THE EFFECTS OF ITRACONAZOLE ON THE IMMUNE RESPONSES IN ICR MICE

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ABSTRACT — Effects of itraconazole (ICZ) on the immune responses were studied in ICR mice. Mice were divided into 5 groups (10 mice/group), and ICZ at doses of 10, 20, 40 and 80 mg/kg were orally administered to mice once a day for 21 days. Mice were immunized and challenged with sheep red blood cells (SRBC). The body weight gains and the relative weights of spleen and thymus were dose-dependently increased following ICZ treatment. However, Plaque forming cells (PFC) and hemagglutination (HA) titers to SRBC were significantly suppressed in mice dosed at 80 mg/kg ICZ, as compared with those in controls. Delayed-type hypersensitivity (DTH) reaction to SRBC, phagocyte activity and circulating leukocytes also were significantly decreased in mice dosed at 40 and 80 mg/kg ICZ. These studies demonstrate that ICZ treatment results in a marked suppression in both humoral and cell-mediated immune responses to SRBC at concentrations producing embryotoxicity.

KEY WORDS : Itraconazole, Immune responses, Mice.

INTRODUCTION

The clinical efficacy of antimicrobial chemotherapy generally requires a functional immune competence to eradicate exogenous pathogens. This is the reason why opportunistic infections in immunocompromised patients are usually so difficult to treat successfully. In such cases, further depression of immune competence by long-term treatment of antimicrobial agents may severely hamper recovery (Bint, 1980; Hauser and Remington, 1982; Targett, 1985). Among them, amphotericin B has remained the mainstay of systemic antifungal therapy (Terrell and Hermans, 1987), but it has been shown to suppress immune defense mechanisms in vivo and in vitro (Bjorksten et al., 1976; Ferrante and Thong, 1980; Roselle and Kauffman, 1980; Alford and Cartwright, 1983). Nitidazole and metronidazole have been clearly shown to produce immunosuppressive properties at therapeutic concentrations (Mahmoud et al., 1975; Grove et al., 1977). Ketoconazole and miconazole, earlier generationazole derivatives, have also been shown to interfere with lymphocytic and natural killer cells functions, and neutrophil chemotaxis (Thong and Ferrant, 1979; Gergely et al., 1984; Rowan-Kelly et al., 1984; Odds and Webster, 1988).

Itraconazole (ICZ) is a new oral antifungal agent, like the other azoles (Van den Bossche, 1985; Lavrijsen et al., 1986), which inhibits the biosynthesis of fungal cell-membrane ergosterol (Van den Bossche et al., 1984; Van den Bossche, 1987). It is the drug of choice for the therapy of chronic mucocutaneous candidiasis (Hay and Clayton, 1987; De Beule et al., 1991) and is now showing promise in particular in the therapy of dermatophytoises, vaginal candidiasis, paracoccidioidomycosis, and systemic mycoses (Borelli, 1987; Restrepo et al., 1987; Tucker et al., 1988).
In extensive trials little toxicity has been observed, and only a small proportion of patients experience vomiting, nausea, and transient changes in liver enzymes (Cauwenbergh et al., 1987; Alcantara and Garibay, 1988). In addition, ICZ does not appear to cause the gastrointestinal and endocrine side-effects in contrast with ketoconazole and miconazole (Feldman, 1986; Cauwenbergh et al., 1987). On the other hand, it can be considered that ICZ also is immunosuppressive in laboratory animals and humans, because its mechanism of action is similar to that of antimicrobial agents, e.g. amphoterin B (Bjorksten et al., 1976; Ferrante and Thong, 1980; Roselle and Kauffman, 1980; Alford and Cartwright, 1983), niridazole (Mahmoud et al., 1975), metronidazole (Grove et al., 1977), ketoconazole and miconazole (Thong and Ferrant, 1979; Gergely et al., 1984; Rowan-Kelly et al., 1984; Odds and Webster, 1988) which have immunotoxic properties. However, little is known about the in vivo immunological effects of ICZ, a recently licensed triazole derivatives with great promise in the treatment of systemic fungal infections.

The present study was undertaken, therefore, to investigate the dose-response relationships for the immunosuppressive effects of oral ICZ in ICR mice.

MATERIALS AND METHODS

**Animals** : Male ICR mice, 6 weeks of age, weighing 17–21 g, were used. Animals were housed individually in each cage and acclimatized for at least 7 days prior to the use. The cages were maintained at 23±2°C and 50–60% relative humidity throughout the whole experimental period. Mice were fed with animal chows (Jeil Ind. Ltd., Korea) and tap water ad libitum but deprived of animal chows for 16 hr prior to sacrifice.

**Materials and treatment** : ICZ was supplied by Janssen Pharmaceutica (Korea) and dissolved in polyethylene glycol (PEG)-400 containing 6 M HCl (10 µl/ml PEG-400). ICZ (10, 20, 40 and 80 mg/kg, respectively) was orally (p.o.) administered to mice through a zonde once a day for 21 consecutive days. Control mice were treated with the same amount of acidified PEG-400.

**Lymphoid Organ and Body Weights** : Mice were sacrificed by cervical dislocation on the next day after the last ICZ treatment, and spleen and thymus were removed and weighed. Lymphoid organ weight ratio to body weight was calculated for each of mice.

**Antigen Preparation** : Sheep red blood cells (SRBC) collected from a single female sheep were kept at 4°C in sterile Alserver's solution (pH 6.1). SRBC were washed three times with phosphate-buffered saline (PBS; Gibco Lab. Co., Grand Island, N.Y., U.S.A., pH 7.4) after centrifugation at 400×g for 10 min and diluted to provide a desired concentration by hemacytometer count.

**Immunization** : All mice were immunized by intravenous (i.v.) injection of 0.1 ml of SRBC suspension (1×10^8 cells/ml) 4 days prior to each assay as described by Lake and Reed (1976). To assess the delayed-type hypersensitivity (DTH) reaction, separate groups of mice were challenged by subcutaneous (s.c.) injection of 0.05 ml of SRBC suspension (2×10^8 cells/ml) into their left hind footpad 4 days after immunization.

**Preparation and Inactivation of Serum** : The blood sample from each mouse was obtained from the carotid artery. The blood was allowed to clot in polyethylene tubes at 4°C for 30–60 min, and then centrifuged at 700 × g for 20 min. The serum was withdrawn and heat-inactivated in polyethylene tubes at 56°C for 30 min.

**Preparation of Spleen Cells** : The spleen cells from each group of mice were washed three times by centrifugation and finally suspended in cold complete medium (RPMI-1640 medium supplemented with 100 unit penicillin/ml, 100 µg streptomycin, and 2 mM L-glutamine) as described by the modified method of Mishell et al. (1980). The cells were counted and the viability was determined by trypan blue exclusion test. Cell viabilities were never less than 95%.

**Hemagglutination (HA) Titers** : HA titer was determined in microtiter trays (Limbro Chemical Co., Inc. New Haven, Connecticut, U.S.A.) using 0.025 ml volume of diluent by serial dilution of inactivated pooled sera in Hanks' balanced salt solution (HBSS; Gibco Lab. Co., Grand Island, NY, U.S.A.) in plastic microtiter plate, which was added on to 0.05 ml volume of 0.5% packed SRBC as described by Yoshikai et
Effects of itraconazole on immune responses in mice

al. (1979). The specified plate (Flow Lab., U. S. A.) was incubated for 18 hr at 37°C. Each titration was performed in duplicate and the mean titer was expressed as log₂.

**Assay of Plaque Forming Cells (PFC):** In order to examine whether ICZ accelerates the antibody production to heterologous antigen or not, the slide technique of Cunningham and Szenberg (1968) was utilized. The number of direct PFC was counted 4 days after the immunization with 10⁷ SRBC i.v. Numbers of PFC were expressed as those per 10⁶ viable spleen cells or per spleen.

**Assay of Delayed-Type Hypersensitivity (DTH) Reaction:** Four days after immunization, mice was challenged s.c. in the left and right hind footpads with 10⁸ SRBC and the corresponding volume of saline, respectively. The footpad swelling was evaluated by measuring the increase in thickness with a microcaliper (Mitutoyo Mfg. Co., Ltd., Japan) displayed in 0.01 mm gradation as described by Titus and Chiller (1981) and Henningsen et al. (1984). At 24 hr after challenge, the extent of swelling was calculated by subtracting the thickness of the saline-injected footpad from that of the antigen-injected footpad.

**Assay of Phagocyte Activity:** Phagocyte assay was determined by the modified method of Biozzi et al. (1954). In brief, for the preparation of colloidal carbon solution, rotting® ink was diluted 1/6 with 1% gelatin and kept in a stoppered tube at 37°C during the experiment. In order to measure the phagocyte activity, separate groups of mice were challenged via the lateral tail vein by using a 1 ml syringe with 26 gauze needle at the dose of 0.01 ml of colloidal carbon solution per gram body weight of mouse. At the interval of 10, 20 and 30 min, 20 μl of blood sample was obtained from the retro-orbital venous plexus. The collected blood samples were expelled into each vial containing 2 ml of 0.1% sodium carbonate, and the contents were well mixed for the lysis of erythrocytes. The absorbance of the colloidal carbon contained in blood was measured directly with spectrophotometer (Bausch and Lomd, Sphen, U. S. A.) at 600 nm. Ten times of density readings were converted to logarithmic scale and plotted against time. The slope of the line is called phagocytic coefficient K. The mice were killed and the weights of spleen and liver were measured. Corrected phagocytic index which is a measure of phagocyte activity per unit weight of tissue. Corrected phagocytic index = [body wt./(spleen wt. + liver wt.)]×3×√K.

**Count of Circulating Leukocytes:** Blood samples for measuring leukocytes in mice were collected from the retro-orbital plexus immediately before assay. Türk's solution was used for staining leukocytes and lysis of un nucleated cells. The number of nucleated cells was counted in hemacytometer chamber under a microscope. Triple counting per sample was carried out and the mean value of the results was calculated. The number was compared with that obtained from control mice.

**Statistics:** Values are expressed as means ± standard error (S. E.). All data were examined for their statistical significances of differences with Student's t-test.

**RESULTS**

The effects of ICZ on body and selected organ weights are shown in Table 1. The body weight gains and the relative weights of spleen and thymus were significantly increased in a dose-dependent manner, as observed in the 80 mg/kg treatment group.

Antibody responses were performed to evaluate humoral immunity following p.o. treatment with ICZ using the T-dependent antigen, SRBC. Direct antibody plaque forming cells (PFC) response of spleen cells to SRBC after immunization was observed in mice given ICZ, and the reduction in PFC per 10⁶ spleen cells was statistically significant in mice dosed at 80 mg/kg ICZ (1,040±27, P<0.001 as compared with those in control mice (1,540±46). A tendency toward suppression of the PFC response was also observed in mice dosed at 40 mg/kg ICZ but not in those dosed at 10 and 20 mg/kg ICZ. Their PFC per spleen also showed similar alterations (Table 2). Hemagglutination (HA) titers of serum to SRBC were slightly increased dosed at 80 mg/kg ICZ (i.e., 2.57±0.11, P<0.001 at 80 mg/kg dose level as compared with 4.00±0.06 in controls) (Table 2). These results indicate that the highest dose of ICZ (80 mg/kg) suppresses humoral immune responses to SRBC in mice.
Model was utilized to evaluate cell-mediated immunity following p.o. treatment with ICZ, delayed-type hypersensitivity (DTH) reaction to SRBC, which requires T cells. The DTH reactions were significantly decreased in mice dosed at 40 and 80 mg/kg ICZ (1.42±0.13 mm, P<0.05 and 1.27±0.09 mm, P<0.01, respectively), as compared with those in control mice (1.85±0.15 mm) (Table 3). These results indicate that ICZ suppresses cell-mediated immune responses to SRBC in mice at dosages that is apparent toxic effects in laboratory animals.

<table>
<thead>
<tr>
<th>Table 1. Effects of itraconazole on body and organ weights in ICR micea).</th>
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<tr>
<td>Itraconazole dose (p.o.) (mg/kg/day)</td>
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a) Mice were given p.o. daily for 21 days 10, 20, 40 and 80 mg/kg of itraconazole in polyethylene glycol (PEG)-400 containing 6 M HCl (10 μl/ml PEG-400). Control mice were treated with the same amount of acidified PEG-400. Mice were immunized i.v. with 10⁷ SRBC 4 days prior to each measurement. Each value represents the mean ± S.E. of 10 mice. b) Asterisks denote a significant difference compared to the value in control mice that did not receive itraconazole. (* P<0.05; ** P<0.01; *** P<0.001)

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<th>Table 2. Effects of itraconazole on antibody responses in ICR micea).</th>
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<td>Itraconazole dose (p.o.) (mg/kg/day)</td>
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# PFC, plaque forming cells; ## HA, hemagglutination; a) Mice were immunized i.v. with 10⁷ SRBC 4 days prior to each assay. Each value represents the mean ± S.E. of 10 mice. b) Asterisks denote a significant difference compared to the value in control mice that did not receive itraconazole. (** P<0.01; *** P<0.001)

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<th>Table 3. Effects of itraconazole on delayed-type hypersensitivity (DTH) responses in ICR mice.</th>
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<td>Itraconazole dose (p.o.) (mg/kg/day)</td>
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a) DTH reaction was determined by footpad swelling test. Footpad swelling is shown as the difference between the thickness of the footpads challenged with SRBC and saline, respectively. Each value represents the mean ± S.E. of 10 mice. b) Asterisks denote a significant difference compared to the value in control mice that did not receive itraconazole. (* P<0.05; ** P<0.01)
Table 4 shows the effects of ICZ on corrected phagocytic index in ICR mice. Significant decreases in corrected phagocytic index were observed in mice dosed at 40 and 80 mg/kg ICZ (i.e., 3.48±0.12, P<0.05 at 40 mg/kg dose level and 3.06±0.18, P<0.01 at 80 mg/kg dose level), as compared with those in control mice (4.05±0.21).

The effects of ICZ on circulating leukocyte counts to SRBC are shown in Table 5. In mice up to 20 mg/kg ICZ, there were no significant increases in circulating leukocyte counts as compared with those in controls (5.179±132), whereas higher dose levels resulted in a dose-dependent suppression (i.e., 4.661±82, P<0.01 at 40 mg/kg dose level and 4.349±63, P<0.001 at 80 mg/kg dose level). These results indicate that ICZ at high dose levels significantly decreased leukocytes and these reductions may be related to the result of either direct or indirect suppression of lymphocyte activity.

<table>
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<tr>
<th>Itraconazole dose (p.o.) (mg/kg/day)</th>
<th>Corrected phagocytic index$^a$</th>
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<tr>
<td>0</td>
<td>4.05±0.21</td>
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<tr>
<td>10</td>
<td>4.11±0.06</td>
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<tr>
<td>20</td>
<td>4.21±0.08</td>
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<tr>
<td>40</td>
<td>3.48±0.12$^b$</td>
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<tr>
<td>80</td>
<td>3.06±0.18$^*$</td>
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$^a$ Corrected phagocytic index is a constant obtained from a formula relating the cube root K to the ratio of body weight to the weights of the liver and spleen. Each value represents the mean ± S.E. of 10 mice. $^b$ Asterisks denote a significant difference compared to the value in control mice that did not receive itraconazole. ( * P<0.05 ; **P<0.01)

Table 5. Effects of itraconazole on the number of circulating leukocyte in ICR mice$^a$.

<table>
<thead>
<tr>
<th>Itraconazole dose (p.o.) (mg/kg/day)</th>
<th>Number of circulating leukocyte (/mm$^3$)</th>
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<tbody>
<tr>
<td>0</td>
<td>5,179±132</td>
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<tr>
<td>10</td>
<td>5,290±138</td>
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<tr>
<td>20</td>
<td>5,346±123</td>
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<tr>
<td>40</td>
<td>4,661±82$^b$</td>
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<tr>
<td>80</td>
<td>4,349±63$^*$</td>
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</table>

$^a$ Blood samples for measuring leukocytes in mice were collected from the retro-orbital plexus immediately before assay. Each value represents the mean ± S.E. of 10 mice. $^b$ Asterisks denote a significant difference compared to the value in control mice that did not receive itraconazole. ( ** P<0.01 ; *** P<0.001)
DISCUSSION

ICZ has been widely studied for its effects associated with clinical trials on antifungal activity (100–200 mg/day) (Borelli, 1987; Hay and Clayton, 1987; Restrepo et al., 1987; Tucker et al., 1988; De Beule et al., 1991). However, to date its effects in vivo on the immune system have not been investigated. It is widely accepted that ICZ appears to interfere with the biosynthesis of fungal cell-membrane ergosterol which is similar mechanism of action to that of the previously developed azole analogs (Van den Bossche et al., 1984; Van den Bossche, 1987). Peak of plasma ICZ levels, following oral administration of 10 and 50 mg/kg, are reported to range from 0.89 to 1.8 mg/l, respectively, at 4–6 hr in laboratory animals (Faergemann, 1984; Graybill and Ahrens, 1984). ICZ has been reported to obtain plasma levels of 0.07 to 0.14 mg/l after a single oral administration of 50 to 100 mg in humans (Van Cutsem et al., 1984). Preliminary data on in vitro immune functions had indicated that ICZ did not cause significant reduction in the chemiluminescence response of phagocytic cells at concentrations of up to 20 mg/l (Abruzzo et al., 1987). Vuddhakul et al. (1990), on the other hand, showed that ICZ produced suppression of neutrophil random movement, chemotaxis, deoxyglucose uptake and hexosamono-phosphatase shunt activity at concentration of 10 mg/l. Moreover, ICZ suppressed lymphocyte transformation to the same extent as ketoconazole at concentrations as low as 1 mg/l. Due to the lack of data regarding the in vivo effects of ICZ on the immune responses, however, we selected the doses from the toxicological studies of ICZ which have been previously reported by Van Cauteren et al. (1987). They showed that oral administration of ICZ for three months in Albino rats and dogs was without apparent toxic effects at 10 mg/kg, whereas higher dose levels resulted in a dose-dependent toxicity. Embryotoxicity and teratogenicity studies in Albino rats also demonstrated that oral administration of ICZ at 10 mg/kg was a non-toxic effect level. At 40 and 160 mg/kg, dose-dependent maternal toxicity was observed that was associated with embryotoxic and teratogenic effects. On the basis of these findings, the results of the present study tested are discussed as follows.

The humoral immunity of ICZ-treated mice was evaluated by utilizing the T-dependent antigen, SRBC. The T-dependent antibody responses to SRBC, which require functionally competent B cells, macrophages and T cells (Mosier and Coppelson, 1968; Claman and Mosier, 1976), were most affected at the highest dose of ICZ. Previous studies of ketoconazole which is a similar pharmacological action to ICZ had indicated that ketoconazole did not affect serum immunoglobulin levels, serum C3 and C4 complement levels and total haemolytic complement at therapeutic concentrations in human (Van Rensburg et al., 1983) but dose-dependently reduced in vitro B-lymphocyte proliferation to lipopolysaccharide in BALB/c mice (Buttke and Chapman, 1983). In the present study, we observed significant suppression of antibody responses to SRBC along with hypertrophy of the spleen in ICR mice given the highest dose of ICZ, as shown in Wistar rats (Van Cauteren et al., 1987). These findings indicate that ICZ treatment results in a significant suppression in the humoral immune responses to SRBC in association with hypertrophy of the spleen at concentrations producing overt embroyotoxicity.

Delayed-type hypersensitivity (DTH) reaction to SRBC was utilized to evaluate cell-mediated immunity following in vivo ICZ treatment. Nirodazole (Mahmoud et al., 1975), metronidazole (Grove et al., 1977) and miconazole (Thong and Rowan-Kelly, 1978; Thong and Ferrant, 1979), chemically related to ICZ, have been shown to suppress cell-mediated immune responses, in particular DTH reaction. A recent report by Mosmann et al. (1986) suggests that mouse T helper (Th) cells are distinguishable into at least two separate subpopulations of Th 1 cells, which secrete interleukin-2 (IL-2) and interferon-γ, and Th 2 cells, which secrete IL-4 and IL-5. Previous studies by this group have shown that only Th 1 cells are involved in mediating DTH reaction (Cher and Mosmann, 1987). Based on these findings, the marked suppression of DTH reactions and hypertrophy of the thymus in mice dosed at 40 and 80 mg/kg ICZ suggest that ICZ may suppress the cell-mediated immune responses to SRBC, at least in part, by blocking the
activation of mouse T cells which are associated with DTH reactions.

Macrophages play a key role in antigen recognition and processing with subsequent interaction with T and B cells to initiate cell-mediated and humoral antibody responses (Felman, 1972; Tam and Hindsill, 1984). Two components of macrophages must be considered. One is the fixed phagocytic cells in the reticuloendothelial system (RES), the other is the mobile macrophages, represented by peritoneal exudate. There are previous reports that B cells are capable of forming lymphokines that can active macrophages (Campbell et al., 1974; Petit and Unanue, 1974). There are also indications that zymosan, a carbohydrate compound present in yeasts, may activate B cells and increase phagocyte activity by the RES (Maeda and Chihard, 1973; Thomas and Chiffelle, 1974). Previous studies of terbinafine which is a similar mechanism of action to ICZ showed that it was capable of stimulating the chemiluminescence response of BALB/cBY spleen cells to the same extent as zymosan at the same concentrations (40 mg/l) (Abruzzo et al., 1987). In the present study, however, we showed that higher doses of ICZ (40 and 80 mg/kg) significantly suppressed phagocyte activity and circulating leukocyte counts in mice. The mechanism by which higher doses of ICZ (40 and 80 mg/kg) suppress macrophage functions is not yet known, but may be related to its antifungal mechanism where it interferes with the biosynthesis of cell-membrane ergosterol.

In conclusion, ICZ administration of 10 or 20 mg/kg did not cause any adverse effects on immunological functions. These doses have also been used in the treatment of systemic fungal infections. However, at higher doses (40 and 80 mg/kg), ICZ resulted in marked suppression of humoral, cell-mediated and non-specific immune functions.

ACKNOWLEDGMENTS

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