Effect of Biphenyl Dimethyl Dicarboxylate on the Cellular and Nonspecific Immunotoxicity by Ethanol in Mice

Joung-Hoon Kim

Professional Graduate School of Oriental Medicine, Wonkwang University, Shinyong-dong, Iksan, Chunbuk, 570-749, Korea. Received April 3, 2000; accepted June 19, 2000

Biphenyl dimethyl dicarboxylate (PMC) has been reported to protect against chronic ethanol toxicity. The present study was conducted to evaluate whether PMC might be accompanied by a reduction of ethanol-induced cellular immunotoxicity. PMC at a dose of 6 mg/kg was orally administered to ICR mice daily for 28 consecutive days, and normal mice were given vehicle. Mice treated with ethanol were given free access to 20% w/v ethanol instead of water. Mice were immununized and challenged with sheep red blood cells (SRBC). Delayed-type hypersensitivity (DTH) reaction to SRBC was increased to normal levels by the combination of PMC and ethanol, compared with the treatment of ethanol alone. Splenic CD4+ cells were also greatly enhanced by PMC treatment as compared with the treatment of ethanol alone. In the case of CD8+ cells, however, a slight reduction was observed by the PMC or ethanol treatment. The natural killer (NK) cell and phagocytic activity used for evaluation of nonspecific immunocompetence were significantly augmented in PMC plus ethanol-treated mice when compared with the treatment of ethanol alone. The number of peripheral leukocytes was significantly decreased by the treatment of ethanol alone, then also restored to normal levels by PMC treatment. These findings indicate that cellular immunotoxicity caused by ethanol consumption is significantly restored or prevented by PMC treatment.

Key words biphenyl dimethyl dicarboxylate; ethanol; cellular immunotoxicity; immunocompetence

Ethanol is a colorless, volatile, and flammable liquid which is used as an industrial and laboratory solvent. While humans are exposed to ethanol in the workplace, consumption of ethanol in alcoholic beverages far exceeds any occupational exposure. The imtertaneous consumption of ethanol is a tremendous social problem of humankind. Ethanol-induced effects are very widespread and include cirrhosis, cancer, and heart disease, in addition to related psychological factors. Decreased resistance to infection is one effect recognized long ago, and which is now well documented. It has been reported that recurrent infections in alcoholic patients may be due to decreased host defense resulting from decreased bone marrow granulocytic reserve and decreased granulocyte functions. Several studies have indicated that ethanol ingestion lowers cell-mediated immune responses in vivo and in vitro. Tenenbaum et al. also showed that chronic ethanol ingestion results in a reduced delayed-type hypersensitivity (DTH) response to dinitrochlorobenzene in rats. Wands et al. found that ethanol reduced natural killer (NK) cell activity in alcoholic patients. However, Meadows et al. reported abnormality of NK cell functions related to ethanol. Roselle et al. found decreases in CD4+ and CD8+ cells or their ratio in patients with alcoholic hepatitis. In addition to peripheral blood lymphocyte abnormality, in animal models ethanol also adversely affects thymic weight and the proliferative response of T cells of antigen/mitogenic stimulation. Abnormality of number and function of thymocytes in rats with fetal alcohol syndrome has also been shown, as has, abnormality of expression of differentiation antigens. Ethanol is thus a powerful immunosuppressive agent and some of the health complications of chronic ethanol consumption should be viewed as consequences of its adverse effects on the immune response.

Biphenyl dimethyl dicarboxylate (PMC) is a substance derived from the synthesis of Schizandra sp. constituents which have been used in traditional Chinese medicine as a tonic and sedative for over 2000 years. It is shown to protect against liver injuries by carbon tetrachloride (CCL4) and b-galactosamine in mice and rats. The mutagenicity of aflatoxin-B and benzo(a)pyrene in Ames test on Salmonella typhimurium and micronucleus test in rats were also antagonized by PMC. It is to be especially noted that the elevated serum glucatemic pyruvic transaminase (SGPT) levels of patients with chronic viral hepatitis dropped to a normal limit after PMC treatment. In an immunological study by Ahn and Kim, PMC restored the suppression of cell-mediated immune functions produced by CCl4 in mice. It was reported that oral administration of PMC has a restorative effect against oral tolerance to ovalbumin in C3H/HeN and BALB/c mice. It has also been shown that PMC significantly restores the suppression of both DTH reaction to sheep red blood cells (SRBC) and nonspecific host defense induced by ketoconazole treatment. A previous study in our laboratory has demonstrated that PMC prevents the humoral immunosuppression caused by ethanol. On the basis of these studies, PMC is believed to be beneficial against cellular immunotoxicity caused by ethanol without adverse effects.

The purpose of the present study, therefore, was to investigate whether PMC might significantly restore or prevent the cellular and nonspecific immune functions suppressed by ethanol. The results indicate that PMC does significantly restore the suppression of both DTH reaction to SRBC and nonspecific immune responses caused by this substance.

MATERIALS AND METHODS

Experimental Animals and Administration Male ICR mice 5 weeks old were purchased from Sam Yuk Laboratory Animal Inc., Korea. Animals were housed individually in cages and acclimatized for at least 7 d prior to use. The cages were maintained on a 12 h light/dark cycle at 23 ± 2 °C and
50±5% of relative humidity throughout the whole experimental period. All experimental mice were fed standard rodent laboratory chow (Jeil Chedang, Korea) and tap water or 20% w/v ethanol solution ad libitum. PMC (dimethyl-4,4'-dimethoxy-5,6,5',6'-dimethylene-dioxibiphenyl-2,2'-dicarboxylate) was supplied by Dongkwang Pharmaceutical Co., Korea and suspended in 2% starch solution. PMC at 6 mg/kg was orally administered to mice daily for 28 consecutive days. This dose has been used for the treatment of type B hepatitis virus. Mice treated with ethanol were given free access to 20% w/v ethanol solution instead of water. The average daily intake of EtOH was 19.2±1.8 g/kg body weight. Normal animals received the appropriate vehicle only and were treated at the same time as the corresponding experimental animals. Ethanol consumption and body weight of each mouse were recorded daily.

**Immunization** SRBC was kept at 4°C in sterile Alsever's solution (Gibco Co., Grand Island, NY, U.S.A., pH 6.1), and was washed three times with phosphate-buffered saline (PBS; Gibco Co., pH 7.4) after centrifugation at 400×g for 10 min and diluted to provide a desired concentration by hemacytometer count. All mice were immunized by intravenous (i.v.) injection of 0.1 ml of SRBC suspension (1×10⁸ cells/ml) 5 days prior to each assay as described by Lake and Reed.34)

**Preparation of Spleen Cell Suspension** 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 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mm L-glutamine) by the modified method of Mishell et al.35) In brief, spleens were minced and gently squeezed into fragments between the frosted ends of two sterile microscope slides in 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 10⁸ SRBC and the corresponding volume of PBS, respectively. The footpad swelling was evaluated by measuring the thickness with a micrometer (Mitutoyo Mfg. Co., Ltd., Japan) displayed in 0.01 mm gradations as described by Titus and Chiller36) and Henningsen et al.37) in the 24th hour after challenge; the extent of swelling was calculated by subtracting the thickness of the PBS-injected footpad from that of the antigen-injected footpad.

**Flow Cytometry Analysis** To stain T cells, monoclonal antibodies were used as described by Pelletier et al.38) Each spleen was removed and washed. Spleen cells were made to the free cells with trypsin (Sigma Chemical Co., St. Louis, MO, U.S.A.), and stained with fluorescein isothiocyanate (FITC) conjugated anti-mouse YTS 177.9 for T-helper/inducer (CD4⁺) cells, with phycoerythrin (PE) conjugated anti-mouse Lyt-2 for T-suppressor/cytotoxic (CD8⁺) cells, or with FITC conjugated anti-mouse MCA 500 F for T cells (CD3). IgG2a (PE) and IgG2a (FITC) were used as negative controls (Serotec Ltd., Oxford, U.K.). Briefly, an appropriate volume of each monoclonal antibody and RPMI 1640 (Gibco Co.) were added to tubes and mixed thoroughly. To this mixture, 50 μl of cell suspension (2×10⁴ cells/ml) was added and reacted in the dark for 40 min. Then, this reactants were washed two times with PBS and fixed with 1% paraformaldehyde (Sigma Chemical Co.) in PBS. Finally, the ratios of CD4⁺ and CD8⁺ cells were measured by flow cytometry (Becton-Dickinson, Mountain View, CA, U.S.A.).

**Assay of NK Cell Activity** NK cell activity was determined by the modified method of Kiesseling et al.39) Briefly, 6 mice from each experimental group were sacrificed, and 6 spleens were pooled in each petridish containing 20 ml cold Hank's balanced salt solution (HBSS; Gibco Co.) then squeezed to make a single cell suspension. All cells were resuspended in complete medium (RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mm L-glutamine) to the desired concentration (2×10⁵ cells/ml). 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 labeled by incubating 2×10⁵ cells in 1 ml medium with 100 μCi of Na₂³⁵CrO₄ (specific activity 283.58 mCi/mg, 1 mCi/ml; New England, Boston, MA) for 1 h at 37°C in a CO₂ incubator. The labeled cells were washed three times with HBSS supplemented with 10% fetal bovine serum (10% FBS-HBSS) and adjusted to the desired concentration (2×10⁵ cells/ml). One hundred microliters of each spleen suspension was mixed with 100 μl of labeled target cells in 96-well flat bottomed microplates (Costar, Cambridge, MA) in triplicate, and incubated for 4 h at 37°C in a humidified atmosphere of 5% CO₂ in air. Most experiments were performed at an effector to target ratio of 100:1. The plates were centrifuged at 500×g for 10 min at 4°C, and 100 μl of the supernatants was harvested from each well and counted in an automatic gamma counter (Beckman, U.S.A.).

The percentage of released isotope was calculated by the following formula: specific ⁵¹Cr 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.) of released targets incubated with the 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 more than 95% of total isotope uptake, and SR was less than 10%.

**Assay of Phagocytic Activity** Phagocytic activity was determined by the modified method of Biozzi et al.40) 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. To measure the phagocytic activity, separate groups of mice were challenged via the lateral tail vein using a 1 ml syringe with a 26-gauge needle at the dose of 0.01 ml of colloidal carbon solution per gram of mouse. At intervals 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 a spectrophotometer (Varioy, Cary 219) at 600 nm using water as a blank. Ten 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. The corrected phagocytic index is a measure of phagocytic activity per unit weight of tissue. Corrected phagocytic index $[\text{body wt.} / (\text{spleen wt.} + \text{liver wt.})] \times \frac{1}{K}$.

**Count of Circulating Leukocytes** Blood samples for measuring leukocytes in mice were collected into tubes containing EDTA (Sigma Chemical Co.) by puncture of the retro-orbital venous plexus of animals on the day after the last PMC treatment. Those samples were diluted with isotonic detergent (Biochemical Systems, U.S.A.), hemoglobin lysing reagent (Biochemical Systems) was dropped into this solution until clear, and leukocytes were measured by WBC counter (Biochemical Systems).

**Statistical Analysis** The values were expressed as the mean ± 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 Ethanol-Treated Mice** The effect of PMC on DTH response in ethanol-treated mice is summarized in Table 1. DTH response of ethanol alone-treated mice was significantly decreased by about 39% compared with the response of normal mice ($p<0.05$), but the decrease in DTH response was significantly restored to normal levels by the combined administration of PMC and ethanol ($p<0.05$). This indicates that PMC has a restorative effect against ethanol on a cell-mediated immune response such as DTH.

**Effect of PMC on T Cell Subset in Ethanol-Treated Mice** Spleen cells obtained from ICR mice receiving PMC or ethanol for 4 weeks were analyzed by flow cytometry for their expression of the cell surface antigen detected using monoclonal antibodies (Table 2). The results indicate that total number of antigen positive cells obtained from the spleen significantly decreased in mice receiving ethanol alone compared with those in normal mice ($p<0.05$). However, their decrease was significantly restored by the combined PMC and ethanol ($p<0.05$). Furthermore, the marked reduction of CD4$^+$ cells/spleen ($\times 10^5$) caused by ethanol was restored to normal levels by the combination of PMC and ethanol (i.e., $18.8\pm0.5$ in the combination of PMC and ethanol, $p<0.01$ as compared with $14.1\pm0.6$ in the ethanol alone-treated group). In addition, lower CD4$^+$/CD8$^-$ ratio was also enhanced to normal levels (i.e., $2.5\pm0.2$ in the combination of PMC and ethanol, $p<0.05$ as compared with $1.9\pm0.1$ in the ethanol alone-treated group). These findings indicate that PMC significantly restores the function of CD4$^+$ cells but not CD8$^+$ cells suppressed by ethanol.

**Effect of PMC on NK Cell Activity in Ethanol-Treated Mice** NK cell activity can be used as a measure of nonspecific tumor killing in the body. This activity was measured and expressed as percent lysis by utilizing $^{51}$Cr release from the target YAC 1 cells on incubation with effector cells. Figure 1 shows the effect of PMC on NK cell activity in ethanol-treated mice. The results show that NK cell activity of ethanol alone-treated mice was markedly decreased by about 43% compared with that of normal mice ($p<0.01$). However, NK cell activity, after treatment with PMC and ethanol, was significantly augmented by about 66% as compared with those in ethanol alone-treated mice ($p<0.05$). These results thus seem to suggest that PMC may have antitumor activity in vivo.

**Effect of PMC on Phagocytic Activity in Ethanol-Treated Mice** The data of PMC on phagocytic activity in ethanol-treated mice are presented in Fig. 2. It is apparent that treatment with ethanol alone significantly decreased phagocytic activity compared with the normal mice ($p<0.05$). However, this decrease in activity was markedly restored up to normal levels by the combined PMC and ethanol ($p<0.01$).

**Effect of PMC on the Number of Circulating Leukocytes in Ethanol-Treated Mice** Figure 3 shows the effect of PMC on the number of circulating leukocytes in ethanol-treated mice. In mice receiving the combination of PMC and ethanol when compared with ethanol alone-treated mice, there was a significant increase in the number of circulating...

---

**Table 1. Effect of Biphenyl Dimethyl Dicarboxylate on Delayed-Type Hypersensitivity Response in Ethanol-Treated Mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>DTH reaction to SRBC ($\times 10^{-2}$ mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>227±18</td>
</tr>
<tr>
<td>PMC</td>
<td>232±25</td>
</tr>
<tr>
<td>EtOH</td>
<td>139±16*</td>
</tr>
<tr>
<td>EtOH+PMC</td>
<td>240±32*</td>
</tr>
</tbody>
</table>

**Table 2. Effect of Biphenyl Dimethyl Dicarboxylate on the Numbers of T, CD4$^+$ and CD8$^-$ Cells, and CD4$^+$/CD8$^-$ Ratio in Ethanol-Treated Mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>Total cells</th>
<th>T cells</th>
<th>CD4$^+$ cells</th>
<th>CD8$^-$ cells</th>
<th>Ratio CD4$^+$/CD8$^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>57.6±4.7</td>
<td>34.2±1.4</td>
<td>20.4±0.7</td>
<td>8.1±0.3</td>
<td>2.5±0.2</td>
</tr>
<tr>
<td>PMC</td>
<td>63.2±4.2</td>
<td>36.2±1.2</td>
<td>22.5±0.7**</td>
<td>9.0±0.2</td>
<td>2.9±0.1</td>
</tr>
<tr>
<td>EtOH</td>
<td>43.9±3.2*</td>
<td>30.5±1.1</td>
<td>14.1±0.6**</td>
<td>7.3±0.4</td>
<td>1.9±0.1*</td>
</tr>
<tr>
<td>EtOH+PMC</td>
<td>55.5±4.1*</td>
<td>33.3±1.5</td>
<td>18.8±0.5**</td>
<td>7.5±0.6</td>
<td>2.5±0.2*</td>
</tr>
</tbody>
</table>

The T cells were isolated and the subsets were characterized as described in Materials and Methods. All mice were immunized with SRBC as described in Table 1. Results represent the mean±S.E. of 6 to 7 mice. Asterisks denote a significant difference ($*p<0.05; **p<0.01$) compared to the values in normal mice not fed EtOH and PMC. Number signs denote a significant difference ($p<0.05; #p<0.01$) between EtOH and EtOH plus PMC groups.
The percentage lysis was determined by a standard 4 h $^{51}$Cr release assay and effector to target ratio was 100:1. Other legends and methods are the same as described in Table 1. Each column represents mean and standard error of 5 to 6 mice. Asterisk denotes a significant difference ($^* p<0.01$) compared to the values in normal mice not fed EtOH and PMC. Number sign denotes a significant difference ($^\# p<0.05$) between EtOH and EtOH plus PMC groups.

**DISCUSSION**

Long-term alcohol ingestion is associated with a wide range of untoward consequences and complications. In this regard, ethanol acts as a harmful influence in the immune system, which may lead to an increased incidence of infections and tumors. On the other hand, PMC was found to protect against CCL_{2} or ketoconazole-induced cellular immunotoxicity in mice. It has also been shown that PMC is a useful modulator of oral tolerance to ovalbumin in C3H/HeN and BALB/c mice. Therefore, the objective of this study was to investigate the protective effects of PMC against the cellular and nonspecific immunotoxicity of ethanol.

We selected the same doses from the humoral immunological study of PMC and ethanol which had been previously reported by Kim et al. They showed that PMC had a protective effect against ethanol-induced humoral immunotoxicity. On the basis of these findings, the measured results of the present study are discussed as follows.

Delayed-type hypersensitivity (DTH) reaction to SRBC was utilized to evaluate cellular immunity following PMC or ethanol treatment in vivo. Ethanol has been shown to suppress cellular immune responses, in particular DTH reaction, as reported by Straus et al. and Synder et al. demonstrating the impairment of DTH in patients with alcoholism. The data obtained in this study are in agreement with the results of Tennenbaum et al. who observed that chronic ethanol administration to rats caused a depression of the DTH reaction to 2, 4-dinitrofluorobenzene. A report by Mosmann et al. 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-γ, and Th 2 cells which secrete IL-4, IL-5, IL-6, IL-10 and IL-13. Previous study by this group showed that only Th 1 cells are involved in mediating DTH reaction. In animal models associated with these reports, Kaplan showed that ethanol inhibited stimulation of T cells by IL-2. Dehne et al. also found a suppression of cutaneous hypersensitivity reaction in rats fed a high dose ethanol diet. In the present study, PMC significantly restored DTH reaction to SRBC in mice immunosuppressed by ethanol (Table 1). Therefore, these findings suggest that PMC can block the inhibition of T cell function caused by ethanol.

It has been suggested that NK cell activity might play the role of host defense against various infectious diseases and cancer. In addition, NK cells produce and secrete lymphokines, which may serve as a feedback mechanism to turn off antibody production of B lymphocytes. It has also been suggested that target cells are destroyed by effector cells such as Th, NK cell and antibody-dependent cellular cytotoxicity etc., the mechanism of which may be regulated by immunoregulatory Th and Ts of T cell subsets. Thus, the flow cytometry measurement of T cell subsets has been determined in splenocytes using monoclonal anti-T cell antibodies. Most researchers have reported decreases in total T lym-
phocytes or their percents in patients with alcoholic liver disease.\textsuperscript{17,59,60} That is to say, while some investigators have found no general changes in either the absolute number of helper or suppressor/cytotoxic cells\textsuperscript{59} or their ratio,\textsuperscript{60} others have found decreases in CD4\textsuperscript{+} and CD8\textsuperscript{+} cells.\textsuperscript{17} Chang et al.\textsuperscript{59} further found that the proliferative response of splenic cells of young and old mice induced by Con A was profoundly suppressed by ethanol in a dose-dependent manner. The results of this study showed that the number of CD4\textsuperscript{+} cells and CD4\textsuperscript{+}/CD8\textsuperscript{+} ratio significantly decreased in mice treated with ethanol, but Th and CD8\textsuperscript{+} cells did not show any significant difference between mice receiving ethanol alone and normal mice (Table 2). These abnormalities may be in part reversible by varying the amount consumed and the duration of exposure to ethanol. Similarly, Meadows et al.\textsuperscript{60} also showed abnormality of NK cell function associated with ethanol. Specifically, the authors have shown that ethanol impairs both baseline and IL-2-stimulated splenic NK cell activity in rodents after one and two-week test periods of higher ethanol ingestion. In view of these reports and the data shown in Table 2 and Fig. 1, it is speculated that PMC may restore the reduction of NK cell activity in ethanol-treated mice by blocking the decrease of Th cell function caused by ethanol.

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. Morland and Morland\textsuperscript{61} found that short exposure of human monocytes to ethanol caused a decrease in functional receptors on the surface of the monocytes in vitro. Watson et al.\textsuperscript{62,63} showed a decrease in percentage of peripheral blood and spleen cells expressing markers for macrophage cell type and their activity after treatment of rodents with ethanol. Bagasra et al.\textsuperscript{64} also found that ethanol reduced both number and function of macrophages, at least in animal models. Our previous study of PMC has shown that it significantly restored phagocytic activity and leukocyte counts depressed by ketoconazole.\textsuperscript{32} In the present study, further, it has been shown that PMC combined with ethanol also significantly restored phagocytic activity and circulating leukocyte counts in ethanol-immuno-suppressed mice (Figs. 2 and 3). Therefore, 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 suppression of RES by ethanol. In addition, Fukui et al.\textsuperscript{65} confirmed that the splenic macrophages are important for endotoxin uptake, which may lead to excessive production of tumor necrosis factor (TNF) in rats given large amounts of ethanol. The effect of PMC on cytokine activity, such as TNF, is probably of equal or greater importance, though more data are required before final conclusions can be drawn. Further work is required to clarify the mechanism of action of PMC related to ethanol on activity of cytokines, and also the differences in mechanism according to animal species.

In conclusion, these studies demonstrate that PMC significantly restores the suppression of both DTH reaction to SRBC and nonspecific host defenses caused by ethanol treat-

Acknowledgements This work was supported by the Brain Korea 21 Project. The author is grateful to Dongkwan Pharmaceutical Co., Korea, for the supply of biphenyl dimethyl dicarboxylate.

REFERENCES