

DEOXYSPERGUALIN THERAPY IN AUTOIMMUNE MRL/lpr MICE SUFFERING ADVANCED LUPUS-LIKE DISEASE

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The present study was designed to evaluate the therapeutic activity of a novel immunosuppressive agent, deoxyspergualin (DSG, NKT-01) in male MRL/MpJ-*lpr/lpr* (MRL/lpr) mice suffering advanced systemic lupus erythematosus (SLE)-like lesions. Treatment with DSG in the early phase of the disease at doses of 1.5 and 3 mg/kg strongly suppressed the development of SLE-like lesions. When DSG was administered from week 21 through 29 to MRL/lpr mice in advanced phases of the disease, a daily iv dose of 3 mg/kg (5 days/week) markedly reduced the symptoms, whereas a dose of 1.5 mg/kg did not. Moreover, DSG treatment at a dose of 3 mg/kg, started at the time when the blood urea nitrogen levels were over 50 mg/deciliter, significantly prevented deterioration of the hyperuremia. Taking these findings into consideration, DSG was found to be a promising agent for curing such established autoimmune disease.

MRL/MpJ-*lpr/lpr* (MRL/lpr) mice develop systemic lupus erythematosus (SLE)-like lesions. This disease is characterized by massive lymphadenopathy, development of antibodies to self antigens, and glomerulonephritis¹). As a result, mice suffering from this autoimmune disease offer a good model to evaluate or screen therapeutic activities of immunosuppressive agents. Indeed, some studies have demonstrated the efficacy of immunosuppressive agents in retarding these SLE-like lesions^{2~5}).

Deoxyspergualin (DSG)⁶) is an analogue of spergualin^{7,8}) which is a metabolite of *Bacillus laterosporus* and has been found to have potent immunosuppressive activities in animals^{9~12}). Our group and others have shown that DSG is highly effective in suppressing the development of SLE-like lesions in the early phase of the disease in MRL/lpr mice^{13,14}). The present study was designed to evaluate whether there is any response to DSG in MRL/lpr mice suffering advanced SLE-like lesions.

Materials and Methods

Animals

Male MRL/lpr mice were obtained from Charles River Japan (Atsugi, Kanagawa, Japan). The mice were maintained in specific pathogen-free conditions.

DSG

DSG (1-amino-19-guanidino-11-hydroxy-4,9,12-triazanonadecane-11,13-dione trihydrochloride)¹⁵) was supplied by Takara Shuzo Co., Ltd., Kyoto, Japan, dissolved in saline and sterilized by passing through a 0.22- μ m filter.

Measurement of Anti-DNA Antibody by Enzyme-linked Immunosorbent Assay (ELISA)

Serum from individual animals was assayed for the presence of autoantibodies against DNA using

the following ELISA method¹⁶⁾: 0.3 ml of 0.05% aqueous solution of poly-L-lysine was incubated in Nunc ELISA plates for 1.5 hours at room temperature. The plates were washed using phosphate buffered saline containing 0.1% gelatin (PBS-G) as the washing and diluting buffer, and 0.2 ml of calf thymus DNA (5 μ g/ml PBS-G; Sigma Chemical Company, St. Louis, U.S.A.) was added. After incubating overnight at 37°C, all free sites were blocked by incubating with 0.2% bovine serum albumin for 1 hour at 37°C, then 0.2 ml of 1/200-diluted mouse serum was added and the plates were incubated for 1.5 hours at 37°C. The plates were then washed, and 0.2 ml of 1/1,000-diluted goat antimouse IgG serum (Cappel, Cochranville, U.S.A.) was added and incubated for 1.5 hours at 37°C. After washing 0.2 ml of 1/1,000-diluted alkaline phosphatase-conjugated rabbit anti-goat IgG serum (Cappel) was added and incubated for 1.5 hours at 37°C. Finally, 0.2 ml of *p*-nitrophenyl phosphate (Sigma), 2 mg/ml in 10% diethanolamine buffer, pH 9.8, was added and after 1 hour incubation at room temperature, the absorbance of *p*-nitrophenyl was measured at 410 nm.

Measurement of Blood Urea Nitrogen (BUN)

BUN was estimated by the urease-indophenol method using a Rapid Blood Analyzer Super (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan).

Peripheral Blood Cell Counts

Peripheral red blood cell (RBC), white blood cell (WBC) and platelet counts were performed using a standard hemocytometer.

Statistical Analysis

Data were analyzed by Student's *t*-test.

Results

Prevention by DSG on the Development of SLE-Like Lesions

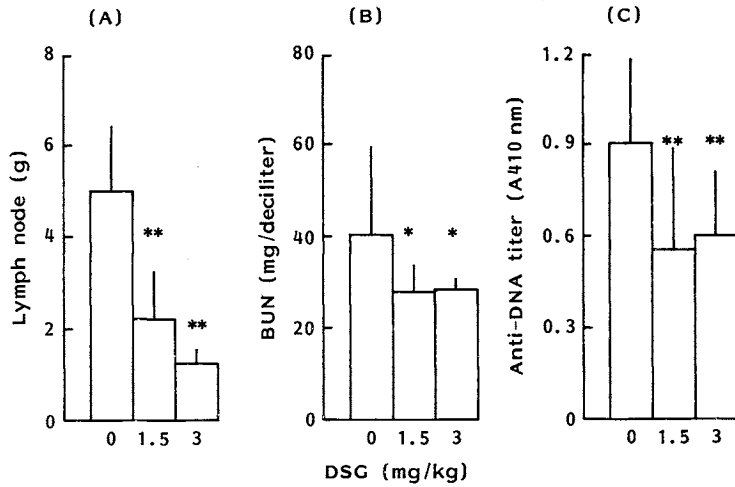
MRL/lpr mice spontaneously develop massive generalized lymphadenopathy with proliferation of nonmalignant lymphocytes, and also characterized by the appearance of circulating antibody to DNA and the increase of BUN accompanying with the development of lupus nephritis. DSG was administered iv at daily doses of 1.5 and 3 mg/kg (5 injections/week) from week 13 through 20. The weight of the lymph nodes, serum anti-DNA titer and BUN were measured (Fig. 1). DSG dose-dependently suppressed the enlargement of the lymph nodes. There was also a significant prevention in the increase of anti-DNA titer and BUN in the mice receiving either doses of DSG. As shown in Fig. 2, control mice had markedly higher WBC counts accompanied by the development of massive lymphadenopathy, while the DSG-treated mice had a significant decrease in WBC counts. Similarly, a significant decrease of RBC counts was observed in the mice which received DSG at a dose of 3 mg/kg. Unexpectedly, DSG induced a dose-dependent increase of platelet counts.

Since the administration of DSG (3 mg/kg) induced anemia as described above, the effects of 3 and 5 injections per week of the same dose of DSG on the development of the lesions and the peripheral blood cell counts in the same experimental setup were compared (Fig. 3). The mice responded well to both regimens, and significantly lowered WBC counts and increased platelet counts were observed with either 3 or 5 injections per week. It was important that no appearance of anemia was observed in the mice given DSG in the regimen of 3 injections/week.

DSG Therapy of Advanced SLE-Like Lesions

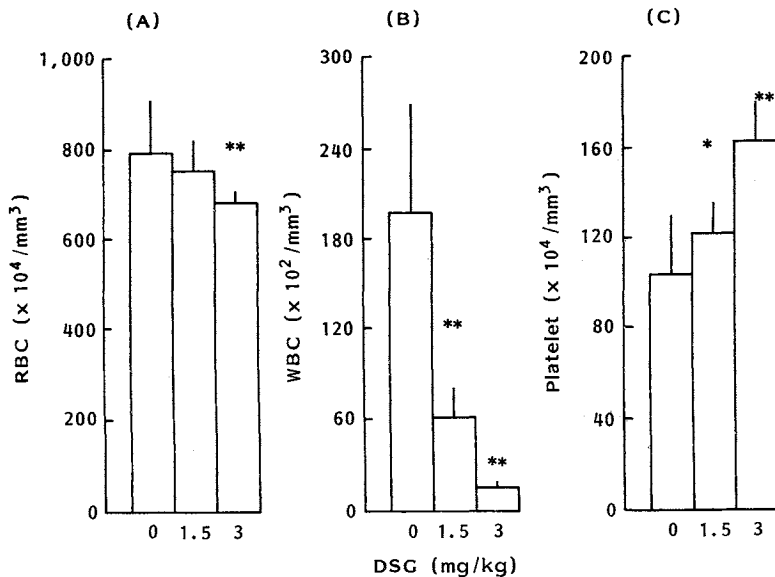
Examining at first the therapeutic activity of DSG in MRL/lpr mice suffering advanced SLE-like lesions, we found that the dose of 3 mg/kg could significantly inhibit not only the enlargement of lymph

Fig. 1. Effect of DSG on the development of lupus-like lesions in MRL/lpr mice.
 * $P < 0.05$, ** $P < 0.01$.



DSG was iv administered 5 injections per week from week 13 through 20. A set of control MRL/lpr mice receiving saline were included. The mice were sacrificed after the completion of the DSG administration, and lymph nodes and blood were removed. The mesenteric, axillary, elbow, inguinal, subaxillary and iliac lymph nodes were pooled individually. Data are shown as mean with SD from 10 to 18 mice per group.

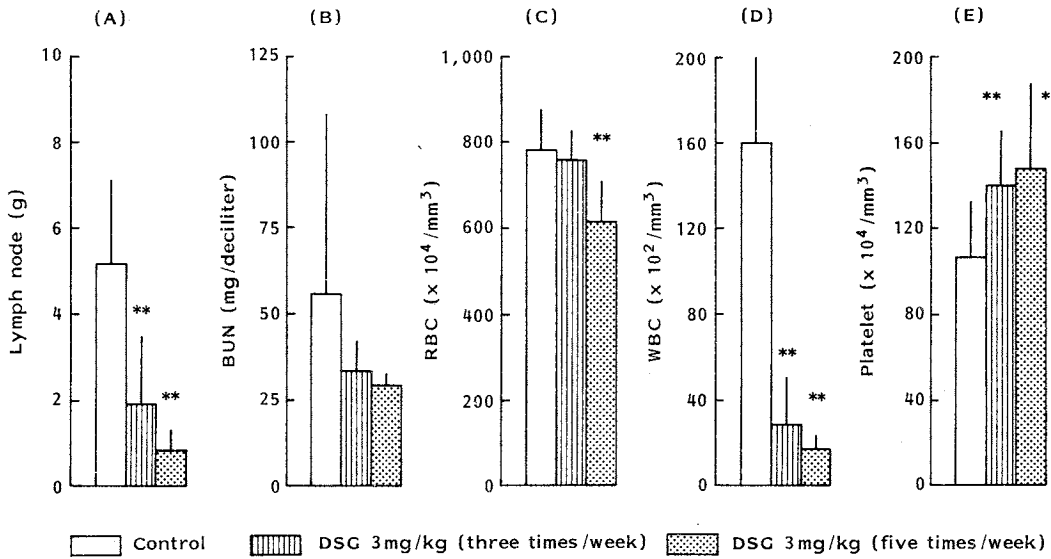
Fig. 2. Peripheral blood cell levels in MRL/lpr mice after the administration of DSG.
 * $P < 0.05$, ** $P < 0.01$.



All experimental procedures were the same as described in the legend of Fig. 1.

Fig. 3. Effect of administration schedule of DSG on the development of SLE-like lesions in MRL/lpr mice.

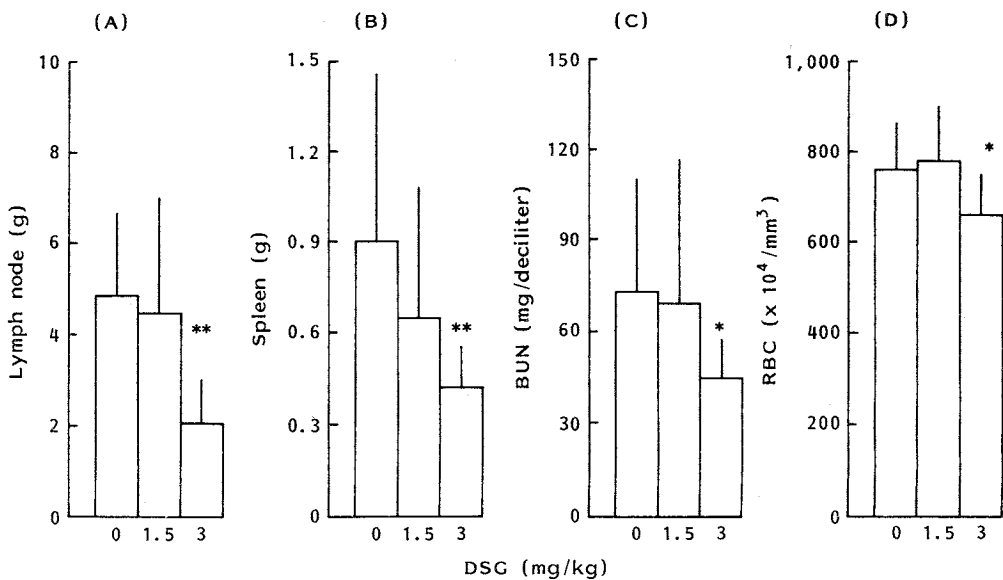
* $P < 0.05$, ** $P < 0.01$.



DSG was iv administered at a daily dose of 3mg/kg 3 or 5 injections per week from 13 through 20. Other experimental procedures were the same as described in the legend of Fig. 1. Data are shown as mean with SD from 9 to 10 mice per group.

Fig. 4. Therapeutic effect of DSG in MRL/lpr mice with advanced disease.

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



DSG was iv administered 5 injections per week from week 21 through 29. The mice were sacrificed after the completion of the DSG administration, and lymph nodes, spleens and blood were removed. Other experimental procedures were the same as described in the legend of Fig. 1. Data are shown as mean with SD from 14 to 15 mice per group.

Table 1. Survival rate of MRL/lpr mice with advanced SLE-like lesions by DSG administration.

Group	Median survival time (days)	Survivors ^a /total	Survival rate (%)
Control	289	6/35	17.1
DSG	316	17/47	36.2

DSG was iv administered at a daily dose of 3 mg/kg (3 injections/week) from week 21 through 33. A set of control mice receiving saline was included.

^a Survived > 384 days (= 55 weeks).

nodes and spleens but also the development of glomerulonephritis, as was demonstrated by the significant decrease of BUN levels, whereas the dose of 1.5 mg/kg was not significantly different from control (Fig. 4). The DSG treatment was started when the animals were 21 weeks old, and continued (5 injections/week) until the mice were 29 weeks of age, and then the experiment was terminated. By this time, 5 out of 14 control mice and 4 out of 15 mice treated with a dose of 1.5 mg/kg of DSG were moribundly sacrificed due to severe hyperuremia (> 100 mg/deciliter of BUN level). In contrast, five of the 15 mice given DSG at a dose of 3 mg/kg decreased body weight below 40 g and $600 \times 10^4/\text{mm}^3$ of RBC, in comparison with 50 g mean body weight and $760 \times 10^4/\text{mm}^3$ mean RBC in the control mice, and one mouse was moribundly sacrificed due to weight loss and anemia, but had neither severe hyperuremia nor marked lymphadenopathy.

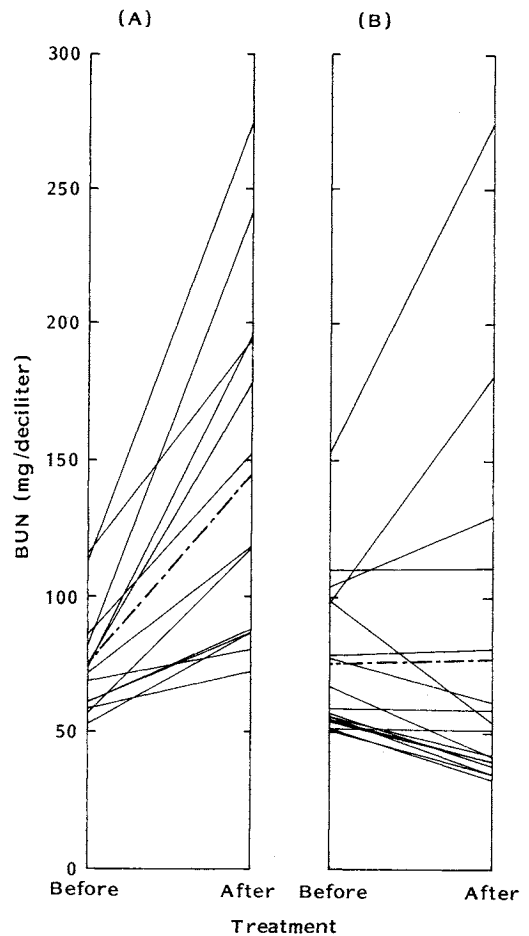
A further study was carried out for examining whether DSG was able to induce a prolongation of life span in MRL/lpr mice with advanced SLE-like lesions (Table 1). Treatment with DSG (3 mg/kg) started with 21-week old mice using the regimen of 3 injections/week, which failed to induce anemia as shown in Fig. 3, and continued until the animals were 33 weeks of age. This therapy slightly increased the survival rate of the MRL/lpr mice, but there was no significant difference ($p=0.068$ by generalized Wilcoxon, $p=0.056$ by Mantel-Cox).

Therapeutic Activity of DSG in Hyperuremic MRL/lpr Mice

Almost all of MRL/lpr mice die due to chronic renal insufficiency which is glomerulonephritis and renal angitis, and can be characterized by severe hyperuremia. We tried 20 day-administration of DSG (3 mg/kg) started at the time when individual BUN levels were more than 50 mg/deciliter. As shown in Fig. 5, the DSG-untreated mice had an initial mean BUN level of 71.3 mg/deciliter and finally their levels rose significantly ($P<0.01$) to a mean of 149.2 mg/deciliter. In contrast, a nonsignificant increase in the BUN

Fig. 5. DSG therapy in hyperuremic MRL/lpr mice.

(A) Control, (B) DSG. — Individual BUN value, - - - - mean BUN value.



MRL/lpr mice having more than 50 mg/deciliter of BUN value were given DSG at an iv daily dose of 3 mg/kg about for 20 days.

level was observed in the DSG-treated mice (an initial mean of 74.9 mg/deciliter; a final mean of 77.0 mg/deciliter).

Discussion

Our previous report demonstrated that treatment of 8-week old male MRL/lpr mice with DSG at an ip dose of 1.5 mg/kg markedly suppressed the development of SLE-like lesions¹⁴). Therefore, we could expect that when the administration of DSG (5 injections/week of 1.5 mg/kg, and 3 or 5 injections/week of 3 mg/kg) started at 13 weeks of age, the development of the lesions might be significantly prevented at 20 weeks of age. However, the regimen of 5 injections/week of 3 mg/kg of DSG induced slight anemia. As previously reported¹⁷), it was found that normal C3H mice receiving DSG at an immunosuppressive dose of 6.25 mg/kg for 15 days developed significant erythrocytopenia and leukocytopenia during the administration period. Therefore the observed anemia could be predicted. In contrast, unexpected thrombopoiesis was observed in the mice which received DSG. Our previous paper showed that DSG enhanced the ability to release interleukin 3, a multipotential hematopoietic factor, from the spleen cells of MRL/lpr mice¹⁴). This DSG-induced thrombopoiesis may correlate somewhat with the increasing production of interleukin 3 induced by the DSG treatment. However, since renal transplant patients receiving DSG showed leukocytopenia and thrombocytopenia¹⁸), these observations might not apply to man. It was of particular interest whether this agent could ameliorate autoimmune disease in MRL/lpr mice suffering advanced SLE-like lesions. When it was administered at doses of 1.5 and 3 mg/kg (5 injections/week) for 8 weeks from 21 weeks of age, the smaller dose was ineffective but 3 mg/kg was highly effective. On the other hand, weight loss and anemia were observed in the mice receiving DSG at a dose of 3 mg/kg, and one mouse was moribundly sacrificed due to the DSG toxicity such as weight loss and anemia. It was likely that the grade of the toxicity induced by the treatment of DSG (3 mg/kg, 5 injections/week), which was slight when started at 13 weeks old, became moderate when started at 21 weeks of age. This appeared to be the result of the more advanced renal disease of the MRL/lpr mouse, because almost all DSG is excreted through the kidneys, when injected systemically. However, there was no drug accumulation observed during the treatment period. These findings may suggest that the long-term administration of DSG under the regimen of 5 injections/week is not appropriate for therapy in mice with insufficient renal function. It was further found that the therapeutic effect of DSG was equivocal, if the regimen of 3 injections/week was used in place of the regimen of 5 injections/week. Accordingly, the short-term administration for 20 days of DSG (3 mg/kg) was performed in the further therapeutic study using hyperuremic mice. It was demonstrated that these hyperuremic mice responded well to DSG therapy.

In conclusion, the present study demonstrated that MRL/lpr mice with advanced SLE-like lesions benefited from the DSG therapy. These findings suggest that DSG has possible efficacy for the treatment of human SLE. Since the long-term administration of DSG alone may predispose to anemia and other complications in clinical use, it will be needed to investigate whether combined therapy with other immunosuppressive drugs has benefit in MRL/lpr mice with advanced renal disease.

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