Prodigiosin 25-C Suppression of Cytotoxic T Cells in Vitro and in Vivo Similar to That of Concanamycin B, a Specific Inhibitor of Vacuolar Type H⁺-ATPase

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The effects of prodigiosin 25-C (PrG) which preferentially suppresses cytotoxic T cells (CTL), was examined in comparison with concanamycin B (CMB), a specific inhibitor of vacuolar type H⁺-ATPase (V-ATPase). PrG and CMB directly inhibited the cytotoxic function of CTL and neutralized acidic organelles of CTL in vitro. In addition, PrG or CMB was injected in C57BL/6 mice after immunization with an allogeneic mastocytoma, P815. PrG and CMB inhibited the killing activity of CTL against the tumor and reduced the population of CD8⁺ cells without affecting CD4⁺ and B220⁺ populations in the spleen. PrG and CMB had only a negligible effect on antibody production induced by sheep red blood cells (SRBC) and mitogenic responses of lymphocytes. These results suggest that PrG and CMB have similar immunosuppressive properties at least through their inhibitory effects on acidification of intracellular organelles required for the effective function of CTL.

Prodigiosin 25-C (PrG; Fig. 1) is a red pigment that was isolated from Streptomyces hoshimensis during the course of screening for new immunomodulators based on the selective inhibitory effect on the blastogenesis of T lymphocytes by concanavalin A (Con A). PrG also inhibited the generation of allo-specific CTL without affecting the function of helper T cells and B cells in vivo, and suppressed T cell-mediated reactions including delayed-type hypersensitivity and rejection of allogeneic skin grafts. We have found that reagents that inhibit intra-organelle acidification selectively inhibited the mitogenic response induced by Con A. In addition, we have reported that PrG raises intracellular pH by inhibiting proton pump activity of vacuolar type H⁺-ATPase (V-ATPase) without affecting its ATPase activity.

Concanamycin B (CMB; Fig. 1) is a macrolide antibiotic initially isolated from Streptomyces diastochromogenos S-45 as an inhibitor of Con A-induced proliferative response of mouse spleen cells, and the physico-chemical properties of CMB are very similar to those of concanamycin A (CMA; Fig. 1). In structure, an ethyl group attached to C-8 of CMA was replaced by a methyl group in CMB. They are

![Prodigiosin 25-C](image1)

![Concanamycin A and B](image2)

**Fig. 1.** Structures of PrG and Concanamycins.

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**Abbreviations:** CMA, concanamycin A; CMB, concanamycin B; Con A, concanavalin A; CTL, cytotoxic T cells; DAMP, 3-(2,4-dinitroanilino)-3- amino-N-methylpropyramine; FCS, fetal calf serum; LPS, lipopolysaccharide; mAb, monoclonal antibody; 2-ME, 2-mercaptoethanol; MIC, minimum inhibitory concentration; PBS, phosphate-buffered saline; PE, phycoerythrin; PHA, phytohemagglutinin; PrG, prodigiosin 25-C; SRBC, sheep red blood cells; V-ATPase, vacuolar type H⁺-ATPase.
structural analogues of bafilomycins and both of them specifically inhibit the ATPase activity of V-ATPases without affecting F-type and P-type ATPases. The V-ATPase acidifies the interior space of endosomes, lysosomes, and Golgi vesicles, and acidification is important in the transport, proteolysis, and the sorting of glycoproteins in acidic organelles.

In this paper we compared the effects of PrG and CMB on cytotoxic T cells (CTL) to address the relationship between immunosuppression and the inhibition of acidification.

Materials and Methods

Chemicals. PrG was prepared as reported previously and kept at -20°C until use. PrG was dissolved in DMSO (10mg/ml) and suspended in phosphate-buffered saline (PBS, pH 7.6) containing 0.2% Tween 80 immediately before injection. CMB was kindly provided by Dr. K. Mizoue, Taisho Pharmaceutical Co., Ltd. (Tokyo, Japan). CMB was suspended in PBS from a stock solution (10mg/ml in ethanol, -20°C) and 0.2 ml of the solution was injected i.p. Phycoerythrin (PE)-labeled anti-L3T4 and anti-Ly1 monoclonal antibodies (mAbs) were purchased from Boehringer Mannheim GmbH (Mannheim, Germany), and PE-labeled B220 mAb from CALTAG Laboratories (San Francisco, CA). Con A, phytohemagglutinin (PHA), and lipopolysaccharide (LPS; from Salmonella enteritidis) were purchased from Sigma Chemical Company (St. Louis, MO), Wellcome Diagnostic (Beckenham, UK), and Difco Laboratories (Detroit, MI), respectively. 3(2,4-Dinitroanilino)-3-amino-N-methylpropylamine (DAMP) and monoclonal anti-dinitrophenol mAb were purchased from Oxford Biomedical Research Inc. (Oxford, MI).

Cell culture. Cells including P815, a murine mastocytoma, were cultured in 5% CO2 atmosphere at 37°C using RPMI 1640 medium with 10% fetal calf serum (FCS), 50 µg/ml mercaptoethanol (2-ME), 50 µg/ml kanamycin, and 8 µg/ml tylosin tetrarate.

Induction of CTL. C57BL/6 mice (H-2b, 6 to 8 wk old, female. Japan Charles River Co., Ltd., Yokohama, Japan) were immunized i.p. with P815 (H-2b, 2 x 10^6 cells/mouse). Splenocytes were removed on day 10 or 11 after P815 immunization and used for the assay of killing activity of CTL and mitogenic responses.

Production of antibody. Mice were immunized i.v. with SRBC (1 x 10^9 cells/mouse). Serum was collected on day 10 and incubated with or without 2-ME for 60 min at 37°C to inactivate IgM. Antibody titer was measured by agglutination assay as described previously.

Cytotoxic assay. Cytotoxic activity of splenocytes was measured as described previously. P815 was labeled with 18.5 MBq/ml of [51Cr]-sodium chromate (Amersham International, Buckinghamshire, UK) for 45 min with tapping every 15 min, or with [3H]thymidine (ICN Biomedicals Inc., Costa Mesa, CA) by incubating overnight at the final concentration of 9.25 kBq/ml. After being washed three times with medium, target cells (1 x 10^6 cells, 0.1 ml) were added to effector cells (0.1 ml) before centrifugation (300 x g, 3 min) and incubated for 4 h at 37°C in a round-bottom microtiter plate. The amount of radioactivity (cpm) in 0.1 ml of supernatant was measured by a gamma counter for 51Cr-labeled P815 and a liquid scintillation counter for 3H-labeled P815. Each measurement was done in triplicate cultures. Percent lysis was calculated as follows: where spontaneous cpm and maximum cpm were radioactivities released without effector and that with 1% SDS, respectively. % specific lysis = [(experimental cpm-spontaneous cpm)/(maximum cpm-spontaneous cpm)] x 100.

Flow cytometry. Splenocytes (1 x 10^6 cells) were incubated with 40 µl of staining buffer (Hank's balanced salt solution containing 2% FCS and 0.1% sodium azide) plus PE-labeled mAb (25 µg/ml) on ice for 30 min. Cells were subsequently washed twice with the staining buffer and analyzed by a flow cytometer (EPICS Elite, Coulter Co., Hialeah, FL).

Mitogenic response. Splenocytes (5 x 10^4 cells/well, 200 µl) were plated in a flat-bottom microtiter plate in triplicate cultures. The cells were cultured with 1 µg/ml Con A, 1 µg/ml PHA, or 2 µg/ml LPS for 48 h. Four hours before harvesting, [3H]thymidine (0.25 kBq/well) was added to each well.

The cells were harvested on a glass filter and the radioactivity was measured by a liquid scintillation counter.

Indirect immunostaining of acidic granules by DAMP. T cells were isolated from spleenocytes by the method reported by Julius et al. with slight modifications. DAMP staining was done by the manufacturer's protocol by using PE-conjugated goat anti-mouse mAb (Tago, Inc., Burlingame, CA) as a secondary mAb.

Results

Effects on CTL in vitro

CMA suppresses cytotoxic activity of a CTL clone in vitro through the inactivation of cytotoxic molecules in the lytic granules. Figure 2 shows the effects on the killing function of CTL induced by allo-immunization in vitro. PrG and CMB inhibited the killing activity of effector CTL in a dose-dependent manner without affecting spontaneous release of radioactivity. Minimum inhibitory concentrations (MIC) were 10 µM for PrG and 100 nM for CMB. PrG and CMB inhibited killing of target cells labeled with [3H]thymidine as well as those labeled with [51Cr]sodium chromate.

Effects on intra-organelle acidification in CTL are shown in Fig. 3 using DAMP, which accumulates in acidic compartments. PrG and CMB inhibited acidification of intracellular granules at their MIC for in vitro CTL suppression, although inhibition by PrG was incomplete as compared to CMB.
Effects on immunized mice in vivo

Allo-specific CTL was generated by immunizing C57BL/6 mice with an allogeneic mastocytoma P815, and CTL activity in the spleen was assessed 10 days after the immunization. These mice were treated with PrG or CMB with a single injection i.p. on day 7. CMB (0.4 mg/kg) as well as PrG (1 mg/kg) completely suppressed the induction of CTL (Fig. 4). The mice were also i.v. immunized with SRBC and anti-SRBC titer in the serum was assessed by the agglutination assay. The same treatment on alloantigen immunized mice with PrG or CMB had only a negligible effect on the production of 2-ME sensitive antibody (mainly IgM) and 2-ME resistant antibody (mainly IgG) against SRBC (Table I). A slight reduction in body weight was noted at these doses and some mice died when treated with higher doses (data not shown).

Table II shows populations of splenocytes. The most evident change in immunized mice was an increase in CD8⁺ cells to approximately twice that of normal spleen, suggesting a significant induction of CD8⁺ CTL against P815. The treatment with PrG and CMB returned the population to the normal level. These treatments had no effects on the population of B220⁺ and CD4⁺ cells except for the slight increase of B220⁺ cells in PrG-treated splenocytes.

Mitogenic responses of splenocytes of P815-immunized mice are shown in Table III. Mitogenic responses against

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Fig. 3. Inhibition of Intra-organelle Acidification of CTL by PrG and CMB.
CTL was induced as described in Materials and Methods and incubated without (A) and with 10 µM PrG (B) or 100 µM CMB (C) for 90 min and further incubated with DAMP for 30 min.

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Fig. 4. Selective Suppression of CTL in Mice Immunized with Alloantigen.
Mice were immunized with alloantigen and treated with PrG (A; 0.4 mg/kg, open squares; 1 mg/kg, filled squares) or CMB (B; 0.2 mg/kg, open triangles; 0.4 mg/kg, filled triangles) on day 7 after the immunization as described in Materials and Methods. Filled circles and open circles show immunized mice and normal mice, respectively. Mice were killed on day 10 and CTL activity of splenocytes pooled from 3 mice was measured using [³H]thymidine-labeled P815. Each point represents a mean of triplicate cultures.

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Table I. Effects on Antibody Induction

<table>
<thead>
<tr>
<th>Mice</th>
<th>Treatments</th>
<th>2ME of antiserum (log₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>None</td>
<td>N.D.</td>
</tr>
<tr>
<td>P815 immune</td>
<td>PrG (0.4 mg/kg)</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>PrG (1.0 mg/kg)</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>CMB (0.2 mg/kg)</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>CMB (0.4 mg/kg)</td>
<td>7.9</td>
</tr>
</tbody>
</table>

Sera of mice in Fig. 2 were pooled and antibody titer against SRBC was measured in the absence or presence of 2-ME.
N.D., not detected.

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Table II. Effects on Lymphocyte Population in Spleen

<table>
<thead>
<tr>
<th>Mice</th>
<th>Treatments</th>
<th>Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CD4⁺</td>
</tr>
<tr>
<td>Normal</td>
<td>None</td>
<td>24.2</td>
</tr>
<tr>
<td>P815 immune</td>
<td>PrG (0.4 mg/kg)</td>
<td>23.9</td>
</tr>
<tr>
<td></td>
<td>PrG (1.0 mg/kg)</td>
<td>19.2</td>
</tr>
<tr>
<td></td>
<td>CMB (0.2 mg/kg)</td>
<td>22.3</td>
</tr>
<tr>
<td></td>
<td>CMB (0.4 mg/kg)</td>
<td>20.7</td>
</tr>
</tbody>
</table>

Splenocytes were prepared as described in the legend of Fig. 2 and their population was analyzed with flow cytometry.
Table III. Effects on Mitogenic Responses of Splenocytes

<table>
<thead>
<tr>
<th>Mice</th>
<th>Treatments</th>
<th>[³H]Thymidine incorporation (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ConA</td>
<td>PHA</td>
</tr>
<tr>
<td>Normal</td>
<td>None</td>
<td>11,220 ± 712</td>
</tr>
<tr>
<td>P815 immune</td>
<td>None</td>
<td>758 ± 36</td>
</tr>
<tr>
<td></td>
<td>PrG (0.4 mg/kg)</td>
<td>643 ± 47</td>
</tr>
<tr>
<td></td>
<td>PrG (1 mg/kg)</td>
<td>805 ± 82</td>
</tr>
<tr>
<td></td>
<td>CMB (0.2 mg/kg)</td>
<td>802 ± 79</td>
</tr>
<tr>
<td></td>
<td>CMB (0.4 mg/kg)</td>
<td>305 ± 14</td>
</tr>
</tbody>
</table>

Splenocytes were prepared as described in the legend of Fig. 2. Proliferative responses of splenocytes in the absence or presence of mitogens were measured in triplicate cultures.

A B cell mitogen, LPS, and T cell mitogens, PHA and Con A, decreased in immunized mice as compared to normal ones. CMB and PrG did not affect the decreased responses of immunized mice. This result is consistent with the above results that indicate the selective suppression of CTL by PrG and CMB.

Taken together, it is suggested that CMB, like PrG, selectively suppresses CTL without affecting functions of helper T cells and B cells.

Discussion

We have shown that PrG selectively inhibited CTL generation in vivo with a different mechanism from cyclosporin A and FK506.3,17 These inhibit IL-2 production of helper T cells through inhibition of calcineurin, a serine- and threonine-specific protein phosphatase. We also found that inhibitors of intra-organelle acidification had biological properties similar to PrG in vitro and that PrG uncouples V-ATPase.7 These observations prompted us to compare the effects of PrG on immune system in vivo as well as in vitro with those of CMB, which has a different mechanism of inhibition of V-ATPase and a different chemical structure.

We have shown that CMA inhibited the killing activity of a CTL clone without affecting binding to target and granule exocytosis of CTL.20 Instead, it changed the structure of lytic granules and inactivated cytotoxic molecules in the granules such as perforin, a pore-forming protein.20 CMB also inhibits acidification of endosomes and lysosomes in macrophages.12 Concentrations of PrG and CMB for the inhibition of cytolytic activity in vitro appear to be comparable to those for the inhibition of acidification. These inhibitory properties of CTL imply that PrG and CMB inhibited CTL killing through the inhibition of acidification similarly to CMA. However, it was observed that PrG inhibited granule acidification of CTL incompletely while CMB inhibited it completely, thus further analysis of PrG and CMA on lytic granules is necessary to draw a conclusion.

In our experimental system of allo-immunization in vivo, however, maximum activity of CTL was completed on day 10 or 11 after the immunization, and PrG and CMB were effective by a single injection of day 7 when killing activity had almost reached a significant level. This suggests that PrG and CMB do not act during the induction phase of CTL, but on effector CTL after the induction.

Because PrG and CMB showed similar immunosuppressive effects in vivo as well as in vitro, it is most likely that the inhibition of intra-organelle acidification is responsible for the suppression of CTL in vivo. We cannot rule out the possibility that PrG and CMB have the same target(s) other than V-ATPase involved in the suppression of CTL.

The selective suppression of CD8+ cells may be due to the lytic granules that are mostly present in CD8+ cells but not in CD4+ cells and B cells. Because the lytic granules contain abundant serine proteases called granzymes, and it has been reported that injection of proteases into cytoplasm can induce apoptosis,21 granular proteases may be involved in the decrease of CD8+ cells. We speculate that PrG and CMB induce the drastic morphological changes of the lytic granules observed with CMA,22 and the granular proteases may be leaked into cytoplasm, which results in the apoptosis of CTL and a marked decrease in CD8+ cells.

Since cyclosporin A and FK506 suppress the induction phase of graft rejection through the inhibition of helper T cells, immunosuppressants that suppress effector phase are expected to provide immunosuppressive agents with new aspects. Our results demonstrate that inhibition of acidification of intracellular organelles could provide a unique target for such effector phase suppression.

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References