EFFECTS OF DEOXYSPERGUALIN ON HEMATOPOIESIS: STUDIES OF MURINE HEMATOPOIETIC PROGENITOR CELL AND PERIPHERAL BLOOD CELL LEVELS

KYUICHI NEMOTO, MICHIKO HAYASHI, YUMI SUGAWARA, FUMINORI ABE, TOMOHISA TAKITA and TOMIO TAKEUCHI†

Research Laboratories, Pharmaceuticals Group, Nippon Kayaku Co., Ltd., 3-31-12 Shimo, Kita-ku, Tokyo 115, Japan
†Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

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The effect of a novel immunosuppressive agent, deoxyspergualin (DSG), on hematopoiesis in mice was studied with measurements of peripheral blood counts and assays of granulocyte-monocyte colony-forming cells (cfu-C) and spleen colony-forming cells (cfu-S) in bone marrow, during and after successive intraperitoneal administration of DSG. When DSG was administered at a strong immunosuppressive dose of 6.25 mg/kg daily for 15 days, mice developed significantly decreased peripheral blood counts and decreased bone marrow cells (BMC) during administration. After the completion of DSG administration a marked rebound in leukocytosis was observed and BMC returned to normal. In contrast, total cfu-C in the femur significantly increased during the DSG administration, and subsequently returned to normal. Moreover, total cfu-S in the femur were normally sustained in contrast with a decrease of BMC during the DSG administration. These findings suggest that DSG does not show generalized overt cytotoxicity against hematopoietic stem cells and freezes the ability of the stem cell in the proliferation or the differentiation.

Deoxyspergualin (DSG) is a derivative of spergualin, a metabolite of *Bacillus laterosporus*[1]. We previously reported that this derivative[2], with a molecular weight of 497 and a moiety bearing a spermidine and a guanidinic group, suppressed the development of graft-versus-host disease in mice[3] and the rejection of skin allografts in rats[4]. DSG was also found to ameliorate ongoing rejection in rat heart[5] and dog kindey[6] allo-transplantation, and advancing graft-versus-host disease in mice[7]. Furthermore, we found that DSG enhances the function of spleen cells in blastogenic response, and the release of interleukins 1 and 2 in skin allografted rats[8]. DICKNEITE *et al.* reported that DSG suppressed the function of monocytes in the release of lysosomal enzymes and superoxide anions in rats given skin allografts[9], while MASUDA *et al.* showed that administration of DSG did not impair the function of peritoneal macrophages in normal mice[10]. The mechanisms responsible for the immunosuppression elicited by DSG remain to be unravelled. Since these data were very encouraging we decided to evaluate the effect of DSG on the hematopoietic system, which could be the main dose-limiting factor of the new drugs.

Materials and Methods

Animals

Female C3H/He mice, 9 to 11 weeks old, from Shizuoka Laboratory Animal Center, Hamamatsu, Japan, were used.

DSG

DSG was supplied from Takara Shuzo Co., Ltd., Kyoto, Japan. It was dissolved in saline and

sterilized by passing through a 0.22-μm filter.

Counts of Peripheral Blood Cells and Bone Marrow Cells (BMC)

Peripheral red blood cells (RBC) and white blood cells (WBC) were counted using a standard hemocytometer. WBC differentials were determined by enumerating 100 or 200 cells on May-Gruenwald-Giemsa-stained smears. Femoral marrow cells were counted using a Türk solution.

Assay of Granulocyte-monocyte Colony-forming Cells (cfu-C)

The number of cfu-C was determined as previously reported[11]. Femoral cells (1×10^5 cells/ml/dish) were incubated in McCoy's 5A medium containing 0.32% agar, fetal bovine serum 20% and L_{929} cell 5% conditioned medium in a 35-mm petri dish. After the incubation at 37°C under 5% CO_2 in air for 7 days, aggregates of 50 or more cells were counted as colonies.

Assay of Spleen Colony-forming Cells (cfu-S)

The spleen colony assay of Till and McCulloch[12] was used. Briefly, irradiation was delivered by a Hitachi MRB-1505R X-ray unit (140 kV, 4 mA) through 2-mm aluminum. C3H mice that had been irradiated with a lethal dose of 950 rad iv received 5×10^4 femoral cells from syngeneic mice. Spleens were removed 7 days after grafting and fixed in Bouin's solution in which the macroscopically visible nodules were counted.

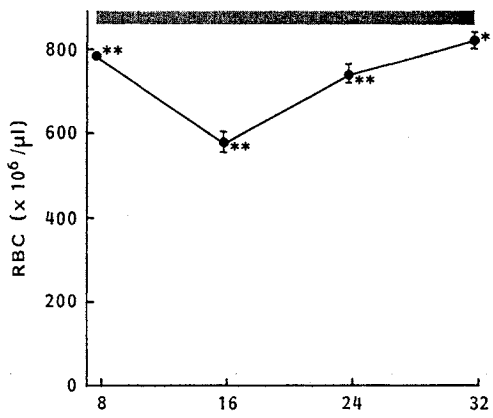
Statistical Analysis

Data are analyzed by Student's t-test.

Results

In time-related experiments, DSG was administered ip at a dose of 6.25 mg/kg daily for 15 days (day 1 to 15). The following hematological parameters were examined on day 8, 16, 24 and 32: RBC, WBC including cell differentiation, BMC and cfu-C. As shown in Fig. 1, the mice developed significant erythrocytopenia (34% decrease compared to control) on day 16. By day 32 the decreased RBC counts gradually recoverd to near normal. WBC counts gradually declined, and were 38% less than control by day 16. A significant rebound leukocytosis (315% of control) was observed on day 24 (Fig. 2A). A similar rebound was observed in the granulocyte counts on day 24 (Fig. 2B). Among WBC counts lymphocyte counts were reduced by only 20 to 37% of control between day 16 and 32 (Fig. 2C). Thus, the leukocytosis mainly depended on granulocytosis. BMC counts in the femur markedly decreased to 34 and 31% of control on day 8 and 16, respectively (Fig. 3A). By day 24, BMC counts had returned to normal. Of greater concern, cfu-C counts per 10^5 BMC (cfu-C ratio) greatly increased to 609 and 333% of control on day 8 and 16 (data not shown), respectively, so that total cfu-C counts in the femur significantly increased on day 8 (214% of control) and 16 (108% of control). After completion of the

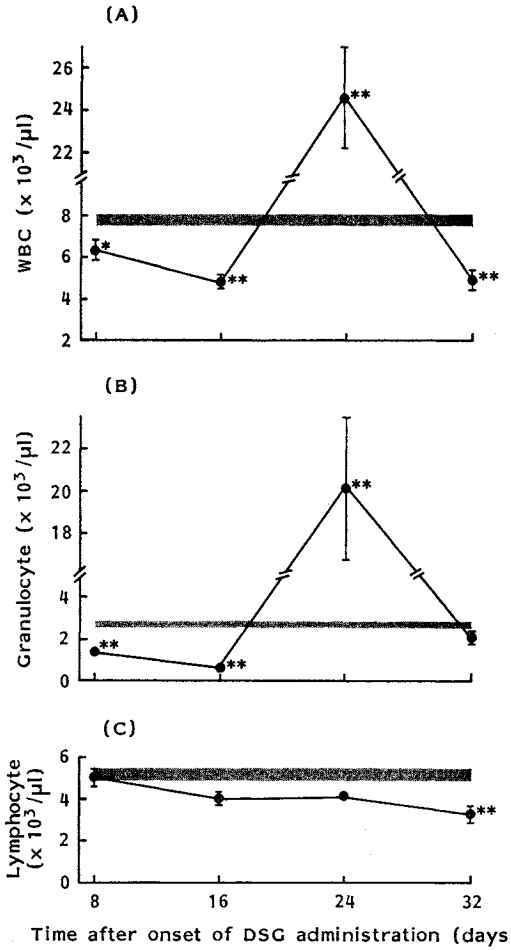
Fig. 1. RBC counts during and after administration of DSG (6.25 mg/kg).



DSG was administered ip at a dose of 6.25 mg/kg once a day for 15 days (day 1 to 15). A set of control mice received saline. Each point from 8 mice is shown as mean with SE. Shaded area represents mean with SE of all control values ($(878\pm11)\times10^6$ cells/μl, $n=32$).

* $P<0.05$, ** $P<0.01$.

Fig. 2. WBC (A), granulocyte (B) and lymphocyte (C) counts during and after administration of DSG (6.25 mg/kg).



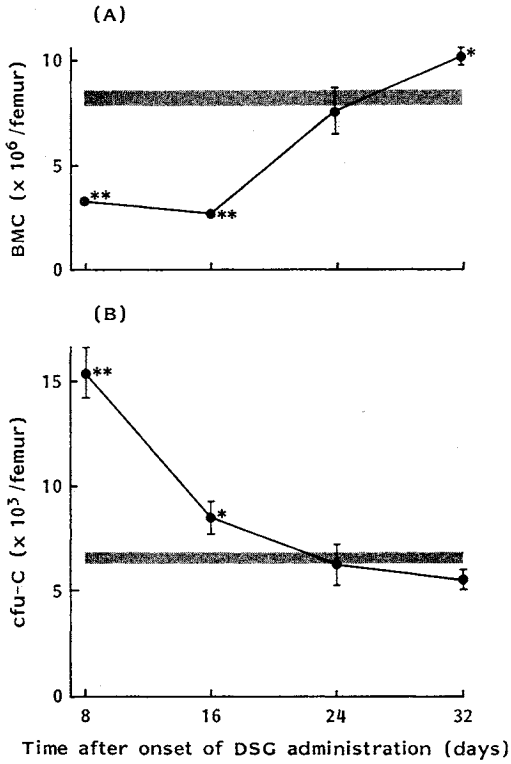
All experimental procedures were the same as described in the legend of Fig. 1. Each shaded area represents mean with SE of control values for WBC=(73±3)×10² cells/μl (n=32); granulocytes =(27±1)×10² cells/μl (n=26); lymphocytes=(51 ±3)×10² cells/μl (n=26).

* P<0.05, ** P<0.01.

DSG administration the total cfu-C counts returned to normal (Fig. 3B).

In the femur increases in the total cfu-C counts were demonstrated in contrast with the

Fig. 3. BMC (A) and total cfu-C (B) counts in the femur during and after administration of DSG (6.25 mg/kg).



All experimental procedures were similar to the legend of Fig. 1. Each point from 5 mice is shown as mean with SE. Each shaded area from 20 mice represents mean with SE of control values for BMC=(8.2±0.4)×10⁶ cells/femur and total cfu-C=(6.7±0.3)×10³ cells/femur.

* P<0.05, ** P<0.01.

Table 1. Hematological findings in the femur from mice administered DSG.

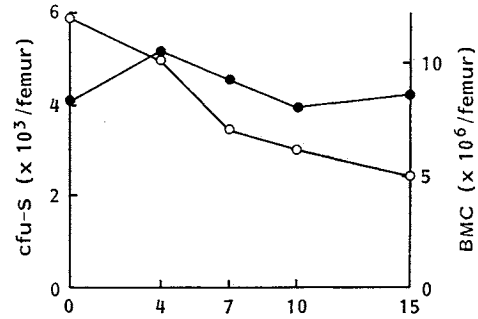
DSG (mg/kg)	BMC counts (×10⁶/femur)	cfu-C counts (/10⁵ BMC)	cfu-C counts (×10³/femur)
0	9.6±1.4	76±11	7.2±0.7
0.78	8.5±0.7	107±3**	9.1±0.6**
1.56	8.2±1.2	115±15**	9.4±1.0**
3.13	6.2±0.6*	250±31**	15.3±0.8**
6.25	3.3±0.5**	462±34**	15.4±2.6**

DSG was administered ip daily for 7 days. Hematological examinations were performed on the day after the 7th administration of DSG. Data are shown as mean with SD from 5 mice.

* P<0.05, ** P<0.01.

decreases in the BMC counts. Therefore, further examinations were carried out using various doses of DSG. The results are shown in Table 1. DSG was given at doses of 0.78 to 6.25 mg/kg for 7 days. BMC counts in the mice administered with DSG at doses of 3.13 and 6.25 mg/kg significantly decreased, while a significant increase in both cfu-C ratio and total cfu-C in the femur was observed at doses of 0.78 to 6.25 mg/kg in a dose-dependent manner. Within this range, there was a significant decrease in RBC, WBC and granulocyte counts (data not shown).

Fig. 4. cfu-S (●) and BMC (○) levels in the femur of mice receiving DSG.



DSG was administered ip at a daily dose of 6.25 mg/kg for 15 days (day 1 to 15) to 4 donor mice. As indicated, the femoral marrow cells were removed and pooled per each group, then 5×10^4 cells were injected iv into 6 to 10 lethally irradiated recipients. Data are shown as mean.

In a second series of experiments, the *in vivo* effect of DSG against pluripotent stem cells, measured as cfu-S, was examined. DSG was administered ip at a daily dose of 6.25 mg/kg for 15 days (day 1 to 15) to donor mice. On day 4, 7, 10 and 15 femoral marrow cells were removed from the mice and assayed for cfu-S. As shown in Fig. 4, in the femur the total cfu-S counts were sustained normally in contrast with the decrease in the BMC during the administration of DSG.

Discussion

DSG was demonstrated to possess a strong immunosuppressive action in many experimental models for both humoral and cell-mediated immunity in several animal species, when successively administered in doses over 1 mg/kg. In the present study, we examined whether immunosuppressive doses of DSG affected hematopoiesis in mice.

During the administration of DSG (6.25 mg/kg daily for 15 days), peripheral blood and BMC counts gradually decreased with time. However, a marked rebound increase in WBC counts was observed following the completion of DSG administration. The rebound was due to the marked increase in the peripheral granulocyte counts. Despite the marked decrease in BMC counts, a significant increase in total cfu-C counts in the femur was found during the administration of DSG. Similarly, a decrease in the total cfu-S counts in the femur did not occur in mice administered DSG at a dose of 6.25 mg/kg for 15 days. Finally, during the administration of DSG, although peripheral blood cell and BMC counts significantly decreased, there was an insignificant decrease of hematopoietic stem cells.

In general, decrease in peripheral blood cell counts which follows the administration of various cytocidal drugs is initiated by a killing of hematopoietic stem cells[13~15]. Our data strongly indicate that perturbation of peripheral hematopoiesis which follows the administration of DSG is not initiated by a generalized killing of hematopoietic progenitors, and that DSG does not kill the cells and is freezing their ability to proliferate and differentiate.

In conclusion, DSG does not show generalized overt cytotoxicity against hematopoietic stem cells. Although it is possible that a decrease of peripheral blood cell counts induced by the long-term administration of DSG could become the main dose-limiting factor in clinical use, its effect is very reversible.

Acknowledgments

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