Original Articles

Protective Activity Induced by Oral Administration of Amphotericin B against Infections due to *Candida albicans* and Vaccinia Virus in Mice

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Abstract

Orally administered amphotericin B (AmB) induced protective activity against infections due to vaccinia virus and *Candida albicans* in mice. This activity was also confirmed in immunocompromised mice. Analyses of the kinetics of peripheral blood cells treated with oral AmB indicated an increased ratio of polymorphonuclear leukocytes (PMN). Enhancement of γ -interferon (IFN) production and of NK-cell activity was also observed. The data were considered to indicate that γ -IFN produced by oral AmB has an important part as one of the factors protecting mice against both candidiasis and vaccinia virus infection.

Key words: amphotericin B, oral therapy, protective activity, experimental infections, *Candida albicans*, vaccinia virus

Introduction

With the recent growing numbers of immunocompromised patients, a concomitant increase in the incidence of opportunistic fungal infections has been reported¹⁾. In those immunocompromised hosts, reduction in the number of lymphocytes, monocytes and polymorphonuclear leukocytes (PMN) or the activity in these immunocompetent cells has been viewed as one of the most important predisposing factors, especially for most fungal infections

^{2,3)}. New azole antifungals have recently been introduced⁴⁾, but these are often ineffective or cannot be used in immunocompromised hosts and major therapeutic gaps remain, particularly for patients with several immunodeficiencies and for those with AIDS³⁾.

Amphotericin B (AmB) has been the first choice for treatment of such infections since it showed a potent fungicidal activity^{4,5)}; its use, however, has frequently been hampered by severe toxicity which makes it necessary to limit dosage⁴⁾. Several strategies have been developed over the past few years in an effort to overcome this disadvantage. These included ⁴⁻⁸⁾: development of [i] new AmB derivatives,

[ii] a new combination method with other antifungal agents, and [iii] design of a novel drug delivery system (DDS) of AmB, including identification of new administration routes. Despite various new AmB derivatives reported, problems still remain^{7,8)}. Combination of AmB with flucytosine has been the drug of choice for cryptococcosis⁹⁾, and recently the combined effect of AmB with new antifungal agents such as cilofungin has been studied and clinical applications are planned¹⁰⁾. Liposomal AmB is believed to hold the greatest promise as a DDS for antifungal chemotherapy and clinical applications are being undertaken⁸⁾.

Intravenously administered AmB was reported to have mitogenic properties under appropriate *in vivo* conditions^{11,12)}. Thomas *et al.*¹³⁾ confirmed this by their findings that some patients with systemic candidiasis were improved by doses of AmB at a level too low to have a candidicidal effect in serum. It was also reported that the survival ratio of mice pretreated with AmB increased after experimental infections with *Listeria monocytogens* or some other intracellular parasites for which AmB has no *in vitro* activity^{5,13)}. No detailed studies on immunomodulating activities of orally administered AmB have been reported, however, and these are described here including the drug's non-antifungal activity.

Materials and Method

Animals:

Male Balb/c mice (age 6 to 8 weeks old) were obtained from the National Institute of Preventive Medicine, Taiwan, ROC, and bred under specific pathogen free environmental conditions. They were housed in groups of five per cage and were fed and given water *ad libitum*.

Reagents:

AmB was reconstituted from an oral suspension (Fungizone, Squibb, Japan) with pyrogen free normal saline. Cyclophosphamide was purchased from Sigma Chemical Co., Ltd. and was dissolved in saline (20mg/ml).

Experimental therapy:

A highly virulent *C. albicans* ATCC 48130 (serotype A) strain was subcultured at 37°C on Sabouraud dextrose agar (Difco, Detroit, U.S.A.) for 18hr and 0.2ml of washed blastospores (5×10^6 cells/ml) was intravenously injected to the mice. AmB (10mg/kg) was subsequently orally administered at various times and the survival rate was noted. Balb/c mice rendered leukopenic by a modification of the procedure of Cryz *et al.*²⁾ were also used. Namely, an intraperitoneal injection of 200mg/kg of cyclophosphamide in 0.2ml of phosphate-buffered saline (pH7.4) was given 4 days before the infection with *C. albicans*.

Vaccinia virus (Lister strain) was grown in BHK-21 cells. The virus titer of the preparation was $10^{6.5}$ TCID₅₀/ml. For *in vivo* antiviral activity against vaccinia virus, groups of 10 mice were inoculated intravenously with 0.1 ml of the vaccinia virus preparations at a site about 3 cm from the tail base. Animals were then treated with cyclophosphamide on day -4, and AmB was orally administered once a day 1 to 3 times. The number of lesions on the tail was counted on day 7 after staining with 1% fluorescein 0.5% methylene blue staining¹⁵⁾.

IFN assay:

γ -IFN titer was determined¹⁴⁾ after oral AmB (10mg/kg) treatment on day 0 using a mouse γ -IFN-ELISA test kit (Genezyme Corporation, Cambridge, MA, U.S.A.).

Preparation and analyses of peripheral blood cells:

Mice were given an oral administration of AmB (10mg/kg) on day 0 and peripheral blood was drawn from the retroorbital plexus¹⁵⁾. The number of white blood cells and populations of monocytes, PMN and lymphocytes were determined microscopically following Giemsa staining.

NK cell activity:

NK-cell activity was assessed by a standard 4hr Cr⁵¹ release assay as described previously¹⁴⁾. Each group consisted of five mice. Cyclophosphamide was administered on day -4.

AmB (10mg/kg) was orally administered on day 0. The spleen cells were depleted of adherent cells by incubation in plastic dishes in RPMI-1640 medium with 5% FCS for 1 hr at 37°C in a 5% CO₂ atmosphere. The mouse lymphoma cell line YAC-1 was used as target cells. The following formula was used to calculate specific lysis of Cr⁵¹: specific lysis (%) = [(cpm of the test group - cpm of spontaneous release) / (cpm of the complete release - cpm of spontaneous release)] × 100.

Statistical analysis:

Statistical significance between control and test group was analyzed by Student's t-test.

Results

Effect of oral AmB therapy on *C. albicans* infection:

We first determined the optimal doses of oral AmB against systemic *C. albicans* infection in mice. Three graded doses (100, 10 and 1 mg/kg) of AmB were orally administered 6 hr after the infection. Ten mg/kg was found to be the most effective and dose-response relationships between the drug and the survival rate were noted (data not shown). To determine the optimal treatment regimen, AmB at a concentration of 10mg/kg was orally administered at various times after infection and the survival rates were determined. The

cumulative mortalities of the mice are shown in Fig. 1. Most non-treated control mice died between days 2 and 4 after infection. The most effective dosing time was found to be at 6 hr and day 1 after the infection, and 90 % of the animals survived throughout the observation period. Treatment on day 2 was less effective and achieved a 60% survival rate, while the survival rates of animals treated on days 3 and 4 were 40% on day 12. Both treatments were thus slightly less effective than that made on day 2. These results indicated that oral AmB treatment is most effective when given within 1 day after infection.

We also tested whether such effects could be seen in immunosuppressed mice. Mice were treated intraperitoneally with the cyclophosphamide (200mg/kg) on day -4. AmB was administered orally 6 hr after infection of *C. albicans*. As shown in Fig. 2, the cyclophosphamide treated mice died much earlier than did control mice. However, it was found that oral AmB is effective even in immunosuppressed mice.

Effect of oral AmB on vaccinia virus infection:

All mice were treated with cyclophosphamide on day -4. Following intravenous inoculation of vaccinia virus on day 0, discrete dermal vesicles began to appear on the tail on day 5.

Fig. 1. Anticandidal activity of oral amphotericin B. Amphotericin B was orally administered at various times after *C. albicans* infection.

Fig. 2. Anticandidal activity of oral amphotericin B in immunosuppressed mice. Cyclophosphamide and amphotericin B were administered on day -4 and on day 1, respectively.

Fig. 3. Effect of oral amphotericin B on vaccinia virus infection in mice. Cyclophosphamide treated mice were considered as control.

*: statistical difference from control value at $p < 0.01$.

The number of lesions caused by the virus reached maximum on day 7 and the average number was about 30. When AmB (10mg/kg) was orally administered on day 0, the number of lesions was much lower (Fig. 3). The inhibition was time-dependent and more activity was apparent several days after AmB treatment when it reached 60%.

Kinetics of cell population:

We also analyzed total white blood cell number and population in the peripheral blood

after AmB administration to mice. As shown in Table 1, the number of white blood cells gradually decreased after oral AmB treatment and reached the lowest level on day 7, then returned to normal on day 14. The time course of cell population in peripheral blood after oral AmB treatment is also shown in Table 1. Throughout the observation period, the ratio of monocytes was constant, although the ratio of PMN gradually increased with time with the maximum observed on day 7; on day 14

Table 1. Effect of orally administered amphotericin B on the white blood cell numbers and ratio of monocytes, lymphocytes and polymorphonuclear leukocytes

Days	WBC number (Mean $\pm$ SD)	Monocytes (%)	Lymphocytes (%)	PMN (%) ^{a)}
0	6300 $\pm$ 125	1	75	24
2	5000 $\pm$ 98	2	66	33
5	4000 $\pm$ 100	1	56	43
7	3500 $\pm$ 88 ^{b)}	2	35	63
10	4100 $\pm$ 150	1	77	22
14	6350 $\pm$ 180	1	74	25

^{a)} : means of two experiments

SD: standard deviation

^{b)} : statistically different from 0 day at $p < 0.01$

Fig. 4. Effect of oral amphotericin B on NK-cell activity. 2 D and 5 D indicate the treatment regimens: (2 D, 2 days' oral treatment, 5 D: 5 days' oral treatment).

*: statistical difference from non-treated and cyclophosphamide treated group at $p < 0.01$.

the ratio returned to normal. Lymphocyte ratio gradually decreased after oral AmB and reached the lowest level on day 7, then rebounded on day 10.

Effect of oral AmB on NK-cell activity:

Effect of oral AmB on the NK-cell activity was determined by radioactivity release assay from target YAC-1 cells. The percent lysis of YAC-1 cells by NK-cells from normal mice

was about 7 % (Fig. 4). Although NK-cell activity of cyclophosphamide (on day -4) treated mice was slightly less than that of non-treated control, the difference was not significant. Oral AmB, on the other hand, increased NK-cell activity in these mice; much higher activity was observed on day 5 than on day 2. Thus, oral AmB did enhance NK-cell activity in the immunosuppressed mice.

Fig. 5. Effect of oral amphotericin B on the production of γ -IFN.

*: statistical difference from control value at $p < 0.01$.

Effect of oral AmB on γ -IFN production:

We also tested the production of γ -IFN in serum after oral AmB administration to mice. As shown in Fig. 5, when AmB was orally administered (on day 0), increased production of the IFN titer was confirmed. This production increased with time and 3 units of IFN activity were observed on day 5.

Discussion

Oral AmB therapy was initiated when the drug was first discovered, but the lower gastrointestinal absorption rate delayed further development of this therapy against various fungal infections¹⁶⁾. With the progress of research on fungal infectious mechanisms, the role of gastrointestinal tract as the first barrier against *C. albicans* infection was clarified¹⁷⁾. The use of AmB to prevent *C. albicans* infection from the gastrointestinal tract was begun, and its action as a preventive drug has benefitted immunocompromised patients and those who have undergone antitumor chemotherapy¹¹⁾. The number of cases in which its use has been successful has been increasing¹⁶⁾. In this study, we confirmed that oral AmB can enhance antiviral as well as antifungal resistance in mice. Enhancement of the former is believed due to the potentiation of non-specific activity, because AmB is known to

have no direct antiviral activity. There is, therefore, a possibility that oral AmB therapy plays some role in resistance to various infections by increasing the host's immunomodulating activity. Our preliminary data indicated that when 10 mg/kg of AmB was administered orally, its maximum serum concentration was around 0.1 to 0.2 μ g/ml. Therefore, it may be reasonable to assume that most of the anti-candida activity by oral AmB is due to the direct antifungal effect of AmB. However, AmB has been reported to be an inducer of TNF, and it was also reported that *C. albicans* cells stimulate the release of TNF from PMN^{18,19)}. Further detailed studies on the role of TNF in *C. albicans* infection appear interesting.

Schafnner et al.²⁰⁾ recently reported that AmB is active against human immunodeficiency virus type 1 *in vitro*. Although the detailed mechanism of this antiviral activity has not yet been elucidated, the following has been postulated: [i] inhibition of virus replication in cells, [ii] selective toxicity to virus infected cells, and [iii] increase in host mediated immunity. Since in the present experiment, antiviral activity was confirmed by the oral administration of AmB, further studies on the mechanism governing increased activity are of interest.

Our results also indicated that oral AmB

augmented resistance to systemic *C. albicans* infection even in immunocompromised mice. Orally administered, the drug's toxicity was clearly reduced (data not shown), and the lower toxicity is considered due to its lower rate of absorption from the gastrointestinal tract. Regardless of whether or not this assumption is correct, it would be valuable to learn whether such stimulated immunomodulatory activity of AmB can be obtained in immunocompromised patients.

AmB has been reported to be a potent murine polyclonal B cell activator^{5,11)} and is known to stimulate macrophage bacterial and tumoricidal activities⁵⁾. It also reportedly stimulated the phagocytosis of PMN and the production of cytokines such as TNF¹⁹⁾. To our knowledge, however, this is the first report indicating that oral AmB stimulates the immunomodulating actions like production of γ -IFN and NK-cell activities. Opportunistic fungal infections, like latent virus or bacterial infections, have a tendency to particularly be stimulated in immunocompromised patients and occasionally result in very severe disease. Attempts have been made in Japan to enhance overall host resistance to infections in these patients by restoring impaired defense mechanisms using whole bacteria (BCG, picivani), lipopolysaccharide derivatives (LPS), polysaccharide (lentinan and crestin) and synthetic compounds (MDP, levamisole, azimexon)²¹⁻²⁸⁾.

AmB derivatives without a free amino group have been found to be less toxic than original AmB²⁾ and, interestingly, they reportedly had the same immunomodulating activities as the parent AmB. Various N-substituted derivatives of AmB actually having increased stimulatory activities have been prepared: N-(1-deoxy-D-fructose-1-yl) AmB was a potent immunostimulator with lower toxicity⁴⁾. These data thus suggest another means of preparing new AmB derivatives which are less toxic and show potent antiviral activity in oral therapy.

One of the present authors^{28,29)} found that both γ -IFN and activated macrophages play a key role in the manifestation of *in vivo* anti-

candida activity in most biological response modifiers (BRMs) mentioned above. Therefore, it may be reasonable to assume that γ -IFN produced by oral AmB has an important part as one of the factors in protecting mice against both candidiasis and vaccinia virus infection.

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