EFFECTS OF DIPHENYL DIMETHYL DICARBOXYLATE ON ORAL TOLERANCE TO OVALBUMIN IN MICE

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ABSTRACT — The effects of diphenyl dimethyl dicarboxylate (PMC) on oral tolerance to ovalbumin (OVA) were investigated in C3H/HeN and BALB/c mice. Mice orally received 20 mg OVA or 2% starch solution were immunized 7 days later with an i.p. injection of 0.1 mg OVA in complete Freund's adjuvant (CFA). The effects of oral OVA and PMC on antibody production were assessed by ELISA of immunoglobulin (Ig) subclass level in serum collected 7 days after immunization. Oral tolerance was obtained enough on day 7 after immunization and was more effective in C3H strain than in BALB strain, associated mainly with decreases of anti-OVA IgG, IgG1, IgG2a and IgM levels. After oral OVA, oral administrations of PMC for 6 days significantly elevated anti-OVA IgG, IgG1, IgG2a and IgM levels in mice hyposensitized by the oral OVA. These findings indicate that PMC is an useful modulator of oral tolerance to OVA in these two strains.

KEY WORDS: Diphenyl dimethyl dicarboxylate, Ovalbumin, Serum immunoglobulin subclass level, Oral tolerance, Modulator, Mice.

INTRODUCTION

Oral administration of soluble protein antigens induces an immunological unresponsiveness, termed oral tolerance (Mattingly and Waksman, 1978; Ngan and Kind, 1978; Richman et al., 1978; Hanson et al., 1979; Kagnoff, 1980; Mowat, 1987). Modulation of the oral tolerance by drugs and chemicals is considered to promote allergic responses via enhanced production of antibodies to the antigen in inhibitory modulation, or to be available for immunotherapy of allergy in stimulatory modulation. Therefore, our previous study established a model system of oral tolerance to ovalbumin (OVA) in mice for detecting modulating effects of the selected substances, e.g., cyclophosphamide (CP), E. coli lipopolysaccharide (LPS) and cadmium (Cd) (Kim and Oh, 1995). These studies demonstrated that oral tolerance was expressed enough on day 7 after immunization in C3H/HeN mice, associated mainly with decreased serum levels of anti-OVA IgG (including both IgG1 and IgG2a mediated by different T helper cells). An i.p. injection of CP prior to oral OVA, or 5 consecutive day-oral administration of LPS and Cd after oral OVA elevated or reduced anti-OVA IgG levels in mice hyposensitized by the oral OVA, respectively. CP recovered anti-OVA IgG2a levels alone, while LPS and Cd suppressed both anti-OVA IgG1 and IgG2a levels. CP also can
enhance delay-type hypersensitivity (DTH) responses through the elimination of suppressor T cells in BALB/c mice hyposensitized by oral OVA (Mowat et al., 1982). These substances, thus, could be modulators of the oral tolerance. Nevertheless, the use of CP as a model for modulation of the oral tolerance in vivo has the disadvantage that CP is a potent pharmacological agent with a wide range of actions, including a potential for damaging the intestine (Sobhon et al., 1977). Thus, it would be important to modulate an animal model of oral tolerance using agents with a more physiological action as well as little adverse side-effects.

Diphenyl dimethyl dicarboxylate (PMC) is a substance derived from the synthesis of Schizandra constituents (Xie et al., 1982) which are utilized for antitussive and tonic purposes in traditional Chinese medicine, and recently for liver-protective purpose (Maeda et al., 1981, 1982; Hikino et al., 1984; Kiso et al., 1985; Takeda, et al., 1985) as well as for antioxidative action (Toda et al., 1988). Ahn and Kim (1993) described that oral administration of PMC to ICR mice significantly restored the suppression of both DTH and antibody responses produced by CCl4. More recently, it has been shown that oral administration of PMC is capable of stimulating serum antibody production after immunization with OVA in BALB/c mice (Kim et al., 1995). Then, PMC offers a potential means for modulating of oral tolerance to OVA without adverse side-effects, thereby leading to enhance total serum Ig subclass level in BALB/c mice.

In the present study, therefore, we have examined whether PMC interferes with oral tolerance to OVA in 2 strains of mice. The data indicate that orally administered PMC leads to significant increases of anti-OVA IgG, IgG1, IgG2a and IgM levels in C3H and BALB mice hyposensitized by the oral OVA.

MATERIALS AND METHODS

Animals: Male C3H/HeN and BALB/c mice, 6 weeks of age, were used. Animals were housed individually in each cage and acclimatized for at least 7 days prior to the use. Mice were fed with animal chows (Jeil Ind., Ltd., Korea) and tap water ad libitum.

Materials and treatment: Ovalbumin (OVA; chicken albumin, Grade V, Sigma Chemical Co., St. Louis, MO. U.S.A.), 20 mg/ml, was dissolved in sterile distilled water for oral administration to mice. Control mice were given the correspondent volume of distilled water alone. PMC (Dimethyl-4,4'-dimethoxy-5, 6, 5', 6'-dimethylene dioxibiphenyl-2,2'-dicarboxylate) was supplied by Tae Rim Pharmaceutical Co. (Korea) and suspended in 2% starch solution. Mice were fed six times with 6 mg/kg PMC at daily intervals 24 h after oral OVA.

Induction of oral tolerance to OVA: For induction of oral tolerance, mice received an oral administration of 20 mg OVA were immunized 7 days later with 0.1 mg OVA in complete Freund's adjuvant (CFA; Sigma Chemical Co., St. Louis, MO. U.S.A.) intraperitoneally. Further 7 days after the immunization, blood samples for antibody determinations were collected by heart puncture. After centrifuging the samples, sera were collected and stored at −70°C until assay of antibody levels.

Spleen weight: Mice were sacrificed by cervical dislocation after blood collection, and spleen was removed and weighed.

Assay of total serum IgG, IgG1, IgG2a and IgM antibodies: Serum samples were analysed with an enzyme-linked immunosorbent assay (ELISA). Wells of a 96-well microplate (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 µl/well of primary antibody appropriately diluted in phosphate-buffered saline (PBS). Namely, as a primary antibody we used affinity-purified goat F(ab)2 anti-mouse IgG (Seikagaku Corp., Japan) or anti-mouse IgM (Cappel, Organon Teknika Corp., West Chester, PA, U.S.A.). The wells were then washed three times with PBS containing 0.05% Tween 20 (PBS-Tween) and blocked with 1% bovine serum albumin (BSA)/PBS at room temperature for 2 h. This buffer solution was also used as a diluent in all subsequent steps of the ELISAs. After three washing of the blocked wells with PBS-Tween, 100 µl of appropriately diluted serum sample was added to duplicate wells. As a standard serum we used ten serial twofold dilutions of a pooled mouse serum standard containing known concentrations of IgG and IgM (ICN, Biomedicals, Inc., Japan), added to duplicate wells (100 µl/well).
The plates were then incubated at room temperature for 1 h before washing, as described above. Aliquots of 100 μl of horseradish peroxidase (HRP)-conjugated goat IgG (MBL, Co., Ltd., Nagoya, Japan), IgG1, IgG2a (Zymed, San Francisco, CA, U.S.A.), or IgM (Cappel, Organon Teknika Corp., West Chester, PA, U.S.A.) diluted in BSA/PBS were added to each plate. The plates were incubated at room temperature for 1 h before washing, as described above. Substrate solution was then added to the various ELISA plates to complete the assays. For the various Ig-ELISAs, 9.6 mg/24 ml of O-phenylene-diamine and 8 μl/24 ml of 30% H₂O₂ was dissolved in citrate-phosphate buffer, pH 5; 150 μl of such a solution was added to each well, followed by incubation at room temperature in the dark for 15 min, and stopping of the reaction with 50 μl/well of 1 M H₂SO₄. The various ELISA plates were then read at 490 nm using a Microplate Autoreader (Nippon Inter Med K.K.). Optical density (OD) readings of blanks, obtained in the wells containing no serum, were subtracted from the OD values in the wells containing standards and unknowns. The concentrations of the various samples were obtained by interpolation on the standard curves. The final concentration in each sample was calculated as the mean of the results of duplicate cultures.

**Determination of anti-OVA IgG, IgG1, IgG2a and IgM antibodies:** For ELISA of anti-OVA antibodies, wells of a 96-well microplate (Nunc, Roskilde, Denmark) were coated with 100 μl of OVA 100 μg/ml) dissolved in PBS, and incubated overnight at 4°C. After washing the microplate three times with PBS-Tween, the wells were blocked with BSA/PBS at room temperature for 2 h. The serum samples were diluted with BSA/PBS at 1/40 for IgG and IgG2a measurements and 1/10 for IgG1 and IgM measurements, in order to give OD reading on a linear curve obtained from serial dilutions. One hundred μl of each sample was added to the plate well and incubated at room temperature for 1 h. After washing again, 100 μl aliquots of HRP-conjugated goat anti-mouse IgG (MBL, Co., Ltd., Nagoya, Japan), IgG2a, IgG1 (Zymed, Laboratories, San Francisco, CA, U.S.A.), or IgM (Cappel, Organon Teknika Corp., West Chester, PA, U.S.A.) diluted with BSA/PBS was added to each well. The plates were further incubated for 1 h at room temperature. After washing, peroxidase activities were assessed as follows: 100 μl of substrate solution (10 mg of O-phenylene-diamine and 10 μl of 30% H₂O₂ in 25 ml of 0.1 M citrate-phosphate buffer, pH 5) was added to each well of the plate. The plates were incubated at room temperature for 15 min, and enzyme reaction was terminated by adding 50 μl of 1 M H₂SO₄. OD at 490 nm of each well was then measured with a Microplate Autoreader (Nippon Inter Med K.K.). OD values of samples were subtracted with those of blanks containing no serum. Serum levels of each anti-OVA Ig subclass were represented as mean OD values at 490 nm of duplicate determination. In this assay, sera obtained from unprimed mice gave OD readings comparable to blanks.

**Statistical analysis:** Results are expressed as the mean (standard error). All data were tested for statistical significance of difference by student's t-test.

**RESULTS**

**Effects of PMC on spleen weight by the oral**

**Table 1.** Effects of diphenyl dimethyl dicarboxylate on spleen weight in oral tolerance to ovalbumin.

<table>
<thead>
<tr>
<th>Group</th>
<th>Spleen wt./body wt. × 100</th>
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<tbody>
<tr>
<td></td>
<td>C3H/HeN</td>
</tr>
<tr>
<td>Control</td>
<td>0.46 (0.02)</td>
</tr>
<tr>
<td>OVA</td>
<td>0.40 (0.00)*</td>
</tr>
<tr>
<td>PMC</td>
<td>0.51 (0.02)</td>
</tr>
<tr>
<td>OVA + PMC</td>
<td>0.47 (0.02)**</td>
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</table>

All groups of mice were immunized with an i.p. injection of 0.1 mg ovalbumin (OVA) in CFA 7 days after 20 mg OVA feeding. Seven days before the OVA immunization, all groups of mice were orally given distilled water alone (control) or OVA alone (OVA), or PMC 6 mg/kg/day for 6 consecutive days since 24 h after oral OVA (OVA + PMC) or PMC alone for 6 days without the oral OVA (PMC). Spleen weights were measured 7 days after the immunization. Results represent the mean (standard error) of 5 to 6 mice. Asterisks denote a significant difference *(P<0.05)* compared to the values in control mice not given feeding of OVA. Section marks denote a significant difference between OVA and OVA plus PMC groups *(P<0.05) ; *(P<0.01).*
administration of OVA: Table 1 shows the effects of PMC on oral tolerance to OVA in spleen weight. As anticipated, mice fed 20 mg OVA have suppressed the relative weights of spleen compared with controls, with 13% suppression in C3H/HeN mice (P<0.05) and 11% suppression in BALB/c mice (P<0.05). C3H or BALB mice receiving PMC after oral OVA, on the other hand, had the relative weights of spleen which were almost identical to control value and were significantly greater than those of mice only fed OVA (P<0.01 and P<0.05, respectively). PMC itself had no effect on the spleen weights of C3H and BALB mice compared with controls. Then, it is thought that PMC may modulate oral tolerance to OVA by enhancing spleen weight suppressed by OVA.

Ig levels in the serum of PMC-treated mice: It is investigated the effects of PMC on serum antibody production in BALB/c mice for examining the modulator activity on oral tolerance to OVA. The data in Table 2 indicate that BALB mice fed 6 mg/kg/day PMC for 6 days had significant increase of the total serum levels of IgG and IgM compared with control mice (i.e., IgG1, 112±11 μg/ml, P<0.05 as compared with 72±6 μg/ml in the controls; IgM, 325±5 μg/ml, P<0.001 as compared with 270±5 μg/ml in controls).

Then, it is thought that PMC may moderate oral tolerance to OVA.

Detection of modulations by PMC: Table 3 shows the effects of PMC on oral tolerance to OVA in C3H/HeN mice. Having demonstrated that oral tolerance in both C3H and BALB mice fed OVA was obtained enough on 7 days after immunization in our previous study (Kim and Ohsawa, 1995), we examined the modulation of

<table>
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<tr>
<th>Group</th>
<th>Total (μg/ml)</th>
<th>Anti-OVA (O.D.)</th>
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<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgG1</td>
</tr>
<tr>
<td>Control</td>
<td>640 (56)</td>
<td>30 (0.8)</td>
</tr>
<tr>
<td>OVA</td>
<td>480 (26)*</td>
<td>20 (1.3)**</td>
</tr>
<tr>
<td>PMC</td>
<td>1,098 (188)*</td>
<td>34 (1.3)*</td>
</tr>
<tr>
<td>OVA + PMC</td>
<td>1,080 (168)**</td>
<td>32 (3)**</td>
</tr>
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</table>

All groups of C3H/HeN mice were immunized with an i.p. injection of 0.1 mg ovalbumin (OVA) in CFA 7 days after 20 mg OVA feeding. Seven days before the OVA immunization, all groups of mice were orally given distilled water alone (control) or OVA alone (OVA), or PMC (6 mg/kg/day) for 6 consecutive days since 24 h after oral OVA (OVA + PMC) or PMC alone for 6 days without the oral OVA (PMC). Results represent the mean (standard error) of 5 to 6 mice. Asterisks denote a significant difference (*P<0.05 ; **P<0.01 ; ***P<0.001) compared to the values in control mice not given feeding of OVA. Section marks denote a significant difference between OVA and OVA plus PMC groups (§§P<0.01 ; §§§P<0.001).
the oral tolerance in PMC-treated mice after oral OVA. When compared with controls, OVA-fed mice (OVA group) markedly decreased total Ig subclass levels as well as anti-OVA Ig levels. Oral administrations of PMC for 6 days after oral OVA significantly elevated both total and anti-OVA Ig subclass levels compared with hyposensitized C3H mice (OVA group), and these serum antibody levels were similar to those in control mice. PMC treatment without oral OVA (PMC group) produced enhancement in total serum IgG, IgG1 and IgG2a levels, and anti-OVA IgG1 levels. Thus, this result indicates that PMC significantly restored serum antibody production in C3H mice hyposensitized by oral OVA and this recovery may be related to the results of either direct or indirect activation of Th cell populations.

Having demonstrated that the modulation of oral tolerance induced by PMC was effective in C3H mice, we further examined whether BALB/c strain also would be useful for modulating the tolerance by PMC. Table 4 shows the effects of PMC on oral tolerance to OVA in BALB/c mice. Similar to C3H mice, OVA-fed BALB mice had significant suppression of anti-OVA IgG, IgG1, IgG2a and IgM levels, but not total serum Ig subclass level compared with controls. But mice received oral PMC after feeding of OVA (OVA + PMC group) had significantly higher levels of all of total IgG1 levels and anti-OVA IgG1, IgG2a and IgM levels compared with hyposensitized mice (OVA group). This finding indicates that oral PMC restored the tolerance induced after OVA feeding.

DISCUSSION

In general, oral tolerance (oral hyposensitization) has been considered to have a role to prevent allergic responses. Modulation of the oral tolerance by drugs and chemicals can lead to cause or suppress the allergic response. Therefore, the objective of the present study was undertaken to investigate the modulating effects of PMC on oral tolerance to OVA.

Several experimental schedules have been described for the induction of oral tolerance to protein antigens in mice. Earlier studies showed that it took more than a few weeks to induce the tolerance by use of booster of antigen. Thus, we used oral administration of a single dose of 20 mg OVA known the most efficient way to induce oral tolerance, as shown in SWR, BDF1, BALB/c or C3H/HeN mice (Ngan and Kind, 1978; Hanson et al., 1979; Miller and Hanson, 1979; Kim and

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Table 4. Effects of diphenyl dimethyl dicarboxylate on oral tolerance to ovalbumin in BALB/c mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>IgG</th>
<th>IgG1</th>
<th>IgG2a</th>
<th>IgM</th>
<th>Anti-OVA (O.D.)</th>
<th>IgG</th>
<th>IgG1</th>
<th>IgG2a</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>378</td>
<td>60</td>
<td>112</td>
<td>2,206</td>
<td>0.989</td>
<td>(0.093)</td>
<td>(0.138)</td>
<td>(0.033)</td>
<td>(0.094)</td>
</tr>
<tr>
<td></td>
<td>(58)</td>
<td>(6)</td>
<td>(11)</td>
<td>(392)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA</td>
<td>262</td>
<td>46</td>
<td>89</td>
<td>1,446</td>
<td>0.536</td>
<td>(0.146)*</td>
<td>(0.064)*</td>
<td>(0.013)**</td>
<td>(0.034)*</td>
</tr>
<tr>
<td></td>
<td>(17)</td>
<td>(2)</td>
<td>(5)</td>
<td>(298)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMC</td>
<td>422</td>
<td>63</td>
<td>136</td>
<td>2,062</td>
<td>1.063</td>
<td>(0.110)</td>
<td>(0.227)</td>
<td>(0.036)</td>
<td>(0.078)</td>
</tr>
<tr>
<td></td>
<td>(59)</td>
<td>(7)</td>
<td>(17)</td>
<td>(254)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA + PMC</td>
<td>314</td>
<td>55</td>
<td>99</td>
<td>2,180</td>
<td>0.768</td>
<td>(0.055)</td>
<td>(0.151)</td>
<td>(0.027)**</td>
<td>(0.042)**</td>
</tr>
<tr>
<td></td>
<td>(20)</td>
<td>(3)</td>
<td>(13)</td>
<td>(116)</td>
<td></td>
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All groups of BALB/c mice were immunized with an i.p. injection of 0.1 mg ovalbumin (OVA) in CFA 7 days after 20 mg OVA feeding. Seven days before the OVA immunization, all groups of mice were orally given distilled water alone (control) or OVA alone (OVA), or PMC (6 mg/kg/day) for 6 consecutive days since 24 h after oral OVA (OVA + PMC) or PMC alone for 6 days without the oral OVA (PMC). Results represent the mean (standard error) of 5 to 6 mice. Asterisks denote a significant difference (*P<0.05; **P <0.01) compared to the values in control mice not given feeding of OVA. Section marks denote a significant difference between OVA and OVA plus PMC groups (**P<0.05; ***P<0.01).
Ohsawa, 1995). In addition, our previous study has found that oral tolerance to OVA was obtained enough on day 7 after immunization in C3H/HeN and BALB/c mice (Kim and Ohsawa, 1995). In the present study, we have further shown that oral administration of OVA markedly decreased both total and anti-OVA Ig subclass levels as well as spleen weight in C3H and BALB mice (Tables 1, 3 and 4), as shown in C3H/HeN and BALB/c mice (Kim and Ohsawa, 1995). Thus, this model was applied for detecting modulating effects of PMC (Tables 3 and 4).

We chose PMC at concentration with great promise in the treatment of chronic hepatitis to investigate further modulators of the mechanism which produce the tolerance after oral OVA, because of demonstrating the enhancement of antibody production in Table 2 of the present study as well as in the previous study BALB/c mice fed PMC (Kim et al., 1995). Our previous results for detecting modulators of oral tolerance to OVA showed that CP treatment prior to oral OVA, or 5 days-oral administration of LPS and Cd after oral OVA elevated or reduced anti-OVA IgG levels in C3H/HeN mice hypsensitized by oral OVA, respectively (Kim and Ohsawa, 1995). Namely, CP recovered anti-OVA IgG2a levels alone, as demonstrated the enhancement of DTH associated with Th1 cells function through the elimination of suppressor T cells induced by oral OVA to BALB/c mice (Mowat et al., 1982). Whereas LPS and Cd suppressed both anti-OVA IgG1 and IgG2a levels, as shown in mice or rats (Michalek et al., 1982; Wenne-muehler et al., 1982; Khoury et al., 1990). These substances for detecting modulators of the oral tolerance, thus, could be differentiated in association with different response of Th subpopulations. The present study, however, has shown that oral administration of PMC significantly elevated anti-OVA IgG1, IgM, and IgG2a levels associated with Th cells function in BALB and C3H mice hypsensitized by oral OVA (Tables 3 and 4). The activation, proliferation and differentiation of B lymphocytes, on the other hand, is regulated by different lymphokines which are secreted by Th cells (Paul, 1989). The in vivo and the in vitro production of Ig subclass level is also modulated by these cytokines (Paul, 1989). Further, recent murine CD4+ T cells of the Th1 subset mediate DTH and regulate IgG2a production via interleukin-2 (IL-2) and interferon-γ production, whereas CD4+ Th2 cells regulate IgG1 and IgE production via IL-4 and IL-5 (Mosmann et al., 1986; Snapper and Paul, 1987; Pond et al., 1989). Immune responses to most antigens are balanced between antibody production and cell-mediated immunity. However, in some situations, including infections with certain protozoan, bacterial and viral agents, preferential stimulation of Th1-like or Th2-like patterns of cytokine synthesis in vivo leads to immune responses dominated by either antibody production or cell-mediated immunity (Tite et al., 1987; Heinzle et al., 1989; Pond et al., 1989; Scott and Kaufmann, 1991). In the light of these findings, the significant increases of both total and anti-OVA Ig subclass levels as well as that of spleen weight in BALB and C3H mice hypsensitized by oral OVA suggest that PMC can suppress the oral tolerance to OVA, at least in part, by enhancing the activation of Th cells function suppressed by OVA (Titus and Chiller, 1981).

In conclusion, we have shown that after oral OVA oral administrations of PMC for 6 days significantly elevated anti-OVA IgG, IgG1, IgG2a and IgM levels in C3H and BALB mice hypsensitized by the oral OVA. Thus, these findings indicate that mouse model used here can be useful for detecting modulator of PMC on oral tolerance to an antigen.

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