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In vitro lymphoproliferative response and cytokine production in mice with experimental disseminated candidiasis

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ABSTRACT

Objective(s): Systemic candidiasis is an infection of *Candida albicans* (*C. albicans*) causing disseminated disease and sepsis, invariably when host defenses are compromised. We investigated the histopathological changes as well as the lymphoproliferative responses and cytokine production of splenic cells after stimulation with Concanavalin A (Con A) and Pokeweed mitogen (PWM) in mice with disseminated candidiasis.

Materials and Methods: Lymphoproliferative responses were stimulated *in vitro* with Con A (1 µg/ml) and PWM (1 µg/ml) mitogens in Roswell Park Memorial Institute (RPMI) 1640 media, and the production of interferon (IFN)-γ and interleukin-4 (IL-4) in the supernatants was measured by enzyme-linked immunosorbent assay (ELISA).

Results: The results revealed that *C. albicans* organisms multiplied to a greater extent in the kidneys than in the liver and spleen of infected mice. The most predominant forms of *C. albicans* in different parts of the kidneys were yeast mixed with hyphal forms. Infected mice had a significantly increased proliferative response when splenocytes were stimulated with PWM (2.0±0.16) and Con A (1.9±0.19) (P<0.05). PWM and Con A-stimulated production of IFN-γ significantly tended to be higher in infected mice (PWM: 68.4±14.0 pg/ml; Con A: 53.7±17.3 pg/ml) when compared to controls (P<0.05). Stimulation with PWM and Con A showed no differences in IL-4 production between infected mice and controls.

Conclusion: These findings demonstrated a significant increase in both cell proliferation and IFN-γ secretion in supernatants of PWM and Con A- stimulated splenocyte cultures obtained from mice with disseminated candidiasis.

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Introduction

Candida infection became recognised as being of clinical importance over the past two to three decades due to a combination of rising numbers of patients with immunodeficiency illnesses and improvements in general life expectancies. The nature and extent of the impairment of the host defense influence the manifestation and severity of infection (1, 2). The innate and adaptive immune responses are required for development of resistance to disseminated candidiasis. Neutrophils and macrophages can clear the pathogen via phagocytosis, and macrophage activation also leads to the release of several key mediators such as pro-inflammatory cytokines, which are important for protecting the host against disseminated candidiasis (3, 4). In candidiasis, the initial handling of fungal pathogen by cells of the innate immune system plays a major role in determining CD4+ T helper (Th) development.

Th1 and Th2 CD4+ T-cells have been shown to influence the pathway of differentiation of CD4+ T-cell precursors (5). The different cytokine patterns lead to different functions, such as the modulation of anti-fungal effector activity and the regulation of various Th subsets development. Previous studies demonstrated that the development of protective Th1 responses in mice with disseminated candidiasis requires the coordinated functions of several cytokines such as, interferon (IFN)-γ (6), transforming growth factor (TGF)-β (7), interleukin (IL)-6 (8), tumor necrosis factor (TNF)-α (9) and IL-12 (10), in the relative absence of inhibitory Th2 cytokines, such as IL-4 and IL-10, which inhibit the development of Th1 responses (11). The neutralization of Th1 cytokines (IFN-γ and IL-12) in early infection results in immune responses towards the Th2 instead of Th1 cells, whereas neutralization of Th2 cytokines (IL-4 and IL-10) is responsible for a shift of Th2 towards the Th1 responses (12). Thus, Th1 mediates anti-*Candida albicans* host defense through the production of IFN-γ, which is required for optimal activation of phagocytes

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And for helping in the generation of a protective antibody response. Altogether, these data demonstrate that resistance or susceptibility to infections correlates with the levels of *Candida* growth in target organs, as well as with the type of Th cytokine production by specific $CD4^+$ T lymphocytes. It has been hypothesized that a defective Th1 response may be at least partially responsible for the persistence of *Candida* infection in patients with disseminated candidiasis. The aims of this study were to investigate the histopathological changes as well as the lymphoproliferative responses and cytokine production of splenic cells after stimulation with Concanavalin A (Con A) and Pokeweed mitogen (PWM) in mice with disseminated candidiasis.

Materials and Methods

Animal model

Female BALB/c mice, 21 days of age, were purchased from the Razi Institute, Karaj in Iran. Animal experiments were approved by the Animal Experimentation Ethics Committee of the University of Tehran, and were carried out in accordance with the National Health and Medical Research Council's Iranian Code of Practice for the Care and Use of Animals for Scientific Purposes. Mice were housed in standard cages, and provided with food and water *ad libitum*.

Yeast

C. albicans ATCC 10231 was obtained from the Mycology Research Center (MRC), University of Tehran, Tehran in Iran. The yeast cells were cultured in Sabouraud dextrose agar (SDA) (Merck Co, Darmadstat, Germany) at 37 ˚C for 20 hr. Then the blastospores were collected by centrifugation, washed twice with phosphate-buffered saline (PBS), and adjusted to 2×10^6 blastospores per ml in PBS until use (13).

Infection model

Mice were divided into two groups including infected group (15 animals) and control group (10 animals). Infected group was mice infected with *C. albicans* [Intravenously (IV), 2×10^6 cell/ml, 0.2 ml] and control group was mice received normal saline (NaCl 0.9 %) (IV, 0.2 ml/day).

Histopathology

At 7 days post-infection, 5 infected mice were killed and the kidneys, spleen and liver were removed aseptically, weighed and homogenized in 1 ml PBS; dilutions of the homogenates were cultured onto SDA. The Colony forming units (CFUs) were counted after 24 hr of incubation at 37 ˚C and expressed as CFU per gram (13).

Preparation of splenocytes

Spleens were removed from 10 infected mice and 10 control mice, and spleen cells were obtained by pressing through a sterile metal sieve, followed by filtration through an 80-mm nylon mesh. The cells were resuspended in 6 ml of PBS, and lymphocytes were separated on a Ficoll gradient by underlaying the cell suspension with 4 ml Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). The gradient was centrifuged at 340 g for 10 min at room temperature. The buffy coat interface was carefully removed, washed twice in PBS, and resuspended in 1 ml of PBS. Viable lymphocytes were counted in a haemocytometer after staining with trypan blue (Sigma, St Louis, Mo, USA) (14).

Mitogens proliferation

Mitogen proliferation assays were performed by incubating 10^5 splenic cells in RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), L-glutamine and 20% fetal calf serum (FCS). The cells were cultured in triplicate for 72 hr with Con A (1 µg/ml) (Sigma, St. Louis, Mo, USA) and PWM (1 µg/ml) (Sigma, St. Louis, Mo, USA) at 37 ˚C in 5% CO_2. After 72 hr, they received a 6-hr pulse with 0.5 mCi of [3H]thymidine (Sigma, St Louis, Mo, USA) and were then harvested and washed on glass filters. [3H]thymidine incorporation was measured in a liquid scintillation counter (Beckman, Palo Alto, CA, USA). The results were presented as stimulation index (SI) (15).

Cytokines assay

A) IFN-γ assay

The amount of IFN-γ present in the supernatant at 72 hr of culture was determined by using a mouse IFN-γ ELISA kit (BioSource International, Camarillo, Calif., USA). Briefly, 100 ml of supernatant or mouse IFN-γ standard was added in duplicate to each well of a plate precoated with antimouse IFN-γ monoclonal antibody, and the plate was incubated for 1 hr at 24 ˚C. The plate was then washed once with a buffered detergent wash solution, 100 ml of a rabbit polyclonal anti-mouse IFN-γ antibody was added to each well, and the plate was incubated for 1 hr at 24 ˚C. The plate was washed, 100 ml of an anti-rabbit antibody conjugated to horseradish peroxidase (HRP) was added to each well, and the plate was incubated for 1 hr. After washing 4 times, an HRP substrate solution was added for 1 hr. The reaction was stopped by adding 5% sulfuric acid to each well, and the plate was read at 450 nm. The values were calculated by comparison with the standard curve.

B) IL-4 assay

IL-4 was measured in cell culture supernatants at 48 hr by using an IL-4 ELISA kit (Genzyme, Cambridge, MA, USA). A 96-well plate was coated with mouse monoclonal anti-mouse IL-4 and incubated at 4 ˚C overnight. The plate was then washed with a PBS–Tween solution, the IL-4 standards and supernatants were applied to the plate, and the plate was incubated for 2 hr at room temperature. After washing, 100 ml of polyvalent rabbit anti-mouse IL-4 was added to each well and the plate was again incubated for 2 hr at room temperature. The plate was washed, biotin-conjugated goat anti-rabbit immunoglobulin was added, and the

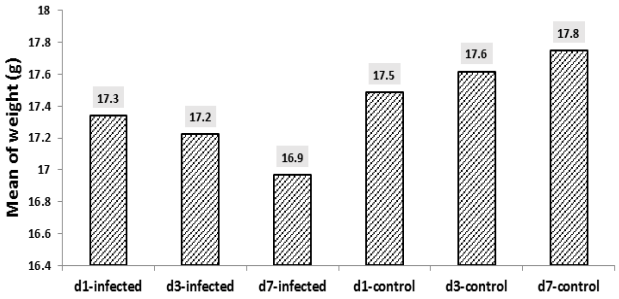

Figure 1. Mean weight of different mice groups at day 7 - d1, d2 and d3: days 1, 2 and 3, respectively

plate was incubated for 45 min. After washing, 100 ml of streptavidin-conjugated HRP was added to each well for 40 min. The plate was washed one more time. The substrate reagent (chromogen with peroxidase) was then added to each well. The reaction was allowed to proceed for 10 min and was then stopped by the addition of 1 M H_2SO_4 to each well; the A 450 was read, and the values were calculated from the standard curve.

Statistical analysis

Statistical evaluation was performed by ANOVA test. Values were considered significant at $P<0.05$.

Results

The results showed that the viable yeast cells in the kidneys reached approximately 5.6 log10 CFU/g at day 7 post-infection, followed by the spleen (3.9 log10 CFU/g) and liver (3.7 log10 CFU/g). The infected mice group had lower weight than control group at days 1, 3 and 7 after infection (Figure 1). No significant differences were observed between infected and control mice groups at different days after infection ($P=0.089$). As shown in Figure 2, the kidneys were the organs with the highest burdens of *C. albicans* throughout the observation period. The kidneys revealed vascular congestion, haemorrhages, tubular degeneration and heterophilic infiltration. The glomeruli showed hypercellularity and glomerular tufts were atrophied in places revealing an expanded Bowman's capsule. The medulla revealed

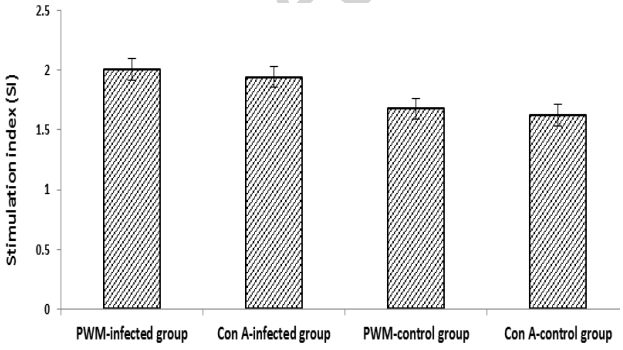

Figure 3. Proliferative response of mononuclear cells from infected and control mice under different stimuli

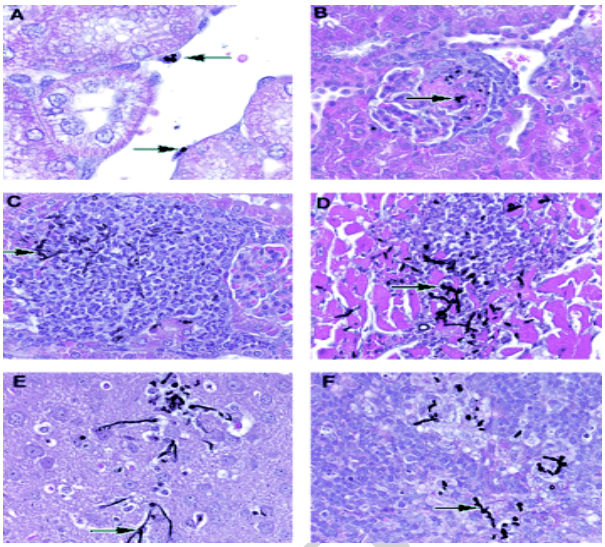

Figure 2. The presence of candidal yeast cells and pseudohyphae in parenchymal tissue of kidneys (GMS)

multiple necrotic foci and an inflammatory reaction initially predominated by neutrophils. The renal papillae and pelvis showed extensive necrosis with infiltrating and macrophages. Fungal elements demonstrated within tissues with Gomori Methanamine Silver (GMS) stain consisted of chains of elongate yeast-like structures and tubular, septate, and branched hyphae.

In infected mice, all animals presented significantly more proliferative responses to PWM (2.0±0.16) and Con A (1.9±0.19) when compared to control mice stimulated with PWM (1.7±0.19) and Con A (1.6±0.15) mitogens ($P<0.05$). The median values demonstrated a clear mitogen stimulatory effect in the following order: PWM > Con A > cell control (Figure 3).

As illustrated in Figure 4, lymphocytes from mice with disseminated candidiasis produced significantly more IFN-γ in response to PWM (68.4±14.0 pg/ml) than controls (PWM: 35.1±9.3 pg/ml) ($P<0.05$). In addition, the mean level of production of IFN-γ after stimulation with Con A was significantly different between infected mice (53.7±17.3 pg/ml) and control (28.9±11.3 pg/ml) groups ($P<0.05$).

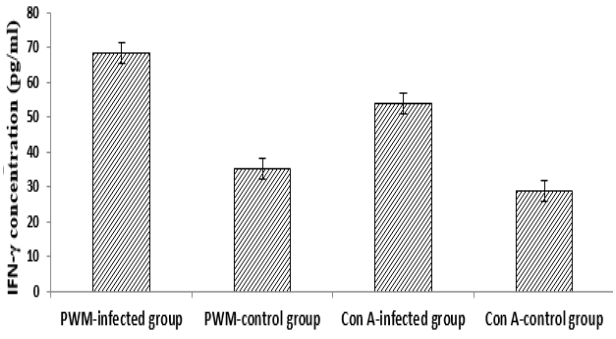

Figure 4. IFN-γ production by stimulated mononuclear cells in culture

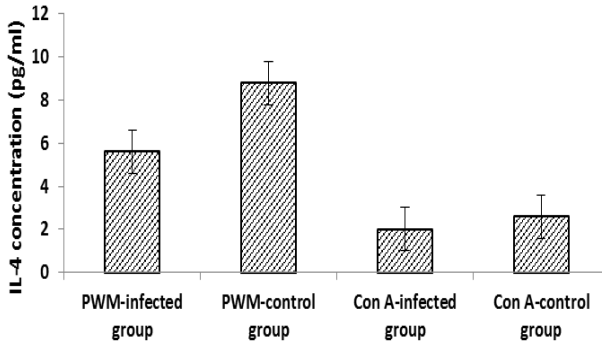

Figure 5. IL-4 production by stimulated mononuclear cells in culture

Figure 5 showed mean values of IL-4 production induced by different mitogens: PWM-stimulated IL-4 production in infected mice resulted in levels of 5.6±1.9 pg/ml when compared to 8.8±2.0 pg/ml for controls, while values detected after Con A stimulation were 2.0±1.2 pg/ml and 2.6±1.1 pg/ml for infected and control groups, respectively (P=0.075).

Discussion

Exploration of immunological events leading to *Candida* resistance or susceptibility has indicated the central role of the innate and adaptive immune systems, the relative contribution of each type of immune responses depends on the site of the primary infection. The results exhibited that viable yeast cells in the kidneys reached approximately 5.6 $\log_{10}$ CFU/g at day 7 post-infection, followed by the spleen (3.9 $\log_{10}$ CFU/g) and liver (3.7 $\log_{10}$ CFU/g). Our results are in consistent with other researchers (13, 16, 17) who reported *Candida* organisms multiplied to a greater extent in the kidneys than in the liver and spleen of animals. A correlation was made between the pathological lesions observed in the groups and the mean mice weight changes. Histopathologic examination revealed pyo-granulomatous inflammation with intralesional fungal organisms in several organs including the kidneys, brain, spleen and liver. The kidneys were the organs with the highest burdens of *C. albicans* throughout the observation period. They revealed vascular congestion, haemorrhages, tubular degeneration and heterophilic infiltration. Fungal elements consisted of chains of elongate yeast-like structures and tubular, septate, and branched hyphae. Khosravi *et al* (13) showed that both the yeast and hyphal forms of *C. albicans* are capable of invading soft tissues and the ability of tissue fluids to interconvert the two morphological forms.

The results of this study demonstrated that host resistance to *C. albicans* infection in a murine model is linked to a particular pattern of cytokine response and an accumulation of lymphocytes. The differences between the colonization patterns of *C. albicans* in "infection-resistant" BALB/c mice and "infection-prone" mice following infection correlated with both T-cell proliferation and the secretion pattern of the cytokines IL-4, IL-12, and IFN-γ (18). The lymphoproliferative responses were analyzed after stimulation with PWM and Con A mitogens. In infected mice, all animals presented significantly more proliferative responses to PWM (2.0±0.16) and Con A (1.9±0.19) when compared to control mice stimulated with PWM (1.7±0.19) and Con A (1.6±0.15) mitogens (P<0.05). The median values demonstrated a clear mitogen stimulatory effect in the following order: PWM > Con A > cell control. In consistence with our results, Katial *et al* (19) demonstrated that PWM was much more effective in proliferating lymphocytes than the other mitogens at a median value above the baseline (cell control) value.

The present study showed that lymphocytes from mice with disseminated candidiasis produced significantly more IFN-γ in response to PWM (68.4±14.0 pg/ml) than controls (PWM: 35.1±9.3 pg/ml) (P<0.05). In addition, the mean level of production of IFN-γ after stimulation with Con A was significantly different between infected mice (53.7± 17.3 pg/ml) and control (28.9±11.3 pg/ml) groups (P<0.05). The increased levels of IFN-γ noted secondary to PWM may have been due to dual stimulation of both T and B lymphocytes. The B cells may have produced an amplification factor which then stimulated the T lymphocytes to increase IFN-γ production.

In primary disseminated candidiasis, IL-4 may limit *C. albicans* infection through promoting effector mediators of immunity, including the differentiation of effector Th1 cells. In particular, IL-4 promotes the development of a protective Th1 response in disseminated candidiasis (20). In our study, detectable levels of mitogens-stimulated IL-4 production were present in both infected and control mice groups. The mean values of IL-4 production induced by different mitogens: PWM-stimulated IL-4 production in infected mice resulted in levels of 5.6±1.9 pg/ml when compared to 8.8±2.0 pg/ml for controls, while values detected after Con A stimulation were 2.0±1.2 pg/ml and 2.6±1.1 pg/ml for infected and control groups, respectively (P=0.075). Mitogens-stimulated responses, although low, were detectable. It has been shown that IL-4- producing cells in the periphery are scarce (21). In our study, the susceptibility of infected mice was lower than that of control mice in view of IL-4 production. In consistence with our results, Kaposzta *et al* (22) revealed low level of IL-4 in mice with disseminated candidiasis. In addition, other studies have shown that mice deficient in IL-4 were more susceptible to acute disseminated infection than normal controls, though no difference in susceptibility to disseminated candidiasis after challenge was noted (23). In contrast, Romani *et al*

(24) demonstrated that neutralization of IL-4 or IL-4 receptor was associated with an increased resistance to *C. albicans*. These paradoxical findings may be explained by different experimental models, different mouse strains, different routes of challenge and doses of *C. albicans* to induce systemic candidiasis, the condition of the infected host and stage of infection. In addition, this difference could be explained by unrelated genetic factors, which influence the immune response and susceptibility of other inbred strains to the growing pathogen.

The explanation of why each mitogen stimulates the cells to produce different levels of cytokines is not clear, although the spectrum of target cells for each mitogen is known to be somewhat different. Perhaps mitogens studied were not potent stimulators for cytokines production, in particular IL-4. Con A is reported to stimulate cytotoxic T cells (25), suppressor inducer T cells (26), or "virgin" T cells (27). PWM, on the other hand, stimulates helper T cells and, in association, B cells (27). Our results showed that cytokine levels vary based on different mitogens.

Conclusion

Our study demonstrated a significant increase in both cell proliferation and IFN-γ secretion in PWM and Con A- stimulated splenocyte cultures from mice with disseminated candidiasis. The results presented above suggest that one needs to be very aware of the culture conditions, the mitogens used, and the clinical state of the animals when performing *in vitro* cytokine measurements.

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Conflict of interest

The authors declared no conflict of interest.

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