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Inhibition of Nitric Oxide Synthase Induction by 15-Deoxyspergualin in a Cultured Macrophage Cell Line, J744A.1 Activated with IFN-γ and LPS

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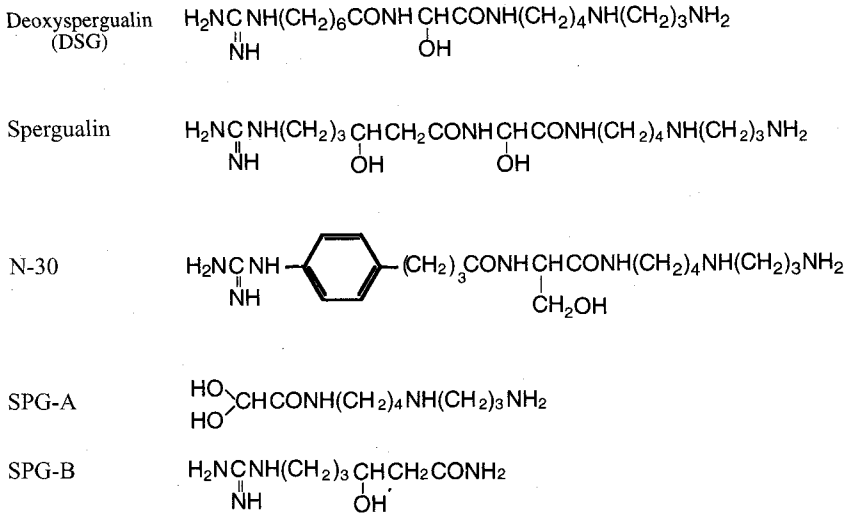
Immunosuppressant 15-deoxyspergualin (DSG) inhibited induction of inducible nitric oxide synthase (iNOS) following stimulation with IFN-γ and LPS in a cultured macrophage cell line, J744A.1. By DSG treatment NO^{2-} accumulation in the medium was blocked, and cellular iNOS protein level decreased as shown by Western blotting.

DSG didn't have any direct effect on iNOS activity. DSG was not used as a substrate of NOS in *in vitro* enzyme systems, and it was too weak an inhibitor of iNOS and cNOS to cause the inhibition of accumulation of NO^{2-}. DSG did not scavenge NO spontaneously generated from NOR.

Structure-activity relationships of analogs and decomposed elements showed that there is correlation between the inhibition of iNOS induction and immunosuppressive activity.

DSG[1] is a more active derivative of spergualin (SPG), an antitumor antibiotic produced by a strain of *Bacillus laterosporus*[2]. DSG has strong suppressive effects on both humoral and cell-mediated immune responses in animals[3~5]. Now it has been used in renal transplantation as a novel immunosuppressant named Gusperims. In spite of its therapeutic effectiveness the mechanism of action of DSG has not been clear. DSG does not suppress IL-2 production[5,6] unlike other immunosuppressants such as cyclosporin A, FK506, and rapamycin. In this paper we aimed at determining interaction of DSG with nitric oxide (NO) functions. DSG is composed of guanidinoheptanoic acid amide and glyoxylspermidine moieties as shown in Fig. 1. DSG might be a substrate or an inhibitor of NOS because of the guanidino group. DSG might be a trapper of NO because of the spermidine moiety. Polyamines are known to react with NO and these zwitterionic polyamine/NO adducts decompose with release of NO[13,14]. Moreover the effect of *in vivo* administration of DSG in animals suggested to us a role of NO in DSG action. DSG has protective effect for irradiation to whole animals[7]. The effect is similar to those of N^G-nitro-L-arginine (NNA, NOS inhibitor) and NO-releasing agent[8,9]. DSG has stronger immunosuppressive effects in normal animals than in tumor-bearing animals[10]. The latter are thought to be in a stage activated immunologically by implantation of cancer cells, when NO generation by macrophages may be induced. The difference of immunosuppressive activity of DSG on normal and tumor-bearing conditions may be explained by that of NO level. IL-2 receptors (IL-2R) in popliteal lymph node cells disappeared without loss of cell numbers by administration of DSG[6]. Transcription of IL-2R α is activated by NF-κB like iNOS[11]. Expression of IL-2R by splenocyte is down-regulated by activated Kupffer cell-derived NO[12]. Thus we tried to determine the effect of DSG on NO generation in macrophages after stimulation with IFN-γ and LPS. DSG inhibited NO generation in the cells and cellular iNOS activity decreased with DSG treatment. We examined the mechanism of the inhibition of NO generation by DSG using an *in vitro* enzyme assay system. DSG is not active as a substrate or a NO scavenger, and is only a very weak inhibitor of NOS. Western blotting of iNOS showed decrease of cellular iNOS protein levels. Thus the inhibitory effect of cellular iNOS activity by DSG was shown to be caused by inhibition of iNOS induction.

Fig. 1. Structure of DSG and related compounds (DSG, SPG, and N-30 are active as immunosuppressant, but SPG-A and SPG-B are inactive decomposition fragments of SPG).



Materials and Methods

Chemicals

DSG and N-30 were provided from Takara Shuzou Co. Ltd. and Nippon Kayaku Co. Ltd., respectively. SPG and its analogs were prepared by ourselves[2,15]. Inducible NOS, cNOS, and NOR4 were obtained from Dojindo Laboratories, Japan. IFN-γ, LPS, N^G-monomethyl-L-arginine acetate (NMMA) and NNA were from Sigma Chemicals Co. St. Louis, U.S.A. Rabbit polyclonal antibody NOS2 and horseradish peroxidase conjugated with anti rabbit IgG were from Santa Cruz Biotechnology, CA, U.S.A. All the other reagents used were special or cell culture grade.

Cell Culture and Treatments

J744A.1 macrophage cell line was given by the Japanese Cell Bank. J744A.1 cells were cultured in RPMI1640 medium supplemented with 10 % fetal bovine serum. Cells were plated in 96-well culture plates at density of $10^4/100\,\mu l$. Cell growth was determined by counting cell number with a Coulter Counter. The macrophage cells were activated by addition of IFN-γ (50 u/ml) and LPS (0.35 ng/ml) for 20 hours.

Nitrite Measurement

Nitrite accumulation in the cell culture medium was measured by DAN assay[16] using freshly prepared sodium nitrite as a standard. Mixture of each 50 μl aliquot from duplicate wells was incubated with 10 μl of 2.3-diaminonaphtalenene (0.05 mg/ml in 0.62 N HCl) for 10 minutes at room temperature. After addition of 5 μl of 2.8 N HCl, fluorescence intensity (Em: 450 nm, Ex: 365 nm) was measured using a Cytofluor 2300 multiwell fluorescence plate reader (Millipore Co.).

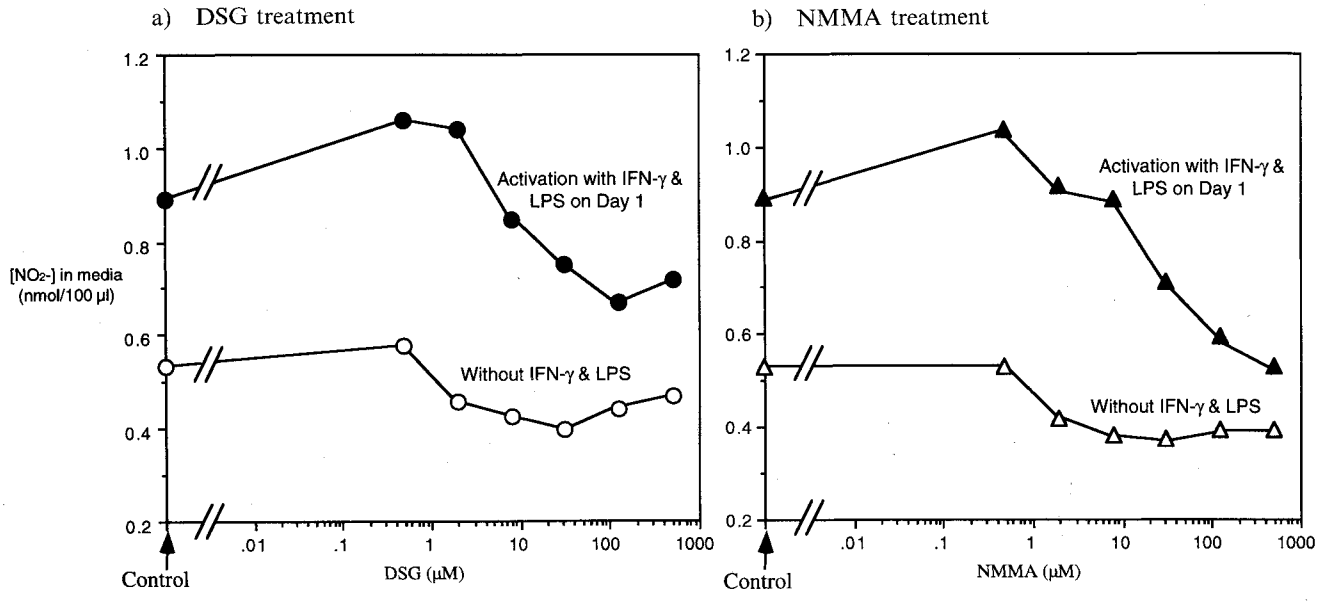
Assay of Nitric Oxide Synthase

Cellular iNOS activity was determined, after removal of media from each well and washing with PBS twice, by incubation with 100 μl of 100 mM L-arginine in PBS with 2 mg/ml glucose for 3 hours at 37°C. After incubation nitrite generated spontaneously from NO was determined using a 50 μl aliquot from the supernatant of each well as described in nitrite measurement.

Incubation mixture (50 μl) for purified iNOS contained 5 mM L-arginine and/or DSG, 100 μM NADPH, 12 μM tetrahydrobiopterin, 167 μM DTT, and 100 units/ml of enzyme in 50 mM HEPES (pH 7.4). That for cNOS (50 μl) contained 50 μM L-arginine and/or DSG, 1 mM $CaCl_2$, 20 μm/ml calmodulin, 120 μM NADPH, 12 μM tetrahydrobiopterin, 167 μM DTT, and 100 units/ml of enzyme in 50 mM HEPES (pH7.4). The reaction was started by addition of enzyme, continued for 30 minutes at 37°C, and generated nitrite was determined by DAN assay.

Fig. 2. Inhibition of NO generation in J744 A.1 cells by DSG and NMMA.

Effect of DSG (a) and NMMA (b) on [NO^{2-}] accumulation in J744A.1 cells with or without activation by IFN-γ and LPS were determined as described in the text.



Assay of NO Scavenging Activity by DSG

Scavenging activity of NO by DSG was examined to determine if the presence of DSG disturbed determination of spontaneous release of NO from NOR4 at pH 7.1. NO release was started by the addition of 10 μl of 10 mM NOR4 solution in DMSO to 190 μl of PBS (pH 7.1) at 37°C. The release was observed for 60 minutes by determining nitrite for 10 μl aliquots at intervals of 10 minutes by DAN method.

Western Blot Analysis of iNOS

Cell lysis and western blotting were carried out according to "Current Protocols in Molecular Biology"[17]. Cells treated with DSG were washed with PBS, collected by centrifugation at 1,000 $\times g$ and resuspended in RIPA buffer (PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml PMSF, 1 mM antipain, and 1 mM leupeptin). Then the cells were homogenized by passage through a 23 gauge needle and sonication. The homogenates were incubated on ice for 30 minutes and microcentrifuged at 15,000 $\times g$ for 20 minutes at 4°C. Cell lysates were mixed with an equal volume of commercial sample buffer (0.25 M Tris-HCl, 2% SDS, 30% glycerol, 10% βME, 0.01% BPB, pH6.8, Daiichi Pure Chemicals, Tokyo, Japan) and heated to 95°C for 2 minutes. After heating, 13 μg of the protein per lane was loaded and resolved on 7.5% SDS/PAGE using minigel. After electrophoresis, the protein was electrotransferred to PVDF membrane (Millipore, Bedford, U.S.A.). The membranes were blocked with 1% BSA/ 0.2% Tween 20 in 10 mM Tris-buffered saline (pH 8.0) and then probed with 1 μg/ml rabbit polyclonal antibody NOS2, and visualized with horseradish peroxidase conjugated with anti rabbit antibody (1 : 10000 dilution) on X-ray film.

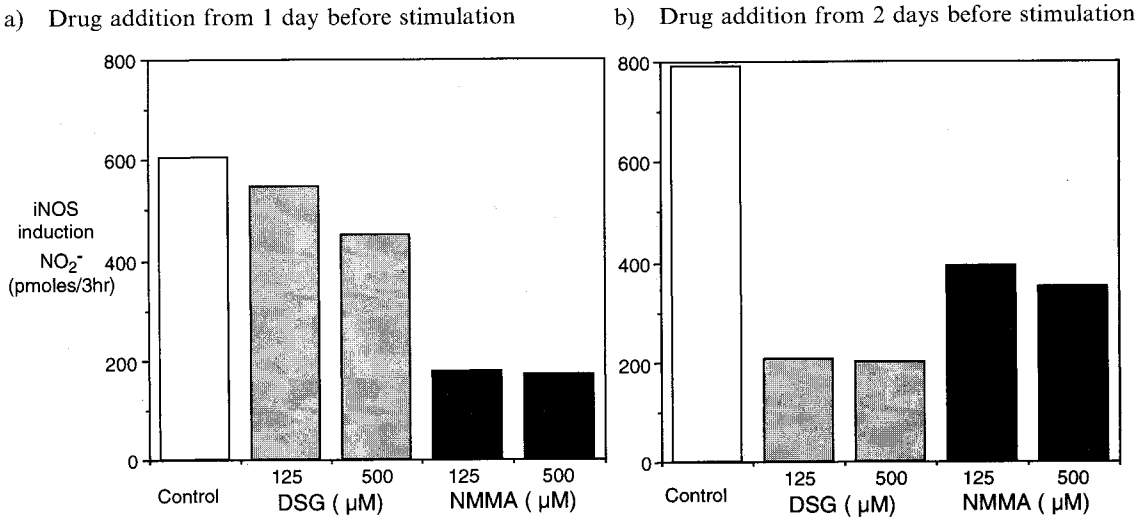
Results

Inhibition of Nitrite Accumulation by DSG in Macrophage Cells Activated by IFN-γ and LPS

J744A1 cells were treated 3 hours after inoculation by various concentrations of DSG or NMMA. After 48 hours IFN-γ and LPS was added for the stimulation group. 24 hours after stimulation, nitrite accumulated in culture media was determined as described in materials and methods. During the 72 hours incubation cell number in the absence or presence of IFN-γ and LPS increased to 16.04×10^4/well and 6.9×10^4/well, respectively. Nitrite in 100 μl of culture media without DSG or

Fig. 3. Decrease of cellular iNOS activity by DSG treatment.

J744.A1 cells were pretreated with DSG from 24 hours (a) and 48 hours (b) before addition of INF-γ (5 units.ml) and LPS (35 ng/ml). At 20 hours after stimulation cellular NOS activity was determined as decribed in "Materials and Methods".



NMMA treatment increased from 0.538 nmoles to 1.64 nmoles by addition of IFN-γ and LPS. DSG treatment significantly decreased the nitrite accumulation dose-dependently as shown in Fig. 2, a). NMMA, an irreversible inhibitor of NOS gave the same result through direct inhibition of iNOS (Fig. 2, b)).

Inhibition of iNOS Activity by DSG in Macrophage Cells Activated by IFN-γ and LPS

Cellular NOS activity in activated macrophage cells pretreated with DSG was determined after removing the media and washing (Fig. 3). Pretreatment of DSG from 24 hours before stimulation showed a slight decrease of cellular iNOS activity, but one from 48 hours decreased NOS activity to 20%. The inhibition of nitrite accumulation by DSG was shown to come from the decrease of NOS activity itself. NMMA, an irreversible inhibitor of iNOS showed strong inhibition in pretreatment from 24 hours before stimulation and less with pretreatment for 48 hours.

DSG is not a NO Scavenger or a Substrate of iNOS, but a Weak Inhibitor

It is known that some polyamine/NO adducts such as NOC compounds release NO in solution[13]. Because DSG has a spermidine moiety, it was thought that DSG may act as a NO scavenger by forming a DSG/NO adduct: But NO release from NOR4, a NO releaser was not disturbed by the presence of DSG (Data not shown.). DSG did not trap of NO.

The release of NO from DSG by incubation with iNOS and cNOS was negligible, so DSG was concluded not to be used as a substrate (Fig. 4). DSG weakly inhibited iNOS and cNOS activity on L-arginine (Fig. 5). This inhibitory effect could not explain the strong inhibition of nitrite accumulation, because NMMA and NNA, direct inhibitors clealy showed dose-dependent inhibition.

Inhibition of Increase of iNOS Protein Level by DSG Followed with Activation by IFN-γ and LPS of Macrophage Cells

DSG did not directly act against iNOS, but decreased cellular iNOS activity. So we determined iNOS protein level by Western blot analysis. As shown in Fig. 6, pretreatment with 10^{-4} and 10^{-6} M of DSG from 1 day before stimulation apparently decreased cellular iNOS protein. DSG was an inhibitor of induction of iNOS in J744A.1 cells.

Fig. 4. DSG's possibility as substrate of inducible and constitutive NO synthase.

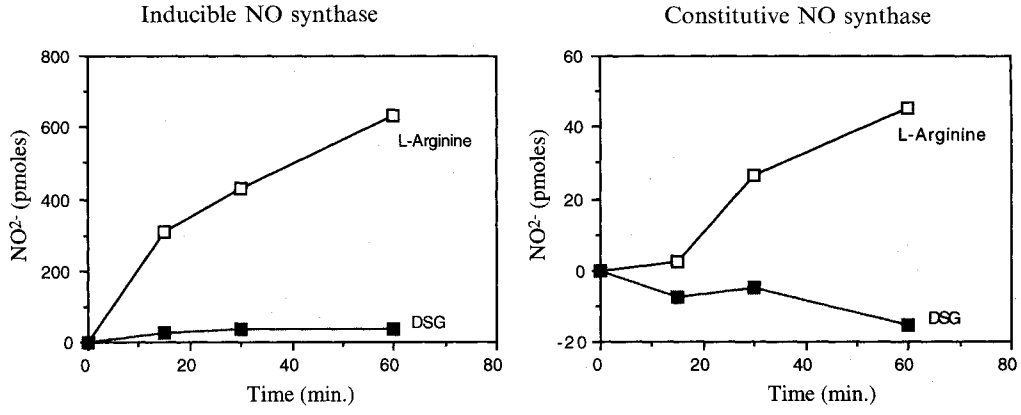
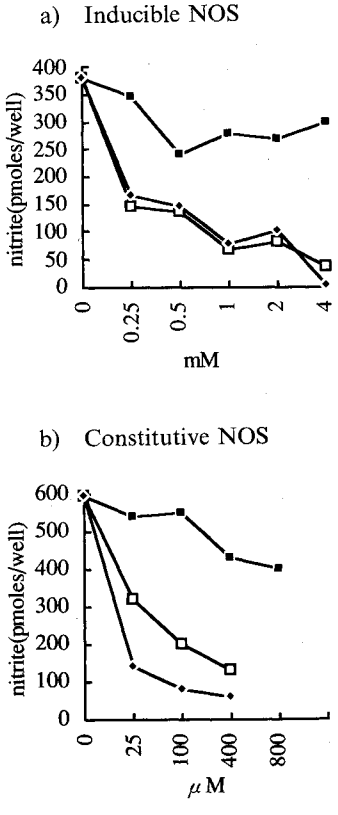


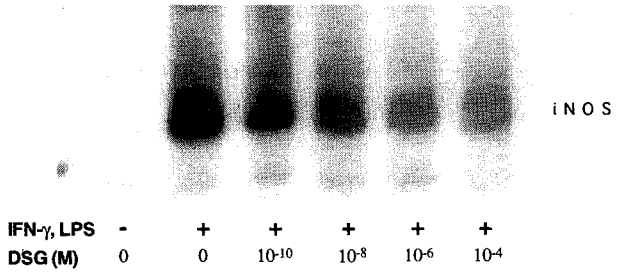
Fig. 5. DSG's possibility as inhibitor of inducible and constitutive NO synthase.

■ DSG, □ NMMA, ◆ NNA.



Inhibitory activity of DSG on NOS were compared with NMMA and NNA, NOS inhibitors.

Fig. 6. Decrease of iNOS protein level by DSG.



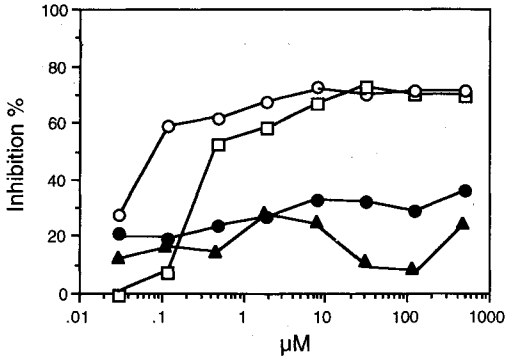
J744A.1 cells were pretreated with various concentrations of DSG from 24 hours before addition of INF-γ (5 units.ml) and LPS (35 ng/ml). At 20 hours after stimulation iNOS of whole cell lysates were immunoblotted by rabbit polyclonal antibody.

Relationship Between Immunosuppression and Inhibitory Activity of iNOS Induction in DSG Derivatives

To determine if the inhibition of iNOS induction by DSG is related to DSG's immunosuppressive activity, active analogs and inactive degradation products of SG (SPG-A and SPG-B) shown in Fig. 1, were tested for their inhibitory activity on iNOS induction. As shown in Fig. 7, SG and N-30 having immunosuppressive activity inhibited the induction of iNOS. But SPG-A and SPG-B did not inhibit iNOS induction. The inhibition

Fig. 7. Effect of DSG's related compounds on induction of iNOS by activated macrophage.

○ Spergualin, □ N-30, ▲ SPG-A, ● SPG-B.



J744A.1 cells were pretreated by active and inactive DSG's related compounds as described in the legend of Fig. 7. and $[NO^{2-}]$ accumulation was determined by DAN assay.

of iNOS induction is thus related to immunosuppressive activity.

Discussion

In spite of DSG's therapeutic effectiveness as an immunosupressant, as reviewed by H. AMEMIYA[18], its mechanism of action has not been clear. Biological activities of DSG *in vivo* and *in vitro* have been reported as follows: antitumor activity[1,9], suppression of humoral and cell-mediated immune responses[3~5], inhibition of angiogenesis[19,20], protection from iradiation[6], but there is little biochemical evidence of a direct target of DSG. DSG has been shown to bind to Hsc70 and Hsp90 and inhibit chaperoning function of Hsc70[21~23]. DSG inhibits antigen processing in monocytes[24]. DSG inhibits κ light chain expression in 70Z/3 pre-B cells by blocking LPS-induced NF-κB nuclear translocation[23]. Hsp70 and Hsp90 are known to exist in multiprotein complexes with steroid and glucocorticoid receptors, immunophilins such as FKBPs that bind FK506 and rapamycin, and cyclophilins that bind cyclosporin A[25~27]. But the actual role of Hsc70 and Hsp90 in immunosuppressive activity of DSG is not clear, because DSG doesn't suppress IL-2 expression[5,6] unlike FK506, cyclosporin A, and rapamycin, and spermidine and derivatives of DSG which are inactive for immunosuppression but can bind to Hsc70[28]. Binding sites of transcription factor NF-κB are found in promoters of iNOS and IL-2R genes. The effect of DSG on induction of iNOS in macrophage cells may be brought about by inhibition of transport of NF-κB into nuclei from cytoplasm by Hsc70 and/or Hsc90.

We showed that action of DSG is related to the effect on the NO pathway by inhibition of induction of iNOS in activated macrophages. The inhibition may occur from inhibition of nuclear chaperoning of NF-κB on the basis of DSG's binding to Hsc70. In rat liver allograft rejection alloimmune response is associated with increase of NO synthesis in grafted liver and DSG treatment suppressed it[29]. Multiple functions of NO have been evidenced in the immune system, neurotransmission, vasodilation, angiogenesis and other biological phenomena. Moreover NO is regulated by complicated networks including induction of various cytokines, change of cyclic GMP level by guanylate cyclase activation by binding of NO, and negative feedback modulation of NOS by itself[30,31]. It may be plausible from the effect on NO pathway that DSG acts as a biological response modifier without remarkable toxicity. Anyhow the result suggests us that NO pathway should be taken into consideration in immune suppression mechanism by DSG.

DSG has different activity in immunosuppression from other immunosuppressants such as cyclosporin A, FK509, and rapamycin besides action mechanism. DSG has protective effects on generation of graft-versus-host-disease in addition to its therapeutic effectiveness. There are many difficulties in investigation of mechanism of action of DSG such as the requirement of long incubation before appearance of DSG's activities, absence of a chromophore usable in histochemistry in DSG, and instability of radioactive DSG. Continuing studies to explain the mechanism of action of DSG may provide new strategies for regulation of the immune system.

Acknowledgment

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