals survived after the treatment. This dose was therefore selected for comparing the antifungal effects of Lip-Amp B with Tuft-Lip-Amp B. Table I shows that the % survival of the infected animals was considerably increased (70–75%) by treating the animals with Tuft-Lip-Amp B. In addition, the animals that had survived by day 7 after the Tuft-Lip-Amp B treatment were virtually free of fungal infection as compared to those treated with Lip-Amp B (Table II). These results demonstrate that the efficacy of Lip-Amp B against *A. fumigatus* infection is considerably increased by grafting tuftsin onto the liposomes surface.

Table II

Fungal load in animals that survived infection after treatment with liposomised Amp B

Organ	Fungal load (CFU)			
	Exp. I	Exp. II	Exp. III	Exp. IV
Animals that survived from the Lip-Amp B-treated group				
Liver	187 ± 88	307 ± 75	258 ± 38	192 ± 21
Lung	625 ± 0	657 ± 50	508 ± 52	692 ± 63
Kidney	625 ± 176	533 ± 74	475 ± 25	483 ± 16
Spleen	2,312 ± 618	1,282 ± 122	1,525 ± 44	1,785 ± 194
Animals that survived from the Tuft-Lip-Amp B-treated group				
Liver	0	0	0	0
Lung	0	0	0	0
Kidney	0	50*	25*	0
Spleen	0	0	25*	0

The fungal load was determined on day 7 post-treatment. Values are means of 3 animals ± S.D. *Fungal load in one animal.

Table III

Effect of Tuft-Lip pretreatment on the efficacy of Lip-Amp B against fungal infections

Treatment	Survival on day 7 post-drug treatment	Fungal load (CFU) 24 h after drug treatment			
		Liver	Lung	Kidney	Spleen
PT(−),DT(−)	0/7	71,042 ± 3,442	53,542 ± 2,602	19,166 ± 2,194	39,170 ± 1,301
PT(−),Lip-Amp B	4/12	23,125 ± 1,654	13,333 ± 954	7,708 ± 360	17,916 ± 954
PT(−),Tuft-Lip-Amp B	9/12	9,167 ± 954	2,291 ± 360	5,208 ± 360	16,875 ± 1,275
PT(+),DT(−)	1/7	29,583 ± 2,525	20,833 ± 2,009	14,375 ± 1,875	33,958 ± 954
PT(+),Lip-Amp B	6/7	2,916 ± 721	1,875 ± 625	4,792 ± 721	13,541 ± 954
PT(+),Tuft-Lip-Amp B	6/7	2,708 ± 360	1,041 ± 360	2,500 ± 625	12,708 ± 360

Drug dose, 0.5 mg/kg (single, i.v.); challenge dose, $\sim 1.8 \times 10^7$ spores/animal. PT, pretreatment; DT, drug treatment. Values are means of 3 animals ± S.D.

Monocytes/macrophages are the key line of host defence against pathogenic fungi [6]. As tuftsin is known to increase the killer activity of these cells [1], the observed increase in the therapeutic efficacy of Amp B after its encapsulation in Tuft-Lip may partly be attributed to macrophage activation. To further examine the validity of this conclusion, we determined the effects of Lip-Amp B on the initial fungal load, which was measured 24 h after the drug treatment, and the % survival (on day 7 post-drug treatment) in mice that were treated with Tuft-Lip prior to the *A. fumigatus* infection. The data shown in Table III indicate that the Tuft-Lip pretreatment not only reduced the initial fungal load but also increased the % survival in Lip-Amp-treated animals. In the pretreated groups, both Lip-Amp B and Tuft-Lip-Amp B were equally effective, confirming that macrophage activation does increase the therapeutic efficacy of the antifungal drug, Amp B. These results thus strongly indicate that the chemotherapeutic efficacy of some antifungal drugs can be markedly increased by administering them along with macrophage activators in liposomes.

Amp B is a potent antifungal drug but its therapeutic use is limited due to its high toxicity. The toxicity has, however, been shown to considerably decrease without affecting the drug's efficacy by encapsulating this drug in liposomes [7–10], thus rendering Amp B suitable for use in the treatment of fungal infections. The present study further increases the scope of liposomised Amp B formulations in the treatment of fungal infections by demonstrating a significant improvement in the Lip-Amp B efficacy against experimental aspergillosis after grafting tuftsin onto the surface of liposomes. This modification of the Lip-Amp B surface, however, did not affect the drug toxicity, as we observed almost identical LD_{50} values (7–8 mg/kg) for both Lip-Amp B and Tuft-Lip-Amp B.

In conclusion, this study shows that the Tuft-Lip-Amp B formulation is considerably better than the Lip-Amp B preparation in the treatment of systemic fungal infections due to its higher efficacy and comparable toxicity. In addition, it suggests that administration of macrophage activators/immunomodulators along with Amp B in liposomes should greatly improve the efficacy of fungal chemotherapy.

Acknowledgements: A part of this study was carried out at the Central Drug Research Institute, Lucknow, India: the authors are grateful to the Director, CDRI, for providing facilities. This study was financially supported by the Department of Biotechnology, New Delhi (Grant BT/TF/03/026/001/88). Mohd. Owais received financial support from the Council of Scientific and Industrial Research, New Delhi, in the form of research fellowships.

REFERENCES

[1] Najjar, V.A. (1987) Drugs Future 12, 147–160.
[2] Singhal, A., Bali, A., Jain, R.K. and Gupta, C.M. (1984) FEBS Lett. 178, 109–113.
[3] Singh, S.P., Chhabra, R. and Srivastava, V.M.L. (1992) Experientia 48, 994–996.
[4] Gupta, C.M., Puri, A., Jain, R.K., Bali, A. and Anand, N. (1986) FEBS Lett. 205, 351–354.
[5] Guru, P.Y., Agrawal, A.K., Singha, U.K., Singhal, A. and Gupta, C.M. (1989) FEBS Lett. 245, 204–208.
[6] Deepe Jr., G.S. (1991) in: Handbook of Applied Mycology, vol. 2 (Arora, D.K., Ajello, L. and Mukerji, K.G. eds.) pp. 205–241, Marcel Dekker Inc.
[7] Mehra, R., Lopez-Berestein, G., Hopfer, R., Mills, K. and Juliano, R.L. (1984) Biochim. Biophys. Acta 770, 230–234.
[8] Graybill, J.R., Craven, C.P., Taylor, R.L., William, D.M. and Magee, W.E. (1982) J. Infect. Dis. 145, 748–752.
[9] Graybill, J.R. and Craven, C.P. (1983) Drugs 25, 41–62.
[10] Lopez-Berestein, G., Mehta, R., Hopfer, R.L., Mills, K., Kasi, L., Mchta, K., Fainstein, V., Luna, M., Hersh, E.M. and Juliano, R.L. (1983) J. Infect. Dis. 147, 939–945.
[11] Gupta, C.M. and Bali, A. (1981) Biochim. Biophys. Acta 663, 506–515.