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Myeloprotective Activity of Deoxyspergualin: Influence on Splenic Colony-forming Cell Injury and Antitumor Activity of Mitomycin C in Mice

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We have examined the efficacy of deoxyspergualin (DSG) in protecting splenic colony-forming cells (CFU-S) from mitomycin C (MMC)-induced damage. The main findings of the study are as follows. (1) When DSG was administered at doses of 1.5 and 3 mg/kg for 7 days before the MMC injection, the decrease of the femoral CFU-S caused by MMC was diminished on the day after the MMC injection. The optimal dose was found to be 3 mg/kg. (2) In animals receiving 3 mg/kg DSG for at least 3 days preceding the MMC injection, the femoral CFU-S was more than 200% of that in the MMC alone group one day after the MMC injection. (3) The number of femoral CFU-S in the mice which received 3 mg/kg DSG for 3 days prior to MMC was significantly restored day by day and reached 70% of normal at 5 days after the MMC injection, while it was only 13% of normal in the MMC alone group. Moreover, the prior DSG administration significantly diminished the MMC toxicity to circulating platelets. (4) DSG administration (3 mg/kg) 3 days prior to MMC did not weaken the antitumor activity against colon 26 adenocarcinoma or P388 leukemia when compared with MMC alone. These findings have shown the ability of DSG specifically to protect the animals against bone marrow toxicity caused by MMC without interfering with the antitumor activity.

Key words: Deoxyspergualin — Hematopoietic cell — Mouse — Mitomycin C

A novel immunosuppressive agent, deoxyspergualin (DSG)[1–3] is an analogue of spergualin (SGL),[4] which is a metabolite of *Bacillus laterosporus*, and contains a spermidine moiety and a guanidinic group. Recent papers have revealed that DSG is an effective agent for preventing graft rejection in renal transplant patients.[5–7] A side effect of DSG treatment in these patients was the appearance of a peripheral leukopenia, which was largely reversible. This observation is in accordance with our previous report[8] that the mice successively given DSG at an immunosuppressive dose of 6.25 mg/kg developed leukopenia during the administration but showed rapid rebound leukocytosis after the termination of DSG administration. The number of bone marrow cells in the femur also decreased during the administration of DSG, while the femoral stem cell compartments such as pluripotent stem cells (colony-forming units in spleen, CFU-S) and granulocyte/monocyte-committed stem cells did not decrease. This finding indicated that the leukopenia caused by DSG was not initiated by a generalized killing of hematopoietic stem cells, but by the suppression of their ability to proliferate.[8,9] The inhibition of stem cell proliferation by DSG is of great interest because of the possible application of DSG to protect against bone marrow injury associated with cancer chemotherapy.

The purpose of the present study was to evaluate the ability of DSG to protect against CFU-S toxicity caused by a single dose of mitomycin C (MMC), as well as to examine the effect of DSG on MMC anticancer activities.

MATERIALS AND METHODS

Animals Female C3H/HeN mice were purchased from Shizuoka Laboratory Animal Center, Shizuoka, and used at 8 to 11 weeks of age. Female BALB/c and DBA/2 mice from Charles River Japan, Atsugi, Kanagawa, were used at 8 weeks of age.

Agents DSG (1-amino-19-guanidino-11-hydroxy-4,9,12-triazanonadecane-11,13-dione trihydrochloride)[10] was supplied by Takara Shuzo Co., Ltd., Kyoto. It was dissolved in saline and sterilized by passing the solution through a 0.22-μm filter. MMC was purchased from Kyowa Hakko Kogyo Co., Ltd., Tokyo.

Hematological examinations Femurs obtained from three to five C3H mice per experimental group were pooled. Femoral single-cell suspensions were prepared in Hanks solution after repeated passage of the cells through a 23-gauge needle, and counted using Turk solution. Assay for CFU-S was performed according to the method of Till and McCulloch.[11] Briefly, 0.5 to 1×10^5 of the pooled bone marrow cells (BMC) described above were intravenously (iv) injected into 6 to 8 syngeneic mice that had previously received whole-body X-irradiation at a dose of 950 rad (Hitachi MBR-1505R X-ray unit; 140 kV, 4.5 mA, 2-mm aluminum). Seven

days later, the spleens were removed and fixed with Bouin's solution, and the surface colonies were counted.

Tumors Colon 26 adenocarcinoma and P388 leukemia cells were maintained in solid and ascitic forms in BALB/c and DBA/2 mice, respectively.

Statistical analysis Statistical analysis of the data was performed with Student's *t* test or the Mann-Whitney U test.

RESULTS

Dose and timing of DSG for effect on CFU-S toxicity of MMC At first, DSG was administered at a daily dose of 6 mg/kg, which is a quarter of the LD_{50} value, before a single injection of MMC. As shown in Fig. 1, there was a significant diminution of the decrease in CFU-S/5×10^4 BMC in the animals receiving DSG at times preceding the MMC injection, in comparison with 1.5 or 3 mg/kg MMC only. We performed further experiments using 3 mg/kg MMC to examine the DSG dose-dependency and timing. DSG was administered at doses of 1.5, 3 and 6 mg/kg for 7 days before the MMC injection (Fig. 2). Both DSG and MMC decreased the number of BMC, so that the BMC number in the MMC plus DSG group was lower than that of MMC alone (data not given). However, a significant diminution of the decrease of CFU-S/10^5 BMC was noted in the mice receiving DSG at all doses used. The CFU-S/femur obtained from the mice given 1.5 and 3 mg/kg DSG were 1.9- and 2.5-fold higher compared with the MMC alone group, respectively. The optimal dose of DSG was 3 mg/kg. Then, the myeloprotective effect of DSG (3 mg/kg) was investigated using different numbers of DSG administrations (one

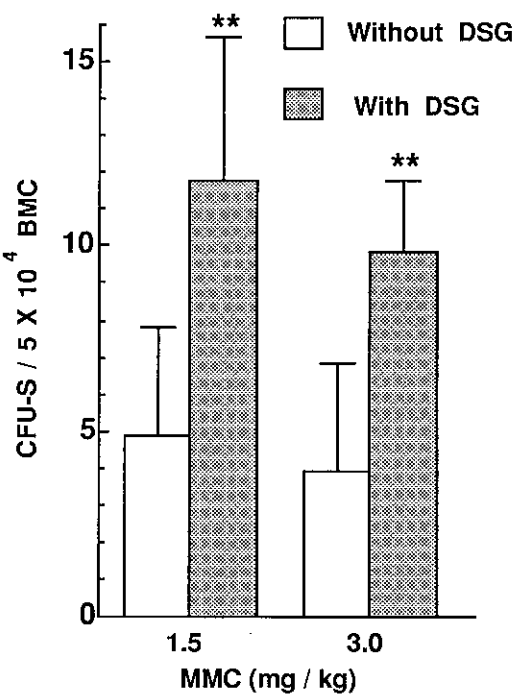

Fig. 1. Effect of prior DSG administration on CFU-S toxicity induced by a single injection of MMC. DSG was ip administered at a dose of 6 mg/kg once a day for 7 days, and MMC was iv injected at a dose of 1.5 or 3 mg/kg on the day of the final administration of DSG. On the day after the MMC injection, the femurs were removed and subjected to the CFU-S assay. Each bar shows the mean with SD. Values of CFU-S/5×10^4 BMC in the DSG alone and normal groups were 35 ± 4 and 18 ± 3, respectively. ** $P<0.01$ compared with MMC alone (Student's *t* test).

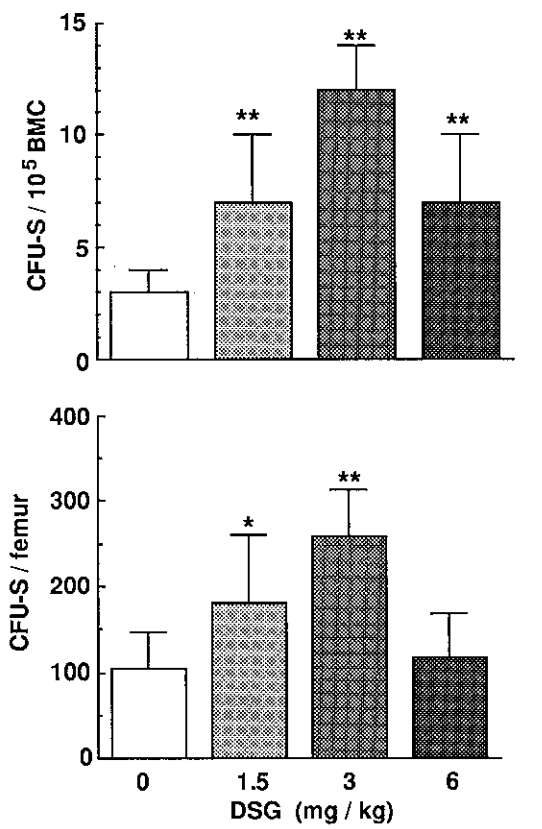

Fig. 2. Dose responses of prior DSG administration for CFU-S injury induced by a single injection of MMC. DSG was ip administered at doses of 1.5, 3 and 6 mg/kg once a day for 7 days, and MMC was iv injected at a dose of 3 mg/kg on the day of the final administration of DSG. Other experimental procedures were the same as in the legend to Fig. 1. Each bar shows the mean with SD from 6 to 8 animals/group. Normal levels: CFU-S/1×10^5 BMC$=35\pm9$; CFU-S/femur$=2091\pm519$. * $P<0.05$, ** $P<0.01$ compared with MMC alone (Student's *t* test).

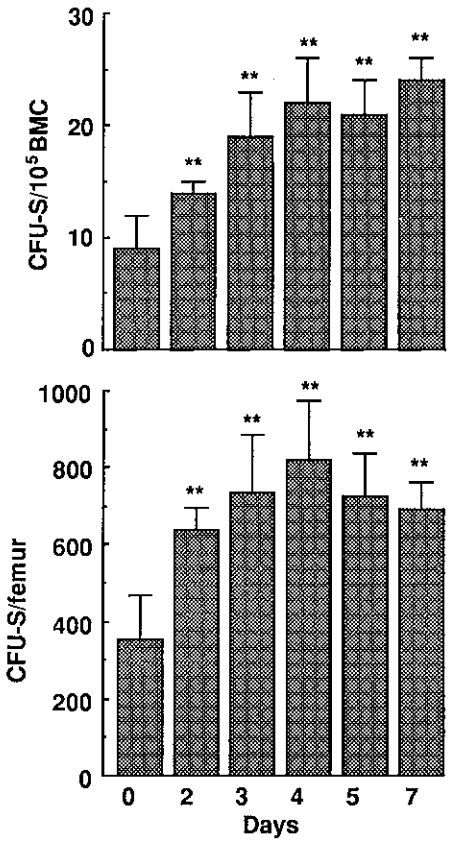

Fig. 3. Effect of the number of prior DSG administrations upon CFU-S injury induced by a single injection of MMC. DSG was ip administered at a dose of 3 mg/kg once a day for 2, 3, 4, 5 and 7 days. MMC was given as described in the legend to Fig. 2. Other experimental procedures were the same as in the legend to Fig. 1. Each bar shows the mean with SD from 6 to 8 animals per group. Normal levels: CFU-S/1×10^5 BMC= 44±7; CFU-S/femur = 2427 ± 394. ** $P<0.01$ compared with MMC alone (Student's t test).

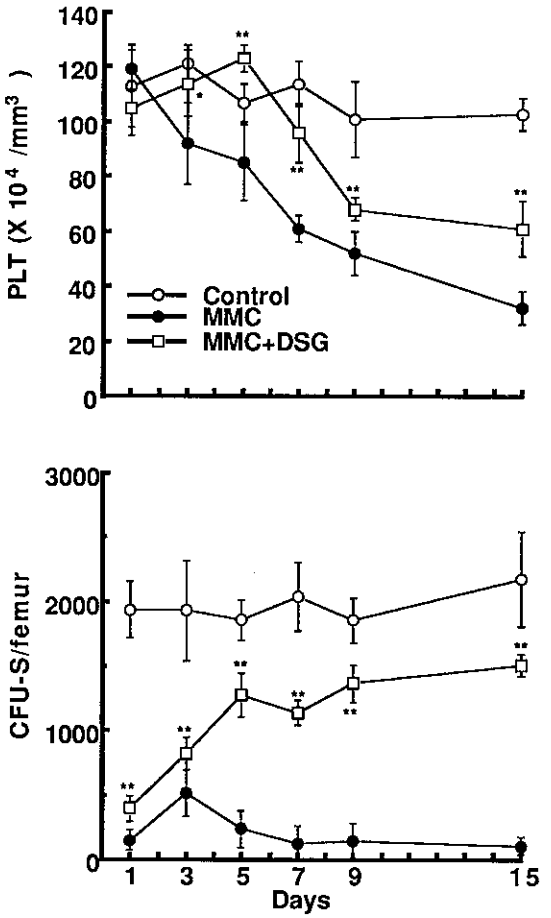

Fig. 4. Effect of prior DSG administration on recovery of bone marrow CFU-S and peripheral PLT after MMC injection. DSG was ip administered at a dose of 3 mg/kg once a day for 3 days. MMC was given as described in the legend to Fig. 2. On the indicated days after MMC injection, the blood and femurs were obtained, and subjected to the circulating blood cell counts and the CFU-S assay. The number of peripheral blood cells was counted using a Celltac MEK-4500 standard hemocytometer (Nihon Koden Co., Ltd., Tokyo). Each symbol shows the mean with SD from 6 to 8 animals per group. Control (○), MMC alone (●), MMC+DSG (□). * $P<0.05$, ** $P<0.01$ compared with MMC alone (Student's t test).

Table I. Femoral CFU-S Contents Following DSG Administration after a Single Injection of MMC

Days after MMC injection	CFU-S/femur	
	MMC alone	MMC plus DSG
Day 4	620±114	564±122
Day 13	460±123	202±43**
Day 20	1507±122	840±156**

MMC (3 mg/kg) was iv injected on day 0. DSG was ip administered at a dose of 3 mg/kg once a day on days 1 to 3. On days 4, 13 and 20, the femurs were removed and subjected to the CFU-S assay. Each value is shown as the mean with SD. Normal levels: BMC=7.78×10^6 cells/femur; CFU-S/femur= 2179±180. ** $P<0.01$ compared with MMC alone (Student's t test)

dose per day) (Fig. 3). Both CFU-S/10^5 BMC and CFU-S/femur doubled compared with MMC alone, when DSG was given for at least 3 days before MMC. Table I depicts the total CFU-S following the administration of DSG, 3 mg/kg, at times following the MMC injection. DSG significantly decreased the CFU-S/femur compared with MMC alone.

Restoring effect of DSG on CFU-S and peripheral blood cells after MMC injection We examined the ability of

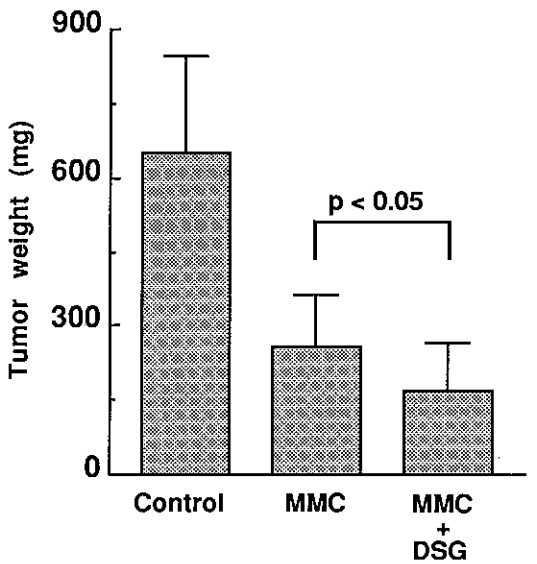

Fig. 5. Influence of DSG on antitumor activity of MMC against colon 26 adenocarcinoma. BALB/c mice were subcutaneously transplanted with 1×10^5 viable cells of colon 26 tumors on day 0, and randomly divided into 3 groups. Each group consisted of 13 or 14 mice. DSG was ip administered at a daily dose of 3 mg/kg on days 1 to 3. A single iv injection of MMC (3 mg/kg) was performed on day 3 (the day of the final administration of DSG). The mice were killed on day 11, and the tumor weights were measured. Each bar shows the mean with SD. Data were analyzed by using Student's *t* test.

DSG to restore the femoral CFU-S contents and peripheral blood cell counts following MMC administration (Fig. 4). The CFU-S/femur obtained from the MMC plus DSG group was significantly restored on each day, being 70%, 56%, 74% and 70% of normal on 5, 7, 9 and 15 days after the MMC injection, respectively. In contrast, the CFU-S/femur in the MMC alone was low (5% to 25% of normal) on all days examined. The circulating platelet (PLT) counts gradually decreased with time. The animals given DSG prior to MMC showed significantly higher PLT counts than the MMC alone group at 3, 5, 7, 9 and 15 days after the MMC injection. There was no difference in the number of peripheral red and white blood cells between the MMC alone group and the MMC plus DSG group in any time point examined (data not given).

Influence of DSG on tumoricidal activity of MMC We examined whether the prior administration of DSG weakened the antitumor activity of MMC against colon 26 adenocarcinoma and P388 leukemia. As shown in Fig. 5, a dose of 3 mg/kg MMC, administered alone or after DSG, showed significant inhibition ($P<0.01$) of colon 26 tumor growth. Interestingly, the mean tumor weight

Table II. Influence of DSG on Antitumor Activity of MMC against P388 Leukemia

Inoculum size of P388 cells	Group	Mean survival days with SD		
3×10^4	Control	10.1±0.3		
	MMC	12.5±0.5**		
	DSG	10.5±0.5*	$P<0.01$	$P<0.01$
	DSG+MMC	13.0±0.0**		
1×10^5	Control	8.3±0.5		
	MMC	11.3±0.5**		
	DSG	9.7±0.5**	$P<0.01$	$P<0.05$
	DSG+MMC	12.0±0.7**		

DBA/2 mice were iv transplanted with P388 leukemia cells on day 0 as indicated. The mice were randomly divided into 4 groups. Each group consisted of 10 or 11 mice. DSG was ip administered at a dose of 3 mg/kg once a day on days 1 to 3. MMC was iv injected at a dose of 3 mg/kg on day 3. Data were analyzed by means of the Mann-Whitney U test.
$*P<0.05$, $**P<0.01$ vs. control.

in the MMC plus DSG group was even smaller than that in the MMC alone group. We further examined the influence of DSG pre-administration on the antitumor activity of the same dose of MMC against P388 leukemia (Table II). DSG alone, MMC alone and DSG plus MMC resulted in a significant prolongation of the mean survival time. The combination of MMC and DSG was significantly more effective than either MMC or DSG alone.

DISCUSSION

The present study proved that DSG can reduce the severity of CFU-S toxicity in murines induced by a single dose of MMC. When the influences of dose and administration time of DSG for protection of animals against the CFU-S toxicity of MMC were investigated, it was found that the mice given 3 mg/kg DSG for 3 days prior to the MMC injection showed values of more than 200% for either CFU-S/10^5 BMC or CFU-S/femur compared with the MMC alone group (Fig. 3). Using the same administration schedule, it was further demonstrated that DSG not only accelerated the recovery of the bone marrow CFU-S but also diminished the degree of thrombopenia after the MMC injection (Fig. 4). In contrast, the administration of DSG following the MMC injection enhanced the CFU-S toxicity of MMC, as expected (Table I).

DSG was found to have *in vivo* antitumor activity against mouse L1210 and P388 leukemias, and to a lesser extent, against mouse solid tumors.[12, 13] To be clinically useful as a myeloprotective agent, DSG must provide protection against bone marrow injury caused by

chemotherapeutic drugs without interfering with their antitumor activity. DSG was demonstrated to fulfill this criterion, because the administration of DSG prior to MMC did not weaken the antitumor activity of MMC against colon 26 adenocarcinoma or P388 leukemia (Fig. 5, Table II).

A recent approach to overcome chemotherapy-induced bone marrow injury has been to administer hematopoietic stimulating factors such as colony-stimulating factor (CSF).[14] Since the role of CSF is to enhance the restoration of neutropenia by accelerating the proliferation of surviving hematopoietic stem cells after treatment with cytotoxic drugs, CSF needs to be applied following chemotherapy. In contrast, the role of DSG is to minimize the damage to these stem cells by keeping them in a drug-resistant stage during the treatment with cytotoxic drugs, so that DSG needs to be used before chemotherapy. The prior application of DSG combined with the post-chemotherapy application of CSF may therefore be expected to prevent the decrease of peripheral blood cells. The great difference between DSG and CSF is that DSG can prevent thrombopenia caused by cancer chemotherapy, whereas CSF can not.

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