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THE EFFECT OF FLUCONAZOLE AND AMPHOTERICIN B ON MACROPHAGE FUNCTIONS

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Abstract: Background: Different antimicrobial drugs inhibit functions of the microbial cells but, unfortunately, some of these drugs can also affect the host cells, including macrophages. Since these cells represent the baseline defense against microbial agents, it is important that they are fully activated.

Materials and Methods: In the present study we investigated the effect of amphotericin B and fluconazole on the functions of peritoneal macrophages from Balb/c mice treated with these antimycotics. For each antimycotic the therapeutic dose used in clinical practice (calculated on the mouse body weight) and two-fold higher doses were administered intraperitoneally once a day for three consecutive days. The control group was treated with saline in the same way. Four different tests, candidacidal assay, nitroblue tetrazolium test, adherence capability and phagocytic capability, were used to determine macrophage functions.

Results: Our results confirmed positive effect of high dose amphotericin B on phagocytic capability ($31.00 \pm 4.46\%$), metabolic changes ($27.93 \pm 6.63\%$) and adherence capacity ($59.24 \pm 8.67\%$). Lower doses of drug amphotericin B (2 mg/kg) significantly increased the adherence index of macrophages ($71.99 \pm 5.55\%$) and intensity of the NBT intracellular reduction ($30.20 \pm 3.83\%$). Maximum dose of fluconazole expressed significantly higher phagocytic index ($30.77 \pm 2.17\%$), metabolic changes ($24.00 \pm 4.07\%$) and candidacidal activities ($27.73 \pm 6.73\%$), while lower doses of fluconazole (15 mg/kg) significantly increased the adherence index of macrophages ($75.58 \pm 5.47\%$) as well as the phagocytic index ($29.23 \pm 2.40\%$). Other results were similar as in control group.

Conclusion: Positive immunomodulatory effects of fluconazole and amphotericin B may be clinically relevant especially in compromised patients who are predisposed to opportunistic fungal infections and re-

quire a long-term antimycotic therapy. Synergistic action of macrophages and antimycotics can influence the course of disorders related to immune suppression.

Key words: Amphotericin B, fluconazole, macrophage functions.

INTRODUCTION

Fungal infections pose a growing medical problem because of the markedly increased numbers of immunologically compromised patients due to human immunodeficiency virus, cancer chemotherapy, bone marrow transplantation or other debilitating conditions (1, 2). These infections are commonly associated with significant morbidity and mortality. Many of these infections are caused by, in normal conditions, harmless fungi. The most common infection associated with this group of patients is candidiasis (3–5). Resistance to fungal infections is based on cell-mediated immunity. Macrophages play a key role in the host immune system. They act as a barrier against microbial invasion and dissemination. Their ability to be mobilized into infectious sites from bone marrow and blood, as well as their competence for phagocytosis and killing of microorganisms is often decisive in determining the outcome of infection (1, 6–8). Once activated, they act directly by destroying bacteria, parasites, viruses, fungi and tumor cells with reactive oxidants and hydrolytic enzymes, or indirectly by releasing specific mediators such as interleukin-1, tumor necrosis factor-alpha and others which can regulate other cells' activities. They are also responsible for processing the antigens and presenting the digested peptides to lymphocytes, while playing the leading role in damage repair (9–11). However, chemotherapeutic agents, corticosteroids, and radiation, disrupt these defense mechanisms. If patients are to survive infections associated with neutropenia

and other compromises in host defense due to use of these therapies, it is important to reverse or at least lessen immunosuppression in these patients (2, 4).

Fungal infections are treated with antifungal drugs. Antifungal agents which are available for treatment of these infections are the polyenes, the azoles and the echinocandins (12–14). Polyene antifungal drug amphotericin B is a standard therapy for invasive *Candida* infections, but high frequency of renal toxicity limits its use. Recently, newer lipid formulations of amphotericin B with less adverse effects and activities similar to that of standard amphotericin B have become available (15). The triazole fluconazole is a leading drug to prevent and to treat candidal infections. This drug displays predictable pharmacokinetics and an excellent tolerance profile in all groups of patients (12–14, 16).

Different antimicrobial drugs inhibit functions of the microbial cells but, unfortunately, some of these drugs can also affect the host cells, including macrophages. Since these cells represent the baseline of defense against microbial agents, it is important that these cells are fully activated (14, 15).

The aim of this study was to investigate the effect of amphotericin B and fluconazole on macrophage functions (adherence, phagocytosis, intracellular killing and digestion of the drug) on experimental mouse model.

MATERIALS AND METHODS

Animals. Female BALB/c mice, 9 weeks old (obtained from the Medical Faculty, University of Rijeka) were used in this study. They were randomly divided in five groups each containing five animals. The animals were kept in plastic cages and were given standard laboratory rodent food and water ad libitum. The Ethical Committee of the Medical Faculty University of Rijeka approved the study and all procedures using mice.

Antimycotics. Two antifungal drugs Amphotericin-B (Amphotericin-B, Bristol-Myers Squibb, Woerden, The Netherlands) and fluconazole (Diflucan, Pfizer, Ambroise, France) were tested.

For each antimycotic, the therapeutic dose used in clinical practice (calculated on the mouse body weight) and twofold higher dose were administered intraperitoneally once a day, for three consecutive days. Standard preparation of amphotericin B was given in doses of 2 mg/kg/day and 4 mg/kg/day while fluconazole was administered in doses of 15 and 30 mg/kg/day. Control group was treated with saline (0,85% NaCl) in the same way.

Peritoneal macrophages. 24 h after receiving the last dose of the antimycotic, the animals were sacrificed by cervical dislocation. Peritoneal macrophages

were obtained after the peritoneal cavity was washed with 4 ml of RPMI medium (Institute of Immunology, Zagreb, Croatia). The injected medium containing peritoneal cells was slowly aspirated. Quantitative analysis of the cell suspension was performed immediately after collection in a Neubauer chamber.

Macrophage adherence assay. Two plastic test tubes (16 x 160 mm) were filled with 0.2 ml of the same cell suspension. The tubes were incubated for 2 hours in a horizontal position at 37 °C in a humidified atmosphere. After gentle removal, the number of nonadherent macrophages was counted in a Neubauer chamber. The results were expressed as the adherence index (AI).

Phagocytic assay. Two plastic rings, 2 mm high and 16 mm in diameter, were stuck with paraffin onto microscopic slides. The wells were filled with the cell suspension containing peritoneal macrophages in RPMI medium and heat killed *Candida albicans*. The wells were covered with cover slips and incubated at 37 °C for 30 minutes in a humid atmosphere. After incubation the cover slips were removed and the liquid was decanted. The plastic rings were removed and the slides were dried, stained with Wright's stain and examined with immersion. The total number of macrophages and the percentage of ingested *Candida albicans* were counted. More than 100 cells per each well were counted. The results were expressed as phagocytic index (PI).

Candidacidal assay. Plastic tubes were filled with 0.1 ml of cell suspension and 0.005 ml suspension of viable *Candida albicans*. The tubes were then incubated at 37 °C for one hour in a humid atmosphere. After incubation, the suspension was treated with cold distilled water for 15 minutes to cause cells lysis. Light microscopic examination showed blue killed *Candida* while the alive ones remained were colorless. Free *Candida albicans* was colored with Tripian blue. More than 300 *Candida* were counted and the results were expressed as percentage of killed *Candida albicans* at 100 microorganisms.

Nitroblue tetrazolium (NBT)-dye assay. NBT-assay expresses metabolic changes in the macrophages after phagocytosis foreign particles. Metabolic changes include reduction of the colorless NBT in the cytoplasm of macrophages in black color formazan. Plastic tubes were filled with 200 µl of the cell suspension and 200 µl of the nitroblue tetrazolium. The tubes were then incubated at 35 °C for 15 minutes in a humid atmosphere and for 15 minutes at room temperature. The suspension was then placed on microscopic slides and dried. Immediately after, dried slides were stained with Wright's stain and examined with immersion. The total number of macrophages and the percentage of formazan positive cells with blue or black inclusions were determined. More than 100 cells per slides were coun-

ted. Results were expressed as percentage of NBT positive cells.

Comparison between groups was performed by Student t-test. Results are expressed as mean and standard deviation. The level of significance was set at 0.05.

All animal experiments were carried out in accordance with Policies and Guidelines for the Care and Use Laboratory Animals. All effort was made to minimize animal suffering.

RESULTS

The effect of fluconazole on macrophage functions

Our results showed that lower dose of fluconazole (15 mg/kg) significantly increased the adherence index of macrophages ($75.58 \pm 5.47\%$) as well as the phagocytic index ($29.23 \pm 2.40\%$), but did not demonstrate any major alteration in *C. albicans* killing assay and NBT intracellular reduction.

Macrophages treated with maximum dose (30 mg/kg) of fluconazole had significantly higher phagocytic index ($30.77 \pm 2.17\%$), metabolic changes ($24.00 \pm 4.07\%$) and candidacidal activities ($27.73 \pm 6.73\%$) comparing to control group ($p \leq 0.05$).

The effect of amphotericin B on macrophage functions

Lower doses of drug amphotericin B (2 mg/kg) significantly increased the adherence index of macrophages ($71.99 \pm 5.55\%$) and intensity of the NBT intracellular reduction ($30.20 \pm 3.83\%$), while other results were similar to the control group.

Treatment of macrophages with higher dose of amphotericin B (4 mg/kg) showed that phagocytic capability ($31.00 \pm 4.46\%$), metabolic changes ($27.93 \pm 6.63\%$) and adherence capacity ($59.24 \pm 8.67\%$) were increased significantly.

Results are summarized in Table 1.

DISCUSSION

The host response to fungal infections requires co-actions of immune system and antifungal drugs to effectively clear the microorganisms. Antifungal drugs may have broad immunomodulatory properties. Cytokines, effector cells, and antifungals appear to work synergistically to oppose fungal growth. Positive immunomodulatory effects of antifungal drugs may be clinically relevant, especially in compromised patients who are predisposed to opportunistic fungal infections and require a long-term antimycotic therapy (17).

In this study, the most commonly used antifungal drugs were studied for their impact on different macrophage functions. Several studies examined the influence of fluconazole on macrophage activity against *Candida*. It appears that the presence of macrophages provides synergistic activity with fluconazole against *Candida* isolates (14). Garcha presented the data that indicate that the combination of two fungistatic agents, fluconazole and macrophages, can synergize for significant anticandidal activity, especially against fluconazole-susceptible isolates (18). In contrast, investigation presented by Yamaguchi et al. suggested that fluconazole had no immunological effect (19). Results of our investigation confirmed synergic activity of as a stimulant on phagocytosis, oxidative burst and microbicidal activity using both fluconazole and macrophages in immune response. We found that fluconazole acted maximum dose and lower dose of fluconazole, although better results were obtained with maximum dose. Fidan and his co-workers concluded in their research that fluconazole has an immunomodulatory effect connected with the presence of different cytokines (17).

It is well known that Amphotericin B has an immunostimulatory effect to macrophages and enhances their function (17, 19). It also has a stimulatory effect on macrophages to produce cytokine tumor necrosis factor alpha (TNF- α) which may be responsible for the immunomodulatory effects of Amphotericin B (20).

Table 1. Adherence index, Phagocytic index, NBT-dye assay and Candidacidal assay for amphotericin B and fluconazole

	Adherence index (AI)	Phagocytic index (PI)	NBT-dye assay (%)	Candidacidal assay (%)
Control	62.21 ± 14.26	22.95 ± 3.55	20.93 ± 1.71	14.10 ± 6.23
Fluconazole (15 mg/kg/day)	$75.58 \pm 5.47^*$	$29.23 \pm 2.40^*$	19.20 ± 3.52	11.40 ± 4.40
Fluconazole (30 mg/kg/day)	68.84 ± 8.68	$30.77 \pm 2.17^*$	$24.00 \pm 4.07^*$	$27.73 \pm 6.73^*$
AmphotericinB (2 mg/kg/day)	$71.99 \pm 5.55^*$	22.67 ± 3.32	$30.20 \pm 3.83^*$	11.13 ± 4.51
AmphotericinB (4 mg/kg/day)	$59.24 \pm 8.67^*$	$31.00 \pm 4.46^*$	$27.93 \pm 6.63^*$	12.60 ± 4.65

Values are expressed as mean \pm SD from each group. Student t-test was used to compare data among the groups.

* Significant increased functions comparing to control group at level $p < 0.05$.

We confirmed that both doses of amphotericin B had effect on macrophages, increasing significantly their phagocytic ability and oxidative burst. Our results are also in correlation with the results obtained by Yamaguchi who concluded that amphotericin B has a profound effect on macrophages in vitro and in vivo, and that this action is mediated by cytokines (19).

Antifungals used to treat infections, especially in immunocompromised patients, could have a potent antimicrobial effect to fungi and positive impact on host defense mechanisms such as macrophage functions.

CONCLUSION

It is preferable that drugs used have a slightly stimulatory, rather than an immunosuppressive effect on

the cells of the immune system. Since some drugs can suppress macrophage functions, it would be appropriate to determine susceptibility of microorganisms to a certain drug since even minor immunosuppressive effect can additionally complicate the course of the disease. Recent investigation suggested that macrophage functions are connected with activation of different cytokines but further studies should be carried out in order to understand better the mechanism by which target host cells react in response to immunomodulators.

Abbreviations:

AI — adherence index

NBT assay — Nitroblue tetrazolium dye assay

TNF- α — tumor necrosis factor alpha

Sažetak

UČINAK FLUKONAZOLA I AMFOTERICINA B NA FUNKCIJE MAKROFAGA

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Uvod: Različiti antimikrobni lekovi inhibiraju funkcije mikroorganizama, nažalost deluju i na ćelije domaćina, uključujući i makrofage. Obzirom da su makrofazi bazna linija odbrane organizma, potrebno je da budu u potpunosti aktivirani.

Materijali i metode: U istraživanju ispitivan je učinak flukonazola i amfotericina B na funkcije peritonealnih makrofaga Balb/c miševa. Za svaki antimikrobni lek korišćena je terapijska doza i dvostruko veća doza. Miševi su tretirani uzastopno tri dana. Kontrolna grupa miševa tretirana je s fiziološkim rastvorom identičnim postupkom. Da bi se odredila funkcija makrofaga korišćena su četiri testa: kandidacidni test, nitroblutetrazolijski test, sposobnost adherencije i fagocitna sposobnost.

Rezultati: rezultati su potvrdili pozitivan učinak amfotericina B na fagocitozu ($31.00 \pm 4.46\%$), metaboličke promene ($27.93 \pm 6.63\%$) i sposobnost adhe-

rencije ($59.24 \pm 8.67\%$). Niža doza amfotericina B (2 mg/kg) značajno povećava indeks adherencije makrofaga ($71.99 \pm 5.55\%$) i metaboličke funkcije ($30.20 \pm 3.83\%$). Viša doza flukonazola značajno povećava fagocitni indeks ($30.77 \pm 2.17\%$), metaboličke aktivnosti ($24.00 \pm 4.07\%$) i kandidacidne aktivnosti ($27.73 \pm 6.73\%$), dok niža doza flukonazola (15 mg/kg) značajno povećava indeks adherencije ($75.58 \pm 5.47\%$) i fagocitni indeks ($29.23 \pm 2.40\%$). Rezultati su slični u kontrolnoj grupi.

Zaključak: Pozitivan imunomodulatorni učinak flukonazola i amfotericina B može biti klinički značajan kod imunološki kompromitovanih bolesnika kod kojih postoji predispozicija razvoja oportunističkih gljivičnih infekcija. Sinergističan učinak makrofaga i antimikotika može uticati na razvoj i tok bolesti.

Glavne reči: Amfotericin B, flukonazol, makrofagne funkcije.

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