Incorporation of amphotericin B in tuftsin-bearing liposomes showed enhanced efficacy against systemic cryptococcosis in leucopenic mice

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Objectives: The role of the immunomodulator tuftsin in enhancing the antifungal activity of liposomal amphotericin B against Cryptococcus neoformans in leucopenic mice was assessed.

Methods: In the present study, we investigated the antifungal activity of amphotericin B liposomes with tuftsin grafted on the surface. Mice were treated with free amphotericin B as well as liposomal formulations after C. neoformans infection. For prophylactic studies, mice were pre-treated with liposomal tuftsin (50 μg/mL) for three consecutive days prior to C. neoformans infection (7 × 10⁵ cfu/mouse). Chemotherapy, with tuftsin-free and tuftsin-bearing amphotericin B liposomes, was started 24 h post C. neoformans infection. The role of tuftsin in immunoaugmentative therapy was assessed by survival and cfu of treated mice.

Results: Amphotericin B entrapped in tuftsin-bearing liposomes showed increased anticryptococcal activity in the murine model. Moreover, tuftsin pre-treatment further augmented the antifungal activity of liposomal amphotericin B in leucopenic mice. Incorporation of tuftsin in liposomes resulted in increased anticryptococcal activity of liposomal amphotericin B compared with amphotericin B deoxycholate and conventional liposomal amphotericin B formulations.

Conclusions: The enhanced anticryptococcal activity of amphotericin B in tuftsin-liposomes can be attributed to the immune-stimulating property of tuftsin. Tuftsin activates the key immune cells, due to the presence of its receptors on macrophages and neutrophils, for a better fight against pathogens. Simultaneous liposome-mediated delivery of amphotericin B to the site of infection kills the pathogens more effectively.

Keywords: immunomodulators, leucocytes, prophylaxis

Introduction

Over the past few decades, Cryptococcus neoformans infections have increased with the increasing frequency of immunocompromised patients due to multiple factors including the AIDS epidemic, the development of antineoplastic therapies, advances in surgery and organ transplantations, and widespread use of broad-spectrum antibiotics.1–3 Besides cryptococcosis, defects in cell-mediated immunity also predispose hosts to other fungal infections such as aspergillosis and histoplasmosis, as well as mucosal candidiasis.

Antifungal drugs such as fluconazole and amphotericin B have shown broad immunomodulatory properties.4 Cytokines, effector cells and antifungals seem to work synergistically to restrict fungal growth in immunocompetent persons.5,6 In immunocompromised hosts, the lack of effector functions that cooperate with antifungal drugs to clear the pathogens seems to be a crucial factor in impeding the effectiveness of the drug.5,7

Antifungal therapy is often ineffective in the setting of immune suppression; hence, immune augmentative therapy is a rational approach for treatment of fungal infections because it is intended to enhance immune functions. An important point before designing a therapy for fungal infections is to analyse the immune status of the host, and whether or not the immunological defect, predisposing to the disease, can be reversed. Antifungal chemotherapy in conjunction with immunostimulatory molecules such as interleukin-12,
interferon-γ and GM-CSF has been found to show enhanced efficacy against many fungal pathogens. 8

Tuftsin, a tetrapeptide (Thr289-Lys290-Pro291-Arg292) sequence of the IgG molecule, has been found to increase macrophage-mediated phagocytosis, the macrophage migration index and splenocyte proliferation. 9 In our previous studies, we demonstrated the immunopotentiating activity of liposomal tuftsin in augmenting the antifungal activity of amphotericin B and nystatin against drug-resistant and drug-susceptible Candida albicans infection, both in normal as well as leucopenic mice. 10–12 Tuftsin-mediated early recovery of leucocytes was observed in cyclophosphamide-treated leucopenic mice. 12 A prophylactic and therapeutic role of tuftsin has also been reported in the treatment of tuberculosis, leishmaniasis and malaria in experimental animals. 13–15

The present study involves the use of the immunomodulator tuftsin in combination with liposomal amphotericin B in the treatment of systemic infection by C. neoformans in temporarily leucopenic BALB/c mice. The results of the present study confirm the immunomodulatory role of tuftsin in enhancing the antifungal activity of amphotericin B against C. neoformans infection.

Materials and methods

Materials

Cholesterol was bought from Centron Research Laboratory, Bombay and used after crystallization with methanol. Egg phosphatidylcholine (egg PC) was isolated and purified according to a previously published procedure. 16 Tuftsin modified at the C terminus was prepared as described previously. 15 Amphotericin B and cyclophosphamide were purchased from Sigma Chemical Co. USA.

Liposomes

Liposomes were prepared from egg PC (49 μmol) and cholesterol (21 μmol) without or with modified tuftsin (7–8% by PC weight) by a sonication method. 10 Egg PC, cholesterol and amphotericin B (drug/lipid, 1:20 molar ratio) were dissolved in a minimum volume of chloroform/methanol (1:1, v/v). The solvents were carefully evaporated under reduced pressure to form a thin lipid film on the wall of a round-bottomed flask. Subsequently, the dried lipid film was hydrated with 2.0 mL of 150 mM sterile saline with centrifuge followed by washing with normal saline. The cells were counted by using a haemocytometer. Each mouse was infected with 7 × 10^5 cfu of C. neoformans suspended in 0.2 mL of normal saline through an intravenous (iv) route.

Role of tuftsin with liposomal amphotericin B against C. neoformans infection in leucopenic mice

Various formulations of amphotericin B (namely free amphotericin B, liposomal amphotericin and tuftsin-bearing liposomal amphotericin B) were tested against C. neoformans infection. Amphotericin B liposomal formulations were used at doses of 1 and 3 mg/kg. Treatment was started 24 h post-infection with C. neoformans. The infected animals were treated with various formulations of amphotericin B on days 1, 3 and 5 post C. neoformans infection. Ten mice were studied in each of the following groups: (i) saline; (ii) sham liposomes; (iii) empty tuftsin-bearing liposomes (sham liposomes); (iv) amphotericin B deoxycholate (1 mg/kg); (v) amphotericin B deoxycholate (3 mg/kg); (vi) liposomal amphotericin B (1 mg/kg); (vii) liposomal amphotericin B (3 mg/kg); (viii) tuftsin-bearing liposomal amphotericin B (1 mg/kg) and (ix) tuftsin-bearing liposomal amphotericin B (3 mg/kg).

Enhanced anticytotoxic activity of amphotericin B in tuftsin liposomes

Animals

Female BALB/c mice weighing 22 ± 4 g were used in the study. The animals were given a standard pellet diet (Hindustan Lever Ltd) and water ad libitum. Mice were checked daily for their mortality and moribundity. The techniques used for bleeding, injection as well as sacrifice of the animals were approved by the Animal Ethics Committee [Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India].

Immunosuppression

Neutropenia was induced by injecting a single dose of cyclophosphamide (250 mg/kg) into each mouse via the lateral tail vein. Cyclophosphamide caused depletion of leucocytes as revealed by the total leucocyte count. Leucocytes (neutrophils and lymphocytes) were counted on day 3 post-cyclophosphamide treatment in both control and tuftsin-treated mice using a haemocytometer. The animals were subsequently exposed to C. neoformans infection on day 3 post-cyclophosphamide treatment with the idea that infection should be established under neutropenic conditions.

Test strain

The strain of C. neoformans (JMCR 102) was obtained from the microbiology section of Jawaharlal Nehru Medical College (JNMC), Aligarh Muslim University, Aligarh, India. SD agar/broth was used for growing patient isolates of C. neoformans. The identity of the clinical isolate of C. neoformans (serotype A) was confirmed in the mycology section of the Department of Microbiology, JNMC, Aligarh, India.

Antifungal susceptibility testing

The MIC of amphotericin B was determined by the broth macro-dilution method according to the guidelines of the NCCLS, document M-27A. 17 A stock solution of amphotericin B was prepared in 5% DMSO. The final range of concentrations was from 0.025 to 2.5 mg/L for amphotericin B. The MIC of amphotericin B for C. neoformans (JMCR 102) was found to be 0.25 mg/L.

Preparation of fungal inoculum for infection

C. neoformans strain for infection was grown in YPD medium (yeast extract 1.5%, peptone 2% and 5% dextrose) at 37°C for 48 h. The cell suspension was centrifuged at 5000 g for 15 min at 4°C in a cooling centrifuge followed by washing with normal saline. The cells were counted by using a haemocytometer. Each mouse was infected with 7 × 10^5 cfu of C. neoformans suspended in 0.2 mL of normal saline through an intravenous (iv) route.

Enhanced anticytotoxic activity of amphotericin B in tuftsin liposomes

Estimation of liposome-intercalated amphotericin B and tuftsin

The intercalation efficiency of amphotericin B in the liposomes was estimated by an HPLC method. 12 Briefly, the sample (20 μL) was injected onto a Hypersil octyldecyl-silane 5 μm particle size analytical column [150 by 4.6 mm (internal diameter)]. Detection was accomplished with a UV-visible-light detector set at 405 nm. The intercalation efficiency of amphotericin B in both plain egg PC and tuftsin-bearing liposomes was found out to be of the same order as estimated by a spectrophotometric method (90 ± 4%). 12 The tuftsin entrapped in the drug-containing liposomes was estimated by the BCA method as modified in our lab. 12 The incorporated tuftsin was found out to be ~95% of the total amount added.
Prophylactic role of liposomal tuftsin in combination with tuftsin-bearing and tuftsin-free liposomal amphotericin B against C. neoformans infection in leucopenic mice

The prophylactic role of tuftsin was also assessed in combination with liposomal amphotericin B in the treatment of murine cryptococcosis. In prophylactic studies, pre-treatment with tuftsin [50 μg/mouse, intraperitoneal (ip)] was started after 12 h of cyclophosphamide treatment for three consecutive days prior to challenge with infection. Mice were treated with tuftsin-free as well as tuftsin-bearing liposomal formulations of amphotericin on days 1, 3 and 5 post C. neoformans infection by an ip route. Animals were divided into six groups and each group consisted of ten mice (where PT stands for pre-treated and DT stands for drug): (i) PT (−) DT (−); (ii) PT (+) DT (−); (iii) PT (−) liposomal amphotericin B (3 mg/kg); (iv) PT (−) tuftsin-bearing liposomal amphotericin B (3 mg/kg); (v) PT (+) liposomal amphotericin B (3 mg/kg) and (vi) PT (+) tuftsin-bearing liposomal amphotericin B (3 mg/kg).

Assessment of fungal burden in brain and lungs

The efficacy of tuftsin-bearing or tuftsin-free liposomal amphotericin B was assessed by monitoring the survival of the animals and determining the clearance of C. neoformans from lungs and brain. For survival studies, mortality of the animals was observed twice each day during 30 days of observation. Quantitative assessment of the fungal burden in brain and lungs was performed following a published procedure.12 Three mice from each group were sacrificed on day 3 post-infection (8 h post second dose of amphotericin B) in different experiments. Mice were euthanized using halothane and vital organs (namely lungs and brain) were taken out under aseptic conditions. The organs were washed extensively with hypotonic buffer to lyse erythrocytes, homogenized and serially diluted with normal saline. The various dilutions of each sample (200 μL) were dispersed on SD agar plates, containing gentamicin and chloramphenicol to avoid bacterial contamination, in triplicate. After incubation for 48–72 h at 37°C, the colonies were counted and the fungal load was calculated by multiplying by the dilution factor.

Statistics

Survival data were analysed by using Kaplan–Meier curves and various groups were compared by log rank test (95% confidence interval) using the Prism software. Fungal burden in organs was analysed by the two-tailed unpaired t-test. A P value of <0.05 was considered statistically significant.

Results

Tuftsin induced early recovery of leucocytes in cyclophosphamide-treated mice

The immunopotentiating effect of tuftsin was analysed in cyclophosphamide-treated mice. Our results showed that tuftsin treatment induced early recovery of neutrophils and lymphocytes. We found that mice treated with cyclophosphamide showed remarkably depleted numbers of leucocytes (125–300 cells/mm³), while mice treated with tuftsin had much higher numbers (1200–2000 cells/mm³) on day 4 post cyclophosphamide treatment.

Tuftsin augments anticytotoxic activity of amphotericin B in leucopenic mice

The role of tuftsin in combination with liposomal amphotericin B was assessed in the elimination of systemic C. neoformans infection from leucopenic BALB/c mice. The antifungal activity of liposomal amphotericin B increased upon incorporation of tuftsin on the surface of the liposomes. The animals were made leucopenic before C. neoformans infection (7 × 10⁵ cfu/mouse). The leucopenic animals treated with tuftsin-bearing liposomal amphotericin B (3 mg/kg) showed an ~50% survival rate. There was only 30% survival of the animals treated with a dosage of 3 mg/kg liposomal amphotericin B formulation (without tuftsin) till day 40 post-infection, while mice treated with tuftsin-bearing liposomal amphotericin B at a dose of 1 mg/kg showed 20% survival. All the animals of other treated or control groups died before day 40 post infection (Figure 1). Out of 10 mice, 5, 4 and 7 mice survived in the group treated with tuftsin-bearing amphotericin B liposomes in three independent experiments, while 3, 3 and 4 mice survived in the group treated with tuftsin-bearing liposomes without tuftsin until day 40 of observation.

Prophylactic use of tuftsin enhances the efficacy of tuftsin-free or tuftsin-bearing liposomal formulations of amphotericin B in leucopenic mice

Mice were pre-treated with tuftsin for three consecutive days after cyclophosphamide administration and before infection with C. neoformans. Pre-treatment with tuftsin was found to augment the efficacy of liposomal amphotericin B in leucopenic mice. Administration of liposomal amphotericin B at a dose of 3 mg/kg in tuftsin pre-treated leucopenic mice resulted in ~60% survival (Figure 2), while tuftsin pre-treatment of mice followed by treatment with tuftsin-bearing liposomal amphotericin B resulted in ~80% survival of the treated mice.
Assessment of severity of infection in the lungs and brain

The severity of *C. neoformans* infection was assessed by culturing the lung and brain homogenates of the infected mice. The cfu count was found to be least in mice treated with tuftsin-containing liposomal amphotericin B in comparison with mice treated with other formulations of amphotericin B (Figure 3). Tuftsin-bearing liposomal amphotericin B (3 mg/kg) was found to be more effective than the same formulation of amphotericin B without tuftsin against *C. neoformans* infection in leucopenic mice ($P < 0.05$).

Tuftsin pre-treatment proved to be more effective in preventing the establishment of *C. neoformans* infection in leucopenic mice. Pre-treatment of mice with tuftsin followed by chemotherapy with tuftsin-bearing amphotericin B liposomes resulted in the greatest reduction in fungal load in the lungs and brain of treated mice (Figure 4). Fungal burden was found to be very low in organs of leucopenic mice pre-treated with tuftsin followed by tuftsin-bearing liposomal amphotericin B treatment in comparison with other groups ($P < 0.05$).

Residual fungal burden

Residual fungal burden in the brain and lungs of the surviving mice was analysed on day 35 post *C. neoformans* infection. It was found to be in the range of 0–200 cfu per organ. It showed that infection in these mice had subsided.

Discussion

Neutrophils, monocytes and tissue-based macrophages are the major cellular components of the innate immune system, which represents the first line of host defence against invading pathogens. On entering the host, yeast cells are first confronted with macrophages and thus active infection and disease depends largely on the competence of the host immune system. Cellular immune mechanisms successfully mediate through the activation of macrophages. Primary interaction between macrophages and yeast cells is a crucial determinant of whether disease ensues or not. Thus the activation of phagocytes (macrophages and neutrophils) is very important for imparting protection against *C. neoformans*.

Amphotericin B has been a reference standard drug for treatment of fungal infections. Tolerance of amphotericin B is limited by its acute and chronic toxicities. Various lipid formulations of amphotericin B are designed to offer the advantage of increased daily dose, better drug delivery to the primary reticuloendothelial organs and reduced toxicity. In the present study, we observed the antifungal activity of liposomal amphotericin B in combination with the immunomodulator tuftsin. Tuftsin-bearing liposomal amphotericin B at a dose of 3 mg/kg was found to impart maximum cure to treated mice against systemic infection of *C. neoformans*. Liposomal amphotericin B in combination with tuftsin was found to impart 50% cure to the treated mice infected with *C. neoformans*. Further, the pre-treatment of mice with tuftsin followed by chemotherapy with tuftsin-containing liposomal amphotericin B (3 mg/kg) increased the survival rate up to 80%. This is concordant
supernatants of cultured splenic and peritoneal cells isolated liposomal amphotericin B (PT(–) tuftsin-bearing liposomal amphotericin B versus PT(+) tuftsin-bearing liposomal amphotericin B (–) liposomal amphotericin B versus PT(+) liposomal amphotericin B (independent experiments. PT(–) DT(–) versus PT(+) DT(–) (organ) of the organisms in the lungs and brain. Values are means ± SEM of fungal load in organ homogenates of C. neoformans–infected BALB/c mice from three independent experiments. PT(–) DT(–) versus PT(+) DT(–) (P = 0.407); PT(–) liposomal amphotericin B versus PT(+) liposomal amphotericin B (P = 0.0078); PT(–) tuftsin-bearing liposomal amphotericin B versus PT(+) tuftsin-bearing liposomal amphotericin B (P = 0.01).

with our previous studies where tuftsin pre-treatment has been demonstrated to enhance the efficacy of polyene antibiotics against C. albicans and Aspergillus fumigatus infections.21 The immunoglobulin heavy chain associated tetrapeptide, tuftsin (Thr-Lys-Pro-Arg), is known to stimulate phagocytosis activity of macrophages.21 Owing to the presence of receptors on macrophages, tuftsin facilitates the targeting of liposomal amphotericin B to macrophages that helps in the elimination of intracellular infection. Tuftsin has been found to increase phagocytosis by PMNs, monocytes-macrophages and NK cells, and modulates their biological activities.22 The grafting of tuftsin on the surface of liposomes would, therefore, enable it not only to home the liposomal drug to the cells possessing receptors but also to stimulate these key cells of the immune system non-specifically against various infections.

Immunocompromised persons are very prone to opportunistic fungal infections. Tuftsin acts as a potential immunorepressor by replenishing the depleted immune cells. This is also confirmed by the results of our present study that show the increased efficacy of liposomal amphotericin B in tuftsin pre-treated leucopenic mice compared with those treated with the same drug formulation without tuftsin pre-treatment. The increased efficacy of amphotericin B in tuftsin pre-treated mice may also be attributed to increased production of tumour necrosis factor- α (TNF-α) in serum and supernatants of cultured splenic and peritoneal cells isolated from tuftsin-treated animals.23 TNF-α plays an important role in preventing fungal infections.24

The biological activity of the immunomodulator tuftsin may be due to the induction of the macrophage respiratory burst phenomenon. Activated macrophages exhibit enhanced levels of NADPH oxidase, O₂, H₂O₂ and myeloperoxidase (MPO). Both O₂ and H₂O₂ damage proteins, nucleic acids and membranes sufficiently to kill the cell or even the whole organism. Nevertheless, for macrophages, hypohalous acid produced by the action of MPO on H₂O₂ has been identified as the major killer agent.25 These phenomena collectively create a hostile environment for the survival of C. neoformans inside the host. At this stage the selective delivery of antifungal drugs proved to be more effective in elimination of the fungal pathogens.

The results of the present study clearly support the use of augmentative immune therapy in combination with antifungal drugs for treatment of fungal diseases particularly in immunocompromised persons. Antifungal drugs reduce the severity of infection by killing the pathogens and immunomodulators potentiate the immune system for a better defence against the invading microorganisms.

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References

Enhanced anticryptococcal activity of amphotericin B in tuftsin liposomes


