Effect of cyclophosphamide on the course of *Candida albicans* infection in normal and vaccinated mice

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ABSTRACT

Objective: To evaluate the immunomodulating effect of cyclophosphamide (Cy) on the course of *Candida albicans* (*C. albicans*).

Methods: We performed this study in the Shiraz Medical School, Shiraz, Iran during April to November 2003. Five groups of 10 mice (vaccinated group) were immunized by 5 equal injections of $2x10^5$, $2.5x10^5$ and $3x10^5$ of the organism intraperitoneally. Then, the group received Cy on day zero and was challenged with lethal doses of *C. albicans* ($7.74x10^5$ colony forming unit) on days zero, one, 3, 6 and 12 post-Cy injection. Another 5 equal groups of 10 mice (non-vaccinated group) received Cy on day zero and similar to vaccinated ones were challenged with lethal doses of the organism too. The control groups received just Cy on day zero and were sacrificed on days zero, one, 3, 6 and 12 days post-Cy injection. We performed the hemogram and the spleen and studied the renal tissues microscopically and macroscopically.

Results: In vaccinated group, we observed an increase in survival time and in spleen and renal weights were visible while in non-vaccinated ones, a significant decrease was also observed on days one and 3 and an increased on days 6 and 12 post-Cy injection. We observed atrophy and necrosis in the spleen while inflammation and necrosis were also observed in the kidneys on days one and 3. We noticed a significant hyperplasia in the white pulp on days 6 and 12 post-Cy injection.

Conclusion: We conclude that hyperplasia in the white pulp of spleen and the increase in peripheral polymorphonuclears due to selective effects of Cy could effectively protect the animal against *C. albicans* infection.

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The yeast *Candida albicans (C. albicans)* is considered as normal flora in most healthy people, where it predominantly colonized in the mucosal surfaces of the gastrointestinal tract. However, acute disseminated candidiasis is a life-threatening disease which may be observed in immunocompromised patients.[1] Despite the introduction of improved antifungal drugs for treatment and prophylaxis, invasive candidiasis is still regarded as a clinical problem. In one population–based active laboratory surveillance study, candida species were reported among 72.8 cases per million populations per year.[2] *Candida albicans* can invade organs deeply in immunocompromised patients, resulting in significant morbidity and mortality.[3] Risk factors for the disseminated form of the disease include

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indwelling catheters, administration of broad-spectrum antibacterial antibiotics, immunosuppressive drug regimens associated with bone marrow or organ transplantation and in cancer chemotherapies.[4,5] Unfortunately, diagnosis of invasive candidiasis is difficult as clinical signs and symptoms of invasive forms of the disease are not specific and currently available serological tests often lack the needed sensitivity or specificity for a rapid and reliable diagnosis,[6] while histopathological study of an infected tissue would be highly specific. Invasive procedures required to obtain deep organ biopsies are not recommended for immunocompromised patients, who may often be thrombocytopenic.[5] Cyclophosphamide (Cy) as an alkylating agent is broadly administrated and induces immunosuppression in bone marrow.[4,5] This selective immunosuppression effect of the drug on immune system made it to be a desirable immunomodulator especially for immunological investigation in laboratory animals.[7,8] We conducted this study to evaluate the immunomodulating effect of Cy on mice immune system before challenging with lethal doses of *C. albicans*.

Methods. One-hundred and fifty white outbred male mice weighing 19-20 grams and aging 30-40 days were selected from the Laboratory Animal Research Center of Shiraz University of Medical Sciences, Shiraz, Iran between April and November 2003. They were divided into 3 equal groups: controls, vaccinated and non-vaccinated and each group was subdivided into 5 equal groups. The control group received Cy (Farmos Group, Finland, each vial contained 500 mg of Cy powder and 225 mg of NaCl while 25 ml of distilled water was added to obtain a solution containing 20 mg of Cy) and in immunization protocol received normal saline (0.9% of NaCl) intraperitoneally. The non-vaccinated group received Cy on day zero and then lethal doses of *C. albicans* (Group A, NCPF-3153 strain from Mycological Reference Laboratory of England in Sabouraud Dextrose Agar, SDA, Oxoid, England) was injected on days zero, one, 3, 6 and 12 post-Cy injection. To determine the lethal dose for 50% (LD 50); 5 equal groups of mice were injected intraperitoneally with live *C. albicans* ranging from 10^4 to 10^7 CFU by the method of Reed and Muench.[9] The vaccinated group received 5 injections of $2x10^5$ of the live organism in a 0.2 ml volume before Cy-injection intraperitoneally. After a 4-days interval, they received 5 injections of 2.5 x 10^5 of the live organism in a 0.25 ml and after a 3-days interval, received 5 injections of 3 x 10^5 of the live organism in a 0.3 ml volume. These immunized groups were then injected with Cy and lethal doses of *C. albicans* on day zero similar to non-vaccinated group. Cyclophosphamide was administered intraperitoneally (200 mg/Kg) for immunosuppression. Hemogram and antibody titration were performed using heparinized capillary tubes to provide blood from orbital veins and after collecting sera, antibody titration was determined by agglutination methods (Ab=1/256). Microplates were used for antibody titration (2-fold dilution of sera in saline) using 2% heat killed whole candida cells as antigen. After incubation of microplates at 37°C for 24 hours, antibody titers were recorded. White blood cells count was performed for all animals when the lethal dose was injected. The animals were challenged with *C. albicans* and were monitored for 60 days. If the animals did not survive, their kidneys and spleen were removed and preserved in formalin buffer to be studied later for histopathological examination. The alive animals were sacrificed after 60 days post-injection of lethal doses of *C. albicans* and preserved in formalin buffer to be studied microscopically (hematoxylin and eosin) and macroscopically. Mean survival times after injection of lethal doses of *C. albicans* were determined. If the animals survived after 60 days, they were considered to be protected against lethal doses of the organism.

The results were statistically analyzed using Student t and Chi-Square tests and a p value less than 0.05 was considered significant. The statistical analysis was carried out using Statistical Package for Social Sciences (version 11.5) software.

Results. The weights of spleens and kidneys decreased significantly on days one and 3 post-Cy injection, while after 6 and 12 days, an increase in their weights was visible (**Table 1**). Atrophy and necrosis were observed in the germinal center on days one and 3, while hyperplasia was noticed on days 6 and 12 post-Cy injection (**Table 2**). In contrast, we observed no considerable changes in kidneys (**Table 3**).

In relation to LD50 of *C. albicans* among outbreed mice during a 30-days period, the mortality rate reached to 80% when CFU was 10^6 and to 100% when CFUs were $5x10^6$ and 10^7. No mortality rate was observed when CFUs were 10^4, $5x10^4$, 10^5 and $5x10^5$. We considered $2x10^6$ CFU as a lethal dose in all experiments based on LD50 value. The mean survival time of non-vaccinated mice after one and 3 days showed a decrease and then after 6 and 12 days increased. A significant correlation was observed in relation to the changes in the weights of spleens and kidneys, white blood cells count and the survival time (**Table 4**).

Table 1 - Changes in spleen and renal weights after injection of cyclophosphamide.

Day after Cy injection	Control group	Day 1	Day 3	Day 6	Day 12
Spleen weights (mg ± SE)	96 ± 5.7	55 ± 10.8	24 ± 1.101	51 ± 2.12	124 ± 16.8
P-value	-	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$
Kidney weights (mg±SE)	196 ± 11.1	197 ± 10.6	175 ± 9.01	183 ± 32.7	207 ± 13.8
P-value	-	not significant	$p<0.01$	not significant	not significant

Table 2 - The effects of *Candida albicans* on white pulp area of spleen tissue in normal and vaccinated mice after injection of cyclophosphamide and lethal doses of the microorganism.

Days for lethal dose inoculation after Cy injection	Day 0	Day 1	Day 3	Day 6	Day 12
Control group	-	No change	Atrophy	Significant hyperplasia	More significant hyperplasia
Non-vaccinated mice	Slight necrosis	Severe necrosis	Severe necrosis + atrophy	Severe necrosis + significant hyperplasia	Slight necrosis + significant hyperplasia
Vaccinated mice	Significant hyperplasia	No change	Atrophy	Significant hyperplasia	More significant hyperplasia

Table 3 - Histopathological changes in the kidney due to *Candida albicans* infection in normal and vaccinated mice after cyclophosphamide injection.

Days for LD inoculation after Cy injection	Day 0	Day 1	Day 3	Day 6	Day 12
Control group	-	No change	No change	No change	No change
Non-vaccinated mice	Large abscess	Widespread microabscess	Slight inflammation	Slight inflammation	Abscess
Vaccinated mice	Large abscess + inflammation + spore	Large abscess	No lesion	Large abscess	Large abscess + inflammation + spore

Table 4 - Changes in the spleen and renal weights, survival time, and white blood cells (WBC) count of non-vaccinated mice after injection of cyclophosphamide (cy) and challenging with lethal doses of *Candida albicans*.

Days for LD* inoculation after Cy injection	Spleen ± SE (mg)	Kidney ± SE (mg)	Survival time ±SE (days)	Differential WBC ± SE (%)						WBC ± SE (mm^2)
				DC	B	E	M	L	PMN	
Control group	54 ± 5.62	196 ± 13.04	11.3 ± 1.29	-	0.1	1.2	4.4	61.8 ± 0.82	32.5 ± 0.91	11125 ± 925
Day 1	36 ± 5.19	160 ± 12.5	5.7 ± 0.72	40.3	-	0.8	3.2	33.2 ± 0.62	22 ± 0.56	4950 ± 411
Day 3	22 ± 1.68	139 ± 16.3	1.5 ± 0.16	81	0.3	0.9	2.1	10.4 ± 0.88	5.3 ± 0.59	1710 ± 102
Day 6	48 ± 11.6	152 ± 13.7	12.1 ± 2.18	41.5	0.8	2.1	8	16.4 ± 0.58	31.2 ± 0.49	5350 ± 230
Day 12	112 ± 16.3	247 ± 18.1	25 ± 4.96	7.3	1.2	4.2	10	19.7 ± 0.58	57.5 ± 0.74	11460 ± 534

*LD - lethal dose for *Candida albicans*, Control groups were inoculated only with 2 x 10^6 of live *Candida albicans*.
PMN - polymorphonuclear leukocytes, L - lymphocytes, M - monocytes, E - eosinophils, B - basophils, DC - degenerated cell

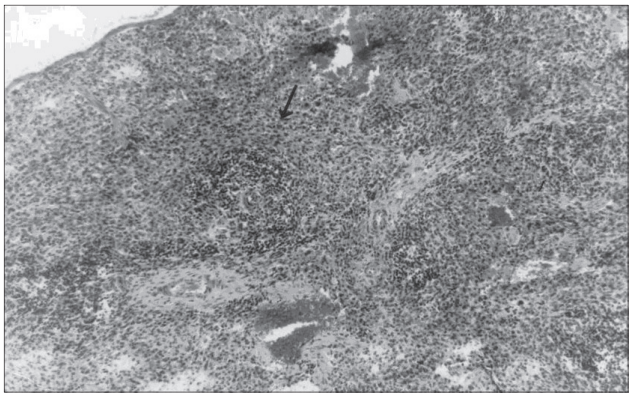

Figure 1 - Histological section of mouse spleen 3 days post-cyclophosphamide injection. Arrow shows atrophy in the white pulp area (Hematoxylin & Eosin x100).

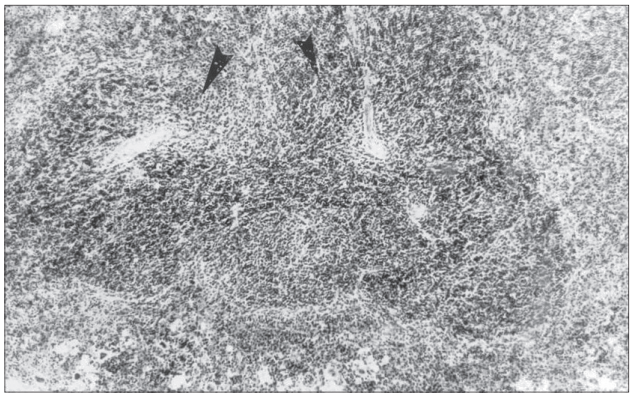

Figure 2 - Histological section of mouse spleen 12 days post-cyclophosphamide injection. Arrows show more hyperplasia in the white pulp area (Hematoxylin & Eosin x100).

Table 5 - Changes in the spleen and renal weights, survival time, differential and total white blood cells in vaccinated mice after injection of cyclophosphamide and challenging with lethal doses of *Candida albicans*.

Days for LD* inoculation after Cy injection	Spleen ±SE (mg)	Kidney ±SE (mg)	Survival time ±SE (days)	Differential WBC±SE (%)						WBC±SE (mm^2)
				DC	B	E	M	L	PMN	
Control group	157 ± 28.5	244 ± 23.6	21 ± 6.14	-	0.6	2.2	5.2	61 ± 0.71	31 ± 0.7	9520 ± 505
Day 1	132 ± 28.4	230 ± 16.6	12.4 ± 3.14	41	-	2.6	4	32.4 ± 1.7	20 ±0.7	5450 ± 221
Day 3	71 ± 6.7	146 ± 5.05	3.1 ± 2.83	5.4	-	1	16	14.2 ± 0.8	7.8 ± 0.96	2450 ± 129
Day 6	164 ± 23.7	247 ± 21.3	25 ± 10.3	41.4	1	3	7	17.4 ± 0.92	30.2 ± 0.66	7675 ± 217
Day 12	160 ± 24.18	227 ± 22.6	29 ± 6.67	9.2	0.8	2.4	8.6	19 ± 1.05	60 ± 1	11280 ± 321

*LD - lethal dose for *Candida albicans,* Control groups were inoculated only with $2x10^6$ of live *Candida albicans*, PMN - polymorphonuclear leukocytes, L - lymphocytes, M - monocytes, E - eosinophils, B - basophils, DC - degenerated cell

Histopathological study of the kidneys showed a considerable invasion of the organism in the tissue causing microabscesses and necrosis (**Table 3**). In spleens revealed necrosis in the white pulp area on days one, 3 which were more significant on days 6 and 12 (**Table 2** and **Figures 1 & 2**).

In vaccinated group, the survival time after one and 3 days decreased, while after 6 and 12 days increased (**Table 5**). Significant correlations were observed between the survival time and increased in the weights of kidneys and spleens, and the white blood cells count (**Table 5**). Vaccination could reduce the mortality rate, and an increase in weights of spleens and kidneys was noticed. Histopathological changes were observed in kidneys (**Figures 3 & 4**), and in the germinal center of the white pulp of the spleens. The tubular coagulative necrosis is visible in kidney (left one-third of **Figure 3**). The white blood cells count in vaccinated mice showed less changes. Vaccinations could increase the spleen weights, while the difference between spleen weights in non-vaccinated and vaccinated mice was significant ($p<0.01$). In vaccinated mice, more increase in the weights of kidneys was observed when compared with non-vaccinated ones and the difference was not statistically significant.

Discussion. Resistance against *C. albicans* infection may be due to several factors. The role of normal flora of human was reported to be a major factor in prevention of invasion by this opportunistic fungus.[10] Some reports pointed to the role of factors in serum such as iron, clamping factor and complement system.[11,12] Some researches confirmed the role of the cell mediated immunity system against *C. albicans* infection,[13] while others demonstrated the

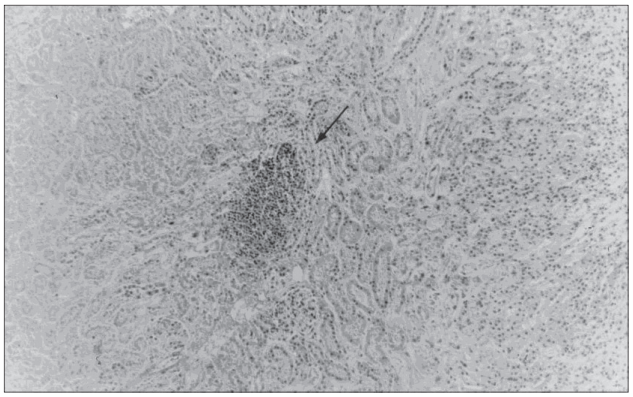

Figure 3 - Section from the kidney after injection of cyclophosphamide and challenging with lethal doses of *Candida albicans*, including cellular infiltration in medulla (Hematoxylin & Eosin x 300).

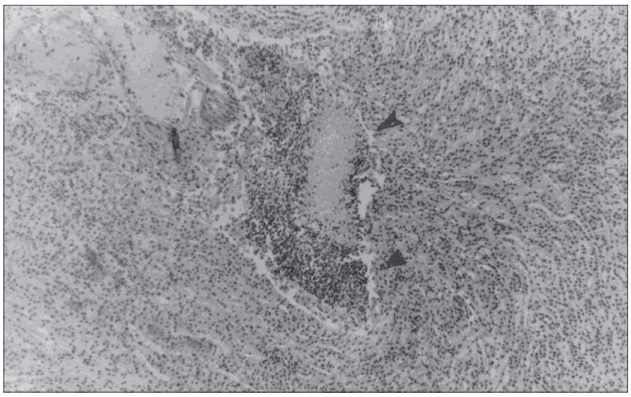

Figure 4 - Section from kidney after injection of cyclophosphamide and challenging with lethal doses of *Candida albicans*, including microabscess in medulla (Hematoxylin & Eosin x 300).

role of humoral immunity.[14] The role of non-specific immunity against this opportunistic fungus was observed in some reports.[15,16] The main target cells for Cy as a temporary immunomodulating drug is replicating cells. It was suggested that inhibition of DNA replication occurred by alkylation of nuclear bases.[17] This drug exerts its effect selectively on different components of the immune system. Thus, by administration of this drug, the correlation between changes of some components of immune system and the resistance to the infection should be clarified.[18] Based on our results, on days one and 3 post-Cy injection, the weights of these organs decreased and then increased after 6-12 days. The histopathological studies of the spleens revealed the effect of Cy on the white pulp and as the spleen is considered as the largest lymphatic organ involved in antigen antibody interactions,[19] Cy could selectively modulate the immune system involved in the spleen.[20] Our results showed a significant correlation between the increase in survival time and the increase in the number of WBCs. However, actual changes were observed in polymorphonuclear leukocytes rather than lymphocytes which may be due to the selective effects of Cy on peripheral polymorphonuclear leukocytes to be increased sharply after 12 days showing the role of peripheral polymorphonuclear leukocytes against *C. albicans* infection.[21] In histopathological studies, the decrease in spleen weights may be due to severe necrosis and atrophy of the white pulp area. Histopathological changes such as microabscesses were noticed in kidneys. Indeed, in groups with longer survival, the blastospore and pseudohypha of fungus could penetrate deeply in corticomedullar junction and cause these pathological changes (**Figures 3 and 4**). In our study, vaccination exerted positive effects when Cy-treated mice were challenged with lethal doses of *C. albicans* as some mice survived during the 60 days of our observation.[22] In addition, the mean survival time of vaccinated groups was higher than non-vaccinated ones. In our study, no significant difference was noticed when WBCs in vaccinated and non-vaccinated mice were compared. Vaccinations could increase the spleen weights due to hyperplasia in germinal center in the white pulp area and the difference between spleen weights in non-vaccinated and vaccinated mice was significant ($p<0.01$). Our results showed that kidneys were more susceptible to *C. albicans* infection similar to the reports of Ito and Tanaka.[23] The antibody titer in vaccinated mice (Ab=1/256) indicated to the role of humoral immunity against *C. albicans* infection as antibody may opsonize the engulfed candidal cells.[24] Sensitization of T and B cells may also cause the release of cytokines and interleukins and indirectly stimulate the immune system.[25] Nevertheless, our results indicated to the role of nonspecific immunity of peripheral polymorphonuclear leukocytes against *C. albicans* infection. Therefore, in vaccinated mice, the release of cytokines and interleukins may activate the proliferation of the polymorphonuclear leukocytes.[25,26] *Candida albicans* is one of the most life-threatening organisms in immunocompromised patients, so, it is suggested that anti-candidal drugs to be administered in treatment regimes of these patients.[27-29] As vaccination could protect some groups of mice against lethal doses of *C. albicans*, vaccination with whole or some components of the microorganism such as mannan or glucan could be alternative approaches to help the immune system of high-risk individuals against invasion of this endogenous opportunistic fungus.[22,30] Our findings may lead to the development of safer strategies during the course of immunosuppression.

We conclude that hyperplasia in the white pulp of spleen and the increase in peripheral polymorphonuclears due to selective effects Cy could effectively protect the animal against *C. albicans* infection.

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