In Vitro Study of Deoxymethylspergualin on Functions of Lymphocytes and Bone Marrow Cells from Healthy Volunteers

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15-Deoxy-11-O-methylspergualin (MeDSG) is an analogue of 15-deoxyspergualin, which has potent immunosuppressive activity. The present study was designed to evaluate the in vitro effects of MeDSG on the functions of peripheral blood mononuclear cells (PBMC) and bone marrow cells derived from healthy volunteers. MeDSG failed to suppress the proliferation of PBMC stimulated with mitogens. In the allogeneic mixed lymphocyte reaction, MeDSG strongly suppressed both the proliferation of lymphocytes and the generation of alloreactive cytotoxic T lymphocytes, but did not affect the cytolytic activity of the established cytotoxic T lymphocytes. MeDSG had no effect on the cytolytic activity of natural killer cells. Concerning positive hematopoietic regulators, MeDSG had a slight enhancing effect on the release of granulocyte-macrophage colony-stimulating factor and a slight inhibitory effect on the release of interleukin-6 and granulocyte colony-stimulating factor from PBMC stimulated with mitogens. Significantly, MeDSG completely suppressed the colony formation of bone marrow cells in the presence of granulocyte colony-stimulating factor.

The novel immunosuppressant 15-deoxyspergualin (DSG) is a derivative of spergualin, which was initially developed as an antitumor agent. DSG was reported to prolong allograft survival in experimental allotransplantation models. It has been difficult to conclusively investigate the in vitro effect of DSG, however, since it is easily and quickly hydrolyzed, and loses its activity in usual culture conditions.

15-Deoxy-11-O-methylspergualin (MeDSG), which was chemically synthesized in the search for stable analogues of DSG, has been shown, like DSG, to possess immunosuppressive activity. Previous in vitro studies on the effect of MeDSG on the function of human lymphocytes showed that (1) in the allogeneic mixed lymphocyte reaction (MLR), MeDSG inhibited both lymphocyte proliferation and the generation of alloreactive cytotoxic T lymphocytes (CTL), but did not inhibit the production of interleukin-2; and (2) MeDSG failed to inhibit the blastogenic responses of lymphocytes stimulated with phytohemagglutinin (PHA), OKT-3 and pokeweed mitogen (PWM).

The present study was performed to examine the effects of MeDSG on various functions of lymphocytes, not excluding those mentioned above, and further to determine whether MeDSG affects the colony formation of bone marrow cells.

Materials and Methods

Immunosuppressant
MeDSG \( \text{L-amino-19-guanidino-11-methoxy-4,9,12-triazanonadecan-10,13-dione trihydrochloride} \)
was kindly provided by Nippon Kayaku Co., Ltd., Tokyo.

Preparation of Peripheral Blood Mononuclear Cells (PBMC)
PBMC were obtained by Ficoll-Hypaque density centrifugation of heparinized venous blood from consenting healthy donors and suspended in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) which was previously treated with 10 mM aminoguanidine at 37°C for 30 minutes to inactivate polyamine oxidase contained therein. This RPMI complete medium was used in the following experiments.

Blastogenic Responses for PBMC
Cultures were performed at 37°C in an atmosphere of 5% CO₂ in air in all experiments. PBMC were cultured in the RPMI complete medium at the concentration of 5 × 10^5 cells/ml for 72 hours in the presence or absence of either 0.1% PHA (Wellcome, Dartford, UK), 10 μg/ml lipopolysaccharide (LPS; Difco, Detroit, MI) or PWM (Sigma, St. Louis, MO) in 96-well culture plates. In the last 6 hours of the culture, each well was pulsed with 18.5 kBq of \(^{3}H\)-thymidine (Amersham, Arlington Heights, IL). After culture, cells were harvested on...
glass-fiber filter using a cell harvester, and the incorporation of $^3$H-thymidine into the cells was measured with a liquid scintillation counter. MeDSG was added at the initiation of culture.

Natural Killer (NK) Assay
PBMC ($1 \times 10^5$ cells) were admixed with $^{51}$Cr-labeled K562 cells ($1 \times 10^4$ cells) in round-bottomed 96-well culture plates in the RPMI complete medium with or without MeDSG. After culture for 4 hours, a fixed volume of supernatant was collected from each well after centrifugation, and the quantity of released $^{51}$Cr was determined with a gamma counter. Spontaneous and total dpm were derived from each supernatant of labeled K562 cells in the RPMI complete medium alone or 0.5% Nonidet P-40 solution. The percentage of lysis calculated according to the following formula was shown as NK activity:

$$\% \text{ lysis} = \frac{\text{experimental dpm} - \text{spontaneous dpm}}{\text{total dpm} - \text{spontaneous dpm}} \times 100$$

MLR Test
Responder PBMC ($1 \times 10^5$ cells) were cocultured with an equal number of stimulator PBMC irradiated with 25 Gy of X-ray in the 96-well plates in 0.2 ml of the RPMI complete medium with or without MeDSG. The cells were cultured for 5 days; 37 kBq of $^3$H-thymidine was added to each well for the last 16 hours of the incubation. After culture, the cells were harvested and $^3$H-thymidine incorporation was measured as above. The percentage of suppression was calculated using the following formula:

$$\% \text{ suppression} = 1 - \frac{\text{experimental dpm}}{\text{control dpm}} \times 100$$

Cell-mediated Lympholysis (CML) Assay
PBMC ($1 \times 10^5$ cells/ml) as responder cells were cocultured with $1 \times 10^5$ cells/ml of irradiated (25 Gy) stimulator PBMC in the RPMI complete medium for 5 days in the presence or absence of MeDSG. After culture, the cells were harvested and used as effector cells. Target cells were prepared by culturing stimulator cells with 0.1% PHA for 2 days. The effector cells ($3 \times 10^5$ cells/ml) were cultured with $1 \times 10^5$ cells/ml of $^{51}$Cr-labeled target cells for 4 hours with or without MeDSG. After the 4-hour culture, the cell-free supernatant was collected and the released $^{51}$Cr was counted as above. The inhibitory effect of MeDSG on cytolytic activity was calculated from the mean of triplicate culture using the following formula:

$$\% \text{ inhibition} = 1 - \frac{\text{experimental dpm} - \text{spontaneous dpm}}{\text{total dpm} - \text{spontaneous dpm}} \times 100$$

Production of Cytokines by PBMC
PBMC were cultured at a concentration of $5 \times 10^5$ cells/ml in the RPMI complete medium for 72 hours under stimulation with 0.1% PHA or 10 μg/ml LPS in the presence or absence of MeDSG. After the culture, the cell-free supernatant was collected and the amount of interleukin-6 (IL-6), granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage CSF (GM-CSF) was measured using enzyme-immunoassay as previously described. 15-17 Cell viability was confirmed by trypan-blue dye exclusion.

Clonal Cell Assay
The colony formation of bone marrow cells was performed according to a previously described method.18 Briefly, $2 \times 10^5$ bone marrow buffy coat cells were cultured in 1 ml of Iscove’s modified Dulbecco medium containing 20% FCS, 0.88% methylcellulose in 35-mm petri dishes. Each dish was supplemented with either various concentrations of G-CSF (Kirin Brewing Co., Ltd., Kakogawa-shi, Japan) or MeDSG. Seven and 14 days after culture, the number of colony-forming unit in granulocyte (CFU-G) or in granulocyte/macrophage (CFU-GM) containing 20 or more cells were counted with an inverted microscope.

Statistical Analysis
Statistical analysis was performed by Student’s t-test.

Results
Effect of MeDSG on Proliferation of PBMC Stimulated with Mitogens
The mean $^3$H-thymidine incorporation was $7,132 \pm 740$ dpm in the non-stimulated culture, and $203,177 \pm 4,300$, $121,747 \pm 12,557$ and $13,889 \pm 1,734$ dpm in the blastogenic responses with PHA, PWM and LPS, respectively; there was a significant difference ($P<0.01$) between the non-stimulated and each mitogen-stimulated groups. MeDSG at a concentration of 10 μg/ml enhanced the proliferation of PBMC stimulated with PWM, inducing an increase of 59% (Fig. 1). In contrast, MeDSG had almost no inhibitory effect on the proliferation of PBMC by PHA or LPS.

Effect of MeDSG on NK Activity
MeDSG at concentrations of 1 and 10 μg/ml had no effect on the lysis of K562 cells in the presence of the effectors (Table 1). This result shows that MeDSG exhibited neither a promotive nor a suppressive effect on NK activity. MeDSG also appeared to prevent the autolysis of K562 cells.

Effect of MeDSG in MLR Tests
MeDSG inhibited the proliferative responses of alloreactive lymphocytes in a dose-dependent manner (Fig. 2). This inhibition was 52% and 71% at concentrations of 1 and 10 μg/ml, respectively.
Fig. 1. Effect of MeDSG on mitotic reaction of mitogen-stimulated PBMC.

Data are shown as the mean±SD of triplicate cultures. * P<0.01 compared with the control culture with PWM alone.

Table 1. Effect of MeDSG on NK activity of PBMC.

<table>
<thead>
<tr>
<th>MeDSG (μg/ml)</th>
<th>With effector</th>
<th>Without effector</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>26.0±4.7a</td>
<td>4.2±1.4</td>
</tr>
<tr>
<td>1</td>
<td>27.8±3.1</td>
<td>0.1±0.1*</td>
</tr>
<tr>
<td>10</td>
<td>27.5±2.8</td>
<td>1.1±0.4*</td>
</tr>
</tbody>
</table>

*a Mean percentage lysis±SD from triplicate assays.
* P<0.05 compared with the control culture without effector.

Effect of MeDSG on Alloreactive CTL Generated in MLR Tests

The addition of MeDSG to the culture during the first 5 days of generation of alloreactive CTL resulted in a remarkable suppression of CTL generation (Fig. 3). In contrast, MeDSG failed to suppress the ability of generated CTL to cause the lysis of target cells when added in the cytolytic phase of the last 4 hours.

Effect of MeDSG on Cytokine Production of PBMC Stimulated with Mitogens

For PBMC cultured in the presence of PHA, MeDSG 10 μg/ml induced an increase of 24% in the release of GM-CSF and a decrease of 19% in the release of IL-6 with respect to the control culture without MeDSG (Fig. 4). The release of IL-6 without PHA was not affected by the addition of MeDSG (data not shown). MeDSG (10 μg/ml) induced a moderate 29% decrease in the ability of LPS-stimulated PBMC to release G-CSF, which was only a small amount below 1 ng/ml. After culture, the trypan-blue dye exclusion test confirmed that cell viability was greater than 95% in each experiment.
Fig. 4. Effect of MeDSG on cytokine release of mitogen-stimulated PBMC.

Data are shown as the mean±SD of triplicate cultures. *P<0.05 compared with the control culture.

Fig. 5. Effect of MeDSG on colony formation of normal bone marrow cells stimulated with G-CSF.

Data are shown as the mean±SD of quadruplicate dishes.

Effect of MeDSG on Colony Formation of Normal Bone Marrow Cells

In the absence of G-CSF, normal bone marrow buffy coat cells spontaneously formed no CFU-G colonies originating from granulocyte-committed progenitor cells, but did form a few CFU-GM colonies (mean 0.75/dish from quadruplicate cultures) originating from granulocyte/macrophage-committed progenitor cells (data not shown). The number of CFU-G colonies increased in a G-CSF concentration-dependent manner when G-CSF was added to the culture (Fig. 5). This colony formation was completely inhibited in the presence of MeDSG at a concentration as low as 1 μg/ml. In contrast, the spontaneous CFU-GM colonies were not affected by the presence of G-CSF, MeDSG or both (data not shown).

Discussion

A previous clinical study proved that DSG is effective and safe in the treatment of graft rejection in patients undergoing kidney transplantation. In this study, the maximum blood concentration of DSG in patients receiving DSG at 180 mg/m²/day was about 4 μg/ml. However, sufficient knowledge about the mechanism of the immunosuppressive effect of DSG has not been obtained, because DSG is hydrolyzed easily and its immunosuppressive activity does not last long in vitro. MeDSG, in contrast, an analogue of DSG, was confirmed to be stable in vitro and to show immunosuppressive activity both in vitro and in vivo. Unfortunately, however, MeDSG showed weaker activity than DSG in preventing graft rejection in rat skin allotransplantation. The present in vitro study was therefore done using MeDSG to help clarify the immunosuppressive mechanisms of DSG. The concentrations of MeDSG (1 and 10 μg/ml) were determined from the maximum blood concentration of DSG in the patients receiving kidney grafts described above.

We first demonstrated that MeDSG failed to suppress the blastogenic responses of PBMC in the presence of mitogens such as PHA, PWM or LPS. Further, in the allogeneic MLR tests, MeDSG inhibited the proliferative responses of lymphocytes and the generation of alloreactive CTL, but not the cytolytic activity of the established CTL. These results agreed with those previously reported. The present study newly found that MeDSG did not affect the cytolytic activity of NK cells.

The major side effect of DSG in renal transplant patients is highly reversible leukopenia. In the present study, we examined the effects of MeDSG on the production of some cytokines by mitogen-stimulated PBMC and on the colony formation of bone marrow cells. MeDSG completely inhibited the colony formation of CFU-G in the presence of G-CSF. Concerning the production of some hematopoietic cytokines by the PBMC stimulated with mitogens, MeDSG tended to partially suppress the release of IL-6 and G-CSF, and enhance the GM-CSF production. These findings imply that MeDSG directly suppresses the ability of granulocyte-committed progenitors to respond to G-CSF and form colonies. This in turn strongly suggests that the leukopenia induced by DSG treatment may be due to the direct suppression of granulocyte-committed progenitors.
References


17) Matsuguchi, T.; S. Okamura, C. Kawasaka & Y. Niho: Production of interleukin-6 (IL6) from human liver cell lines: production of IL6 is not concurrent with the production of a-fetoprotein. Cancer Res. 50: 7457-7459, 1990
