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Efficacy of amphotericin B and fluconazole in the murine mycotic (*Candida albicans)* mastitis model

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Introduction

The increasing threat of fungal infections in immuno-compromised patients such as those suffering from AIDS and cancer, and people undergoing organ transplantation have increased the need for the development of new antifungal compounds. In order to test such compounds the use of discriminative and reliable animal models is required.

The current information in the literature about comparisons of antifungal compounds is difficult to assess due to the varied experimental infection models and treatment regimens that have been used. The variety of animal models used include *e.g.* mice, rats, rabbits, and guinea pigs (*Fidel et al.*, 1997; *Jafari et al,* 1994; *Jensen,*1994; *Jensen et al,* 1991; *Sanati et al.*, 1997; *Witt et al,* 1993; *Yotsuji et al.*, 1997). The animals were challenged orally, subcutaneously, intravenously, intraperitoneally or intramuscularly.

The immune status of the animals used has frequently been modulated e.g. some are estrogen-dependent (*Fidel et al.*, 1997), others are immunocompetent (*Hata et al.*, 1996b; *Nakajima et al.*, 1995) while the majority are immunosuppressed (*Sanati et al.*, 1997; *Hata et al.*, 1996a; *Graybill et al.*, 1995; *Karyotakis et al.*, 1995; *Kullberg et al.*, 1992). The dose of infection used in different studies vary from a few cells of fungi to hundreds of millions of cells. Drug efficacy has been determined in different ways, *e.g.* per cent survival of infected animals (*Hata et al.*,1996b), histopathological screening of different tissues and fungal burden of different organs such as kidneys (*Sanati et al.*, 1997; *Graybill et al*, 1995; *Sugar et al.*, 1995; *Kullberg et al.*, 1992), spleen (*Graybill et al*, 1995: *Kullberg et al.*, 1992), liver (*Kullberg et al.*, 1992), and vagina (*Fidel et al.*, 1997).

While there is still no substitute for animal models of infection, there are widespread research and development activities to refine animal models in studies of infection to introduce earlier end-points (*Collins*, 1998). We have recently reported the sensitivity of the murine mycotic mastitis model (*Guhad et al,* 1995) and used it for investigation of virulence factors in genetically modified *C. albicans* strains (*Guhad et al.,* 1998a; *Guhad et al.*, 1998b). The aim of this study is to compare the effect of intraperitoneal treatment of the two antifungal drugs amphotericin B and fluconazole in the murine mycotic mastitis model.

Materials and Methods

Animals

Male and female BALB/cJ mice, 6 weeks old (M&B, Ry, Denmark) were allowed to acclimatize for two weeks before mating. The mice were housed in groups of four females and one male in size III Macrolone cages (Techniplast, Buguggiate (Varese), Italy) and were individually transferred to size II single cages in a laminar flow cabinet (Scanbur, Køge, Denmark) after confirmation of mating (vaginal plug). The cabinets had negative pressure and the temperature was maintained at 20°C +/- 1. The mice were given pellet diet (R36®, Lactamin AB, Stockholm, Sweden) and tap water *ad libitum*. Light: dark ratio and relative humidity

were maintained at 12 h:12 h and at 50-60%, respectively.

Antifungal drugs

Fluconazole (FLU) (Diflucan®, 2 mg/ml) was obtained from Pfizer, Amboise, France, and stored at 4 °C. Amphotericin B (AmB) (Fungizone®, Pfizer) was obtained as 50 mg powder, dissolved in 10 ml sterile water (Pharmacia, Uppsala, Sweden), divided into 10 parts of 5 mg/ml stock solutions and stored at -20 °C until use. A fresh solution of 1 mg/ml was prepared for use every two days and stored at +4 °C.

Fungal inocula

C. albicans (wild-type strain SC5314), used in our previous studies (*Guhad et al.*, 1998a; *Guhad et al.*, 1998b), were grown on complete culture media, yeast extract/peptone/dextrose (YPD, prepared at the Yeast Genetics Lab, BMC, Uppsala) and were incubated at 36 °C for 48 hours. The cultures were harvested and stock suspensions made in sterile water (Pharmacia AB, Uppsala, Sweden). Decimal dilution of the suspensions were made by adding 0.1 ml of the stock suspension to 0.9 ml of sterile water. One hundred µl suspensions from three different dilutions were incubated on YPD agar plates for 48 hours at 36 °C in order to determine the number of colony forming units per ml (CFU/ml) of the stock suspension, which was stored at +3 °C, until inoculated.

The mycotic mastitis model

Twenty-two females, at the fifth day of lactation, were anesthetized with a mixture of 50 mg ketamine hydrochloride (Ketalar®, Park-Davis Scandinavia AB, Solna, Sweden), 5 mg xylazine (Rompun®vet, Bayer AG, Leverkusen, Germany) and 4.75 ml water for injection (Pharmacia), at a dose of 0.1 ml/10 g body weight administered intraperitoneally. Animals were inoculated through the teat duct at day five postpartum with 50 µl fungal suspension containing 2.5×10^9 *C. albicans* blastospores into the two mammary glands R4 and L4 (L = left and R = right, where 1 is the most anterior and 5 is the most posterior) as described previously (*Guhad et al.*, 1995). Twelve of the infected mice were treated with AmB (4 mg/kg/day) *(Morrison and Stevens*, 1990) and FLU (24 mg/kg twice a day) (*Fidel et al.*, 1997) intraperitoneally (6 animals each) starting immediately after infection. Treatment was continued daily for 4 days before the animals were anaesthetized again and euthanized by intracardiac exsanguination followed by cervical dislocation. Ten animals were treated with sterile water intraperitoneally and acted as controls (half of these were injected twice a day while the other half were injected once a day). These were also anaesthetised, exsanguinated and euthanized by cervical dislocation after 4 days. At death, L4 and R4 glands were removed and examined macroscopically. The brain, liver, spleen, kidneys, uterus, heart, lungs and gastrointestinal tract were removed and, together with R4, fixed in 10% neutral buffered formalin. Following dehydration through graded alcohol concentrations, organs were embedded in paraffin wax, sectioned at 5 µm and stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS), Van Gieson stain and selected sections by Grocott's methenamine-silver (GMS) method. L4 was homogenised with a tissue homogenizer (Tissue Tearor®, model 985-370, Biospec Products, Inc., USA) and the homogenate made up to 2 ml with sterile water. The homogenate was serially diluted tenfold for a final dilution of 10^{-8}. Of each dilution, 100 µl was transferring onto Sabouraud dextrose agar plates (prepared at the Bacteriology Laboratory, Academic Hospital, Uppsala). The plates were incubated for 48 h at 37 °C. By counting the number of viable colonies on each plate the mammary gland fungal density was determined as log CFU/g of tissue.

Statistics

Student's t-test was used to compare each infected and treated group with the infected control group. $P < 0.05$ was considered significant.

Results

Lesions and fungal elements were, at different levels, observed in all inoculated glands of mice in

all three groups (untreated control, AmB and FLU). Six of the control animals had severe lesions not seen in the treatment groups *i.e.*, widespread necroses of the mammary glands in which fungi, in the form of blastospores but especially as hyphae (pseudo-hyphae and true hyphae) were disseminated (Fig. 1). In addition, five of these animals had the following systemic candidiasis: i) one animal had mycotic encephalitis, mycotic myocarditis and mycotic nephritis (Fig. 2) ii) two animals had mycotic encephalitis (Fig. 3) and iii) two animals had necrotizing myocarditis even though fungi were not seen.

The general picture seen in the remaining four control animals and all treated animals was a moderate infiltration by neutrophils and macrophages in and around alveoli and excretory ducts (Fig. 4). Moreover, in some glands, single abscesses were also observed in these mice. Fungi in the form of yeast cells (blastospores) predominated in all these animals.

Quantitative cultures from mammary gland homogenates

FLU treatment did not cause any reduction in the CFU/g of *C. albicans* in the mammary gland compared to the untreated controls. By contrast, treatment with AmB caused significant reductions in the *C. albicans* density in the mammary gland compared with untreated controls ($p < 0.05$) and FLU treated animals ($p < 0.05$) (Fig. 5).

Discussion

In the initial studies that compared oral FLU and oral AmB in systemic *Candida* infections developing subsequent to oral challenge of immunosuppressed mice, 90% of FLU treated mice had culture negative faeces after 3 days of treatment compared to 62% for AmB (*Troke et al.*, 1985). The efficacy of intraperitoneal FLU was compared with that of AmB in a rabbit endocarditis model (*Candida tropicalis* and *C. parapsilosis*) (*Witt et al.*, 1993). Even though the two drugs were equally effective, AmB was more rapidly fungicidal than FLU (*Witt et al.*, 1993). A rabbit model of meningitis was used to investigate the effect of antifungal therapy on the course of *C. albicans* central nervous system infection and inflammation. Again, AmB was more effective than FLU as seen from quantitative cultures of cerebrospinal fluid (*Jafari et al.*, 1994). In the present experiment, we confirm that intraperitoneal AmB is more effective than intraperitoneal FLU in preventing mycotic mastitis when efficacy was assessed from quantitative cultures of mammary gland homogenates.

It is important to consider the immune status of the animal model to be used when comparing the efficacy of antifungal compounds. However, in comparative studies of FLU and AmB in immunocompromised animals, AmB has also consistently been found to be superior to FLU. For example, AmB was superior to FLU in a mouse model of candidiasis immunosuppressed with fluorouracil (*Atkinson et al.*, 1995). In another study, AmB was found to be more effective than FLU in immunocompetent mice but not in neutropenic mice as assessed by CFU cultured from kidneys (*van't Wout et al.*, 1989). In addition, when the efficacy of AmB and FLU were compared in immunocompromised and leucopenic mice models of systemic candidiasis, only treatment with high doses of liposomal AmB was found to be effective in significantly reducing the CFU numbers in kidneys and other organs in leucopenic mice (*van Etten et al.*, 1993).

Oral administration is the recommended method of dosing FLU (*Bennet and Grant,* 1990). However, it has been demonstrated that intraperitoneal administration of FLU was as effective as oral administration against systemic murine candidiasis (*Kawasaki et al.*, 1991). A dose of 20 mg/kg was as effective as at 80 mg/kg when FLU was administered orally in a rat model of systemic candidiasis (*Fisher et al.*, 1989).

Employing the murine mycotic mastitis model, two methods were used to compare the efficacy of AmB and FLU: histopathological evaluation and quantitative cultures from mammary gland homogenates. The histopathological data showed no clear-cut differences between animals treated with AmB and FLU while quantitative cultures from the mammary gland homogenates showed that treatment with AmB caused significant reductions in the *C. albicans* density in the

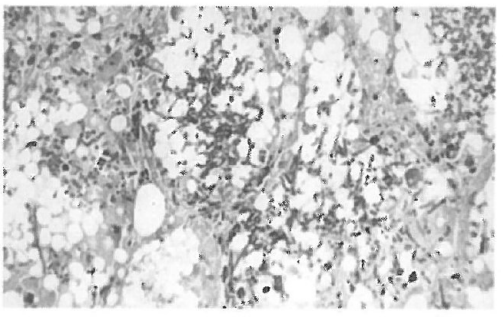

Fig. 1. Murine mammary gland four days after inoculation of 2.5 × 10^9 *C. albicans* blastospores, untreated control group. Widespread necrosis and a predominance of the hyphal forms of *C. albicans* are seen. PAS, obj. × 40.

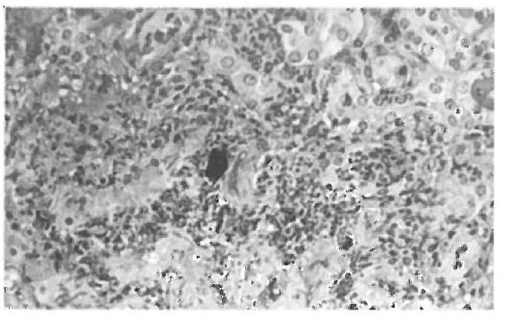

Fig. 2. Kidneys from a mouse four days after mammary inoculation of 2.5 × 10^9 *C. albicans* blastospores, untreated control group. Suppurative embolic nephritis has developed following dissemination from the inoculated mammary gland. Arrow: *C. albicans* hyphae. PAS, obj. × 40.

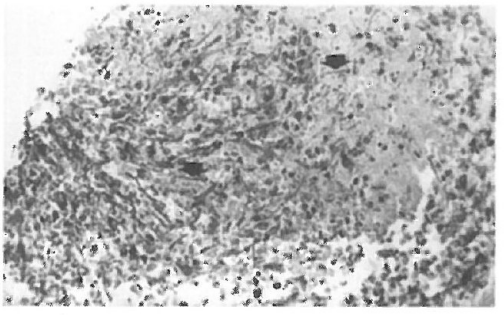

Fig. 3. Brain from a mouse four days after mammary inoculation of 2.5 × 10^9 *C. albicans* blastospores, untreated control group. Necrotising embolic encephalitis has developed following dissemination from the inoculated mammary gland. The lesion is heavily infiltrated by hyphae, the arrows point at some of them. PAS, obj. × 40.

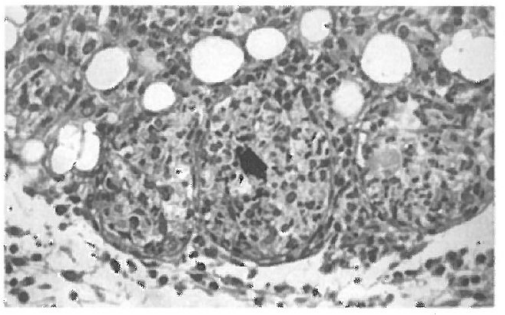

Fig. 4. Murine mammary gland four days after mammary inoculation of 2.5 × 10^9 *C. albicans* blastospores, fluconazole treatment group. Accumulation of macrophages and neutrophils in and between alveoli. Within an alveolus a few blastospores are seen (arrow). PAS, obj. × 40.

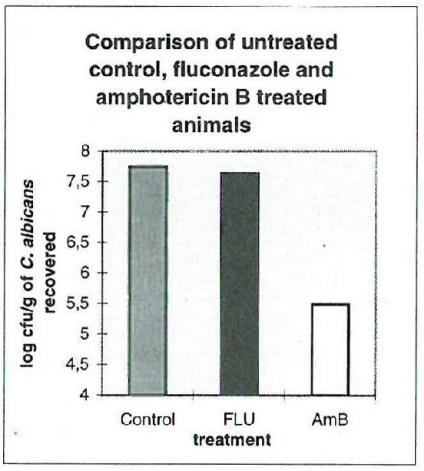

Fig. 5. Treatment with fluconazole (FLU) and amphotericin B (AmB) in experimental murine mastitis induced by *C. albicans* wild-type strain SC5314. FLU was given at a dose of 24 mg/kg administered intraperitoneally at the time of infection and twice daily for 4 days. AmB was given at a dose of 4 mg/kg/day administered intraperitoneally at the time of infection and once daily for 4 days. There was no significant difference between FLU treated and untreated controls. However, there was significant reduction of CFU in AmB treated animals compared to the other two groups (see results and discussion for details). (N = 10 for control and 6 for AmB and FLU).

mammary gland compared with untreated controls and FLU treated animals (see results). This may be explained by the fact that while AmB is fungicidal, FLU is fungistatic, (see *Brajtburg and Bolard*, 1996, for review; *Bennet and Grant,* 1990; *Klepser et al.*, 1997; *Witt et al.*, 1993). Taking the results of this study into perspective, it is hypothesized that if quantitative cultures are compared from organ homogenates of animals treated with the two drugs, more fungi may grow from FLU treated animals than from AmB treated ones while the pathological picture would show that both drugs are equally effective. This demonstrates the importance of using both histopathology and quantitative cultures of organ homogenates in *in vivo* drug efficacy studies.

The post-mortem examination confirmed systemic spread of the *C. albicans* from the mammary glands. This is our first observation of systemic dissemination of the fungi from the mammary glands as the primary site of infection. However, previous infections were performed using fewer yeast cells than inoculated in this study. Because of the high number of yeast cells, the animals' defense mechanisms might possibly have been overwhelmed. From two animals which successfully eliminated infections after 21 days, and from the trend in the fungal burden reducing with time in untreated control animals (*Guhad et al.*, 1998b), it is likely that mycotic mastitis may not kill the animals even at high inoculation doses. These results demonstrate that the murine mycotic mastitis model is a welcome addition to other animal models of fungal infections which have death as the experimental end-point.

Summary

The majority of animal models in antifungal tests use systemic infection and mortality and survival of infected animals as the experimental end-point. We developed a murine model of localised candidiasis (murine mycotic mastitis) and assessed its effectiveness through infection with *Candida albicans* followed by intraperitoneal administration of the antifungal drugs flunazole (FLU) and amphotericin B (AmB). Lactating BALB/cJ mice at day 5 post partum were inoculated (two glands) with a high dose of a human pathogenic *C. albicans* wild-type strain SC5314. Animals were treated immediately after infection with either FLU or AmB intraperitoneally for 4 days and euthanized by intracardiac exsanguination and cervical dislocation following anaesthesia with a mixture of Ketamine and Xylazine. One infected gland was fixed in formalin and examined histopathologically and the other was homogenised for quantitative fungal cultures. There were severe changes in the untreated control animals (some animals had systemic candidiasis) compared to the treatment groups which had milder lesions. Fungal burden, determined as log

[colony forming units (CFU)/g of mammary gland tissue], was similar in the untreated control group (n = 10) and FLU treated group (n = 6). However, there was significantly lower CFU/g in the mammary glands in AmB treated animals (n = 6) compared to both control and FLU treated animals ($p < 0.05$). The results indicate that AmB is more effective in prevention of murine mycotic mastitis than FLU and that the murine mycotic mastitis model may be an attractive animal model for antifungal chemotherapy studies.

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