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Effect of Biphenyl Dimethyl Dicarboxylate on the Cellular and Nonspecific Immunosuppressions by Ketoconazole in Mice

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The effect of biphenyl dimethyl dicarboxylate (PMC) on the cellular and nonspecific immunosuppressions by ketoconazole (KCZ) was investigated in ICR mice. PMC at a dose of 6 mg/kg was administered orally to mice daily for 14 consecutive days. KCZ was suspended in RPMI 1640 medium and orally administered at 160 mg/kg/day 2 hrs after the administration of PMC. Immune responses of the delayed-type hypersensitivity (DTH) reaction to sheep red blood cells (SRBC), phagocytic activity and natural killer (NK) cell activity were evaluated. DTH reaction to SRBC was enhanced to normal level by the combination of PMC and KCZ, compared with treatment of KCZ alone. In the combination of PMC and KCZ, as compared with the treatment of KCZ alone, there were also significant increases in activities of natural killer (NK) cells and phagocytes along with circulating leukocytes. These findings indicate that PMC shows a significant restoration from the immunotoxic status induced by KCZ.

Key words : Biphenyl dimethyl dicarboxylate, Ketoconazole, Allergic reaction, Nonspecific host defense, Mouse

INTRODUCTION

Considering that ketoconazole (KCZ) has been widely used as an antifungal agent (Levine and Cobb, 1978; Bisschop *et al.*, 1979; Dixon *et al.*, 1980; Hay *et al.*, 1980), it is important to know as much as possible about the adverse effects of this drug on the immune system. Recent studies have reported that these adverse effects of KCZ consist of anaphylaxis, gastrointestinal disturbances, thrombocytopenia, gynecomastia, and hepatitis (Feldman, 1986; Cauwenbergh *et al.*, 1987; Wack and Galgaini, 1988; Cohen, 1991). Furthermore, KCZ has been shown to interfere with phagocytic function, neutrophil chemotaxis, random movement, deoxyglucose uptake, and hexomonophosphate shunt activity (Gergely *et al.*, 1984; Vuddhakul *et al.*, 1988). Vuddhakul *et al.* (1990) reported that KCZ also has immunosuppressive properties *in vitro*. It has been further found that the systemic antifungal agents available for clinical use are usually immunotoxic (Thong, 1986; Terrell and Hermans, 1987).

Biphenyl dimethyl dicarboxylate (PMC) is a substance derived from the synthesis of *Schizandra sp.* constituents (Xie, 1982) which have been utilized for antitussive and tonic purposes in traditional Chinese medicine, and more recently for antioxidative action (Toda *et al.*, 1988) as well as for liver-protective purposes (Maeda *et al.*, 1981, 1982; Hikino *et al.*, 1984; Kiso *et al.*, 1985; Takeda *et al.*, 1985). It was reported that oral administration of PMC to ICR mice can restore the suppression of both humoral and cell-mediated immunity produced by CCl_4 (Ahn and Kim, 1993). It has been also shown that PMC is a useful modulator of oral tolerance to ovalbumin in C3H/HeN and BALB/c mice (Kim and Ahn, 1995). Further, a previous study performed in our laboratory focused on the antagonistic effects of PMC on the humoral immune function suppressed by KCZ (Kim *et al.*, 1999). These studies demonstrated that the oral administration of PMC to ICR mice signifi-cantly enhanced the activation of humoral immune function suppressed by KCZ. Thus, it can be considered that PMC restores the cellular and nonspecific immunosuppressions of KCZ without adverse side-effects, suggesting that PMC causes a marked recovery of the humoral immunosuppression produced by KCZ.

In the present study, therefore, it was undertaken to investigate the preventive effects of PMC on the cellular and nonspecific immunosuppressions by KCZ with the aim to gain further insight into mechanism of their action. We report here our findings that orally administered PMC significantly restores the suppression of both cellular

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and nonspecific immune responses produced by KCZ.

MATERIALS AND METHODS

Experimental animals

Male ICR mice of 6 weeks old were purchased from Sam Yuk Laboratory Animal, Korea. Animals were housed individually in each cage and acclimatized for at least 7 days prior to use. The cages were maintained at 23±2°C and 50±5% of relative humidity throughout the whole experimental period. All experimental mice were fed with animal chows (Jeil Ind. Ltd., Korea) and tap water *ad libitum* but deprived of the animal chows for 16 hrs prior to sacrifice.

Materials and treatments

PMC (Bimethyl-4,4'-dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-dicarboxylate) was supplied by Dongkwang Pharmaceutical Co., Korea and suspended in 2% starch solution. PMC (6 mg/kg) was administered orally to mice daily for 14 consecutive days. Ketoconazole (KCZ; Dongkwang Pharmaceutical Co., Korea) was suspended in RPMI 1640 medium and administered at 160 mg/kg orally, 2 hrs after the administration of PMC, daily for 14 consecutive days. Control animals received the appropriate vehicle only and were treated at the same time as the corresponding experimental animals.

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, pH 7.4) after centrifugation at 400×g for 10 min and adjusted 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 (2×10^9 cells/ml) into their left hind footpad 4 days after immunization.

Preparation of spleen cells

Mice were killed by cervical dislocation and their spleens were removed aseptically. Cell suspensions from spleens were prepared in complete medium (RPMI-1640 medium supplemented with 100 unit penicillin/ml, 100 μg streptomycin, and 2 mM L-glutamine) by the modified method of Mishell *et al.* (1980). In brief, spleens were minced and gently squeezed into the fragments between the frosted ends of two sterile microscope slides in a cold complete medium. The cell suspension was passed through nylon mesh to remove major tissue aggregates. The erythrocytes were lysed with 0.83% ammonium chloride solution. The cell suspension was washed three times by centrifugation and finally suspended in a cold complete medium. Cell viability was determined by trypan blue exclusion test.

Assay of delayed-type hypersensitivity (DTH) reaction

Four days after immunization, mice were challenged s.c. in the left and right hind footpads with 10^8 SRBC and the corresponding volume of saline, respectively. The footpad swelling was evaluated by measuring the 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). In the 24 hrs 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 NK cell activity

NK cell assay was determined by the modified method of Kiesseling *et al.* (1975). Briefly, 5 mice from every experimental group were sacrificed, and 5 spleens were pooled in each petri-dish containing 20 ml cold Hank's balanced salt solution (HBSS; Gibco Lab. Co.). After washout twice with HBSS, spleen cell suspensions were prepared in cold HBSS by the gentle teasing of the organ with forceps, and passing it through nylon mesh to remove major tissue aggregates. After allowing the tissue debris to sediment for 5 min in an ice-bath, the cell suspensions in HBSS were layered on Ficoll-Hypaque solution (specific gravity 1.078) and centrifuged at 400×g for 30 min at 18~20°C. The mononuclear cell band was harvested and washed 3 times with HBSS. All cells were resuspended in complete medium to the desired concentration (2×10^7 cells/ml). Complete medium was RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum 100 unit penicillin/ml, 100 μg streptomycin/ml, and 2 mM glutamine. YAC-1 cell line, a cell line of Moloney virus induced lymphoma of A/Sn origin, was used as target cells. The target cells were labelled by incubating 2×0^5 cells in 1 ml medium with 100 Ci of $Na_2{}^{51}CrO_4$ (specific activity 283.58 ml Ci/mg, 1 μCi/ml; New England Nuclear) for 1 hr at 37°C in CO_2 incubator. The labelled cells were washed three times with HBSS supplemented with 10% fetal bovine serum (10% FBS-HBSS) and adjusted to the desired concentration (2×10^5 cells/ml). One hundred microliters of each spleen suspension was then cultured

in triplicate, with 100 μl of labeled target cells in 96-well flat bottomed microplates (Costar, Cambridge, MA) for 4 hrs at 37°C in a humidified atmosphere of 5% CO_2 in air. Most experiments were performed with effector to target ratio of 100 : 1. The plates were centrifuged for 10 min at 500×g at 4 and 100 μl of the supernatants were harvested from each well and were counted in an automatic gamma counter (Beckman, U.S.A.). The percentage of released isotope was calculated by the following formula Specific ^{51}Cr release (%)=(c.p.m. experimental-c.p.m. SR)/(c.p.m. MR-c.p.m. SR)×100, where spontaneous release (SR) was defined as the counts per minute (c.p.m) released ^{51}Cr from targets incubated with medium alone, and maximal release (MR) was determined as the c.p.m in the supernatants after lysis of target with 1% Triton X-100. Throughout the experiments, MR was higher than 95% of total isotope uptake, and SR was less than 10%.

Assay of phagocytic activity

Phagocytic assay was determined by the modified method of Biozzi *et al.* (1954). In brief, for the preparation of colloidal carbon solution, rotring 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 phagocytic 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 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 with spectrophotometer (Varian, Cary 219) at 600 nm using water as blank. Ten times of density readings were converted into logarithmic scale and plotted against time. The slope of the line was called phagocytic coefficient K. The mice were killed and the weights of spleen and liver were measured. Corrected phagocytic index is a measure of phagocytic activity per unit weight of tissue. Corrected phagocytic index=[body wt./(spleen wt.+liver wt.)]×$\sqrt[3]{K}$.

Count of circulating leukocyte

Blood was collected from the retro-orbital plexus of mice. T rk's solution was used for the staining of leukocytes and lysis of unnucleated cells. The number of nucleated cells was counted in a hemacytometer chamber under a microscope. Triple counting per sample was carried out and the mean value of the results was calculated.

Statistical analysis

The values were expressed as means standard error (S.E.). All data were examined for their statistical significance of difference with Student's *t*-test.

RESULTS

Effect of PMC on DTH response in KCZ-treated mice

The effect of PMC on DTH response in KCZ-treated mice is summarized in Table I. DTH responses of KCZ-treated mice were significantly decreased by about 42% as compared with those in controls ($P<0.01$), but the decrease in DTH response was restored to normal value by the combined administration of KCZ and PMC ($P<0.01$). This indicates that PMC has a restorative effect against oral KCZ in cell-mediated immune response such as DTH.

Effect of PMC on natural killer cell activity in KCZ-treated mice

The effect of PMC on natural killer (NK) cell activity in KCZ-treated mice is summarized in Table II. NK cell activity of KCZ-treated mice was significantly decreased by about 40% as compared with those in controls ($P<0.05$). NK cell activity, after treatment with PMC and KCZ, was significantly enhanced by about 51% as compared with that in KCZ-treated mice ($P<0.05$).

Effect of PMC on phagocytic activity in KCZ-treated mice

The effect of PMC on phagocytic activity in KCZ-treated mice is summarized in Table III. Phagocytic activity of KCZ-treated mice was significantly decreased by about 22% as compared with that in controls ($P<0.05$),

Table I. The effect of biphenyl dimethyl dicarboxylate on delayed-type hypersensitivity response in ketoconazole-treated mice

Group	DTH reaction to SRBC ($\times10^{-2}$ mm)
Control	278±26
Ketoconazole	161±11**
Ketoconazole+PMC	280±35
PMC	259±26

Abbreviationsdelayed-type hypersensitivity, DTH. Biphenyl dimethyl dicarboxylate (PMC; 6 mg/kg) was administered orally to ICR mice daily for 14 consecutive days. Ketoconazole was prepared by suspension in RPMI 1640 medium and administered at 160 mg/kg orally 2 hrs after the administration of PMC daily for 14 consecutive days. Mice were immunized i.v. with 10^7 SRBC 4 days prior to each measurement. Footpad swelling is measured as the difference between the thickness of the footpads challenged with SRBC and phosphate buffered saline, respectively. Each value represents the mean± S.E. of 6 to 7 mice. Significantly different from control at **$P<0.01$. Significantly different between ketoconazole and ketoconazole plus PMC groups at $P<0.01$.

Table II. The effect of biphenyl dimethyl dicarboxylate on natural killer cell activity in ketoconazole-treated mice

Group	% Specific lysis of ^{51}Cr-labelled target cell
	Effector Target Cell (100 : 1)
Control	21.79±1.94
Ketoconazole	13.06±2.05*
Ketoconazole PMC	19.78±1.73**
PMC	22.27±2.78

Abbreviations : biphenyl dimethyl dicarboxylate, PMC. The % lysis was determined by a standard 4 hrs ^{51}Cr release assay and effector to target ratio was 100 : 1. Each value represents the mean±S.E. of 4 to 5 mice. Significantly different from control at *P<0.05. Significantly different between ketoconazole and ketoconazole plus PMC groups at **P<0.05.

Table III. The effect of biphenyl dimethyl dicarboxylate on phagocytic activity in ketoconazole-treated mice

Group	Corrected phagocytic index
Control	5.28±0.46
Ketoconazole	4.14±0.19*
Ketoconazole PMC	5.01±0.25**
PMC	5.07±0.32

Abbreviations : biphenyl dimethyl dicarboxylate, PMC. 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 5 to 6 mice. Significantly different from control at *P<0.05. Significantly different between ketoconazole and ketoconazole plus PMC groups at **P<0.05.

but the decrease in phagocytic activity was restored by the combined administration of KCZ and PMC (P<0.05).

Effect of PMC on the number of circulating leukocytes in KCZ-treated mice

The effect of PMC on the number of circulating leukocytes in KCZ-treated mice is summarized in Table IV. The number of circulating leukocytes of KCZ-treated mice was significantly decreased by about 23% as compared with those in controls (P<0.05). But the decrease in the number of circulating leukocytes was effectively prevented by the combined administation of KCZ and PMC as compared with those in KCZ-treated mice (P<0.01).

Table IV. The effect of biphenyl dimethyl dicarboxylate on the number of circulating leukocyte in ketoconazole-treated mice

Group	Number of circulating leukocyte (/mm^3)
Control	6,167±525
Ketoconazole	4,775±347*
Ketoconazole PMC	7,600±647
PMC	7,720±802

Abbreviations biphenyl dimethyl dicarboxylate, PMC. 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 6 to 7 mice. Significantly different from control at *P<0.05. Significantly different between ketoconazole and ketoconazole plus PMC groups at P<0.01.

DISCUSSION

Several investigators have commented on the potentially harmful effects of systemic antifungal agents with immunosuppressive properties, especially when they are used for long-term treatment of patients who are already immunocompromised by their medical conditions (Bint, 1980; Hauser and Remington, 1982; Targett, 1985). In this regard, KCZ has been widely studied for its side-effects associated with immunological studies as well as clinical trials on antifungal activity (Gergely *et al.*, 1984; Feldman, 1986; Cauwenbergh *et al.*, 1987; Wack and Galgaini, 1988; Vuddhakul *et al.*, 1988, 1990; Cohen, 1991). On the contrary, PMC was found to protect against CCl_4-induced immunotoxicity in mice (Ahn and Kim, 1993). It has been also shown that PMC is a useful modulator of oral tolerance to ovalbumin in C3H/HeN and BALB/c mice (Kim and Ahn, 1995). Thus, the objective of this study was to investigate the protective effects of PMC against the cellular and nonspecific immunosuppressions by KCZ. We selected the same doses from the humoral immunological study of PMC and KCZ which had been previously reported by Kim *et al.* (1999). They showed that PMC had a protective effect against KCZ-induced humoral immunosuppression. On the basis of these findings, the results of this study are discussed as follows.

Delayed-type hypersensitivity (DTH) reaction to SRBC was utilized to evaluate cell-mediated immunity following *in vivo* KCZ treatment. Niridazole (Mahmoud *et al.*, 1975), metronidazole (Grove *et al.*, 1977) and miconazole (Thong and Ferrant, 1979), chemically related to KCZ, have been shown to suppress cell-mediated immune responses in particular DTH reaction. Furthermore, our previous study of itraconazole, which has a mechanism of action similar to that of KCZ has also shown that it suppressed cell-mediated immune response such as DTH at higher concentrations (Kim and Ahn, 1994). A recent report by Mosmann *et al.* (1986) suggests that at least two separate subpopulations of T helper (Th) cells exist in the mouse, Th 1 cells, which secrete interleukin-2 (IL-2) and interferon-, and Th 2 cells, which secrete IL-4, IL-5, IL-10 and IL-13. Previous studies by this group have shown that only Th 1 cells are involved in mediating DTH reaction (Cher and Mosmann, 1987). Oral PMC in the present study significantly restored DTH reaction to SRBC in mice immunosuppressed by KCZ alone (Table I). These findings suggest that PMC can restore the cell-mediated immune response to SRBC in KCZ-immunosuppressed mice by blocking the inhibition of mouse T cells, which are associated with DTH reaction.

It has been suggested that the enhancement of natural killer (NK) cell activity might play a role in providing resistance against tumor cell and viral activity *in vitro*

and *in vivo* (Haller *et al.*, 1977; Trichieri and Santoli, 1978; Kasai *et al.*, 1979; Herberman and Ortaldo, 1981; Domzig *et al.*, 1983). In addition, NK cells produce and secrete lymphokines, which may serve as a feedback mechanism to turn off antibody production of B lymphocytes (Abruzzo and Rowley, 1983; Reynolds *et al.*, 1987). In view of these reports and the data shown in Table II, it is thought that PMC may restore the reduction of NK cell activity in KCZ-treated mice by blocking the suppression of lymphokine production by KCZ.

Phagocytes, such as macrophages and polymorphonuclear leukocytes, play a significant part in immunological function, inflammation, infection, autoimmune diseases, tumor necrosis, and other important biological responses. They are known to release many cytokines that play important roles in maintaining homeostasis. It was shown by Gergely *et al.* (1984) that KCZ depressed host resistance mechanism *in vitro*. Our previous study of itraconazole, which is known to have even fewer side effects than KCZ, has also found that it significantly suppressed phagocytic activity and leukocyte counts in mice (Kim and Ahn, 1994). In the present study, we have further shown that PMC significantly enhanced phagocytic activity and circulating leukocyte counts in KCZ-immunosuppressed mice (Tables III and V). Therefore, oral PMC is also likely to more strongly enhance the reticuloendothelial system (RES), including macrophages. The exact mechanism by which PMC enhances phagocytic activity is not yet known, but may be due in part to recovery from the inhibition of RES by KCZ.

Since PMC was shown to reduce the adverse effects of KCZ on the cellular and nonspecific immune system, we suggest that PMC may be used in combination with other antifungal agents. PMC may prove to be especially useful in reducing the leukopenia induced in fungal therapy. It is possible that PMC may provide new approaches to fungal therapy. It is necessary, however, to study further the mechanism of action of PMC and also the differences according to animal species.

In conclusion, these studies demonstrate that PMC significantly restores the suppression of both allergic reaction to SRBC and nonspecific host defenses caused by treatment with KCZ.

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