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Cellular mechanisms: Induction of heart allograft survival in rats by 15-deoxyspergualin

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Abstract. Survival of ACI rat heart grafts in Lewis rat (LEW) recipients treated with a short course of 15-deoxyspergualin (DSG), in a dose of 5 mg/kg daily beginning from day 4 of grafting, was markedly prolonged, with a mean survival time of 29.8 ± 3.0 days. On day 20 after grafting, the cellular mechanism of inducing allograft survival after DSG treatment was analyzed by testing the activation of spleen cells in several assay systems. The results indicate that spleen cells from DSG-treated rats with surviving heart allografts show almost no proliferative response against donor strain stimulator cells in the mixed lymphocyte reaction (MLR) as compared with controls. Their cytotoxic activity was lower than that of spleen cells from rats with heart allograft rejection towards donor strain target cells. Adding various concentrations of spleen cells from DSG-treated LEW rats with surviving ACI heart allografts to the MLR when the responder cells from normal LEW rats were exposed to irradiated ACI or Wistar (third party) stimulator cells revealed a strong suppression, in a cell-dose-dependent manner. Moreover, the transfer of 2.0×10^8 spleen cells from DSG-treated LEW rats with surviving ACI heart allografts to an irradiated grafted host did not prolong the survival either of the ACI heart grafts or of the third party Wistar heart grafts. These results suggest that the proliferative response and cytotoxic activity are lowered and suppressor cells are induced by treatment with DSG, in rats with surviving allografts.

Key words: Immunosuppressive agent – Heart allograft survival – Rejection – Spleen cell

15-Deoxyspergualin, originally developed for its antibiotic and antitumor activity [4], has shown potential as a clinically valuable immunosuppressive agent. Several communications have reported that this agent has immunosuppressive activity against acute rejection in human renal transplantation [2]. In vitro experiments in human subjects have shown that the principle effect is an inhibition of the later phase of the mixed lymphocyte reaction (MLR), mainly by suppression of the expression of the interleukin 2 (IL2) receptors, and cytotoxic T-cell generation [10, 11]. Moreover, many studies in animals have demonstrated that DSG is an effective immunosuppressive agent, capable of inhibiting the immunoresponse in rat skin grafts, rat heterotopic heart transplantation, and dog renal transplantation [1, 5, 17]. These findings indicate that DSG may facilitate the prolongation of allograft survival and emphasize the need for further study of its mechanisms of action in the inhibition of allograft rejection in the rat model. In this present study, experiments were therefore designed to study the cellular mechanisms of allograft survival after a short course of DSG treatment.

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Materials and methods

Animals. Male LEW rats (RT11) weighing 250–300 g were used as recipients. Male ACI rats (RT1a) weighing 150–200 g and Wistar rats (RT1e) weighing 150–200 g were used as donors and third party donors, respectively. The animals were obtained from commercial sources (LEW: Charles River, Japan; ACI: Hishino Experiment Animals, Japan; Wistar: SLC, Japan) and kept under specific-pathogen-free conditions in our animal facility.

Immunosuppression. 15-Deoxyspergualin was supplied by Nippon Kayaku Co. (Tokyo, Japan). The drug was dissolved in physiological saline and stored at −70 °C before use.

Heterotopic heart transplantation were performed using the modified technique of Ono and Lindsey [12]. Survival of the cardiac allograft was determined by daily palpation. Rejection was considered complete at the time of cessation of a palpable heartbeat and confirmed by histological examination.

Preparation of spleen cells. Lymphocytes were obtained from rat spleen. The spleen was isolated and minced, and the red cells were then lysed with buffered hypotonic TRIS-ammonium chloride (0.83%, pH 7.21). Cells were washed twice with RPMI 1640 and then suspended in RPMI 1640 complete medium containing 10%

Table 1. Effect of 15-deoxyspergualin (DSG) on heterotopically transplanted rat hearts

Experimental groups	Survival (days)	Mean ± SD	*P* value
No immunosuppressant (control, $n = 6$)	5, 6 (3), 7 (2)	6.1 ± 0.7	
DSG 5.0 mg/kg daily ($n = 8$)	26, 28, 30, 31, 34	29.8 ± 3.0	< 0.01

Table 2. Mixed lymphocyte reaction (MLR) response and cell-mediated lympholysis (CML) activity of spleen cells from DSG-treated LEW rat with surviving ACI heart graft on day 20 after grafting

MLR	Stimulator cells	Responder cells	cpm[a]
	LEW	LEW	11901 ± 5204
	ACI	LEW	43038 ± 4108
	ACI	DSG-treated LEW	14207 ± 5151
CML	Effector Responder/stimulator	Target	Cytotoxicity (%)[b]
	LEW T R[c] ACI	ACI	20.2 ± 1.8
	LEW T S[d] ACI	ACI	5.7 ± 2.8

[a] Mean of three individual experiments
[b] CML activity was assessed at a 40:1 effector/target ratio
[c] Cells from LEW rat with rejected ACI heart graft
[d] Cells from DSG-treated LEW rat with surviving ACI heart graft

fetal calf serum (FCS), 30 m*M* HEPES, 2.5 m*M* L-glutamine, and 5 μg/ml gentamicin, in various concentrations of cells, for assay.

Mixed lymphocyte reaction. One-way MLR was performed, using spleen cells from DSG-treated LEW rats with surviving ACI heart grafts or from normal LEW rats as responder cells and ACI rats as stimulator cells. Responder cells (0.5×10^6 /ml were co-cultured with 2000-rad-irradiated stimulator cells (1.0×10^6/ml) in 96-well tissue culture plates in RPMI 1640 complete medium. The cells were incubated at 37 °C in a humidified atmosphere of 5 % CO_2 for 4 days and then treated with an 16–20 h tritiated-thymidine (^{3}H-Tde) pulse. The cells were harvested, and ^{3}H-TdR incorporation was measured by a liquid scintillation counter (Packard Tri Carb 4530). The percentage suppression was calculated using the formula:

$$\%\text{Suppression} = [1 - \frac{\text{cpm (experimental)} - \text{cpm (negative control)}}{\text{cpm (positive control)} - \text{cpm (negative control)}} \times 100$$

Cell-mediated lympholysis. Spleen cells from DSG-treated LEW rats with surviving ACI heart grafts as responder cells were co-cultured with 2000-rad-irradiated normal ACI stimulator cells in RPMI 1640 complete medium at 37 °C in a humidified atmosphere of 5 % CO_2 for 6 days. Splenic responder cells from LEW rats with rejected ACI heart allografts were used as the control. After incubation, the cells were harvested and used as effector cells for CML. Target cells were prepared by culturing stimulator cells with 50 μg/ml concanavalin A (ConA) for 2 days. The 4.0×10^6/ml effector cells were cultured with 1.0×10^5/ml ^{51}Cr-labelled target cells for 4 h at 37 °C in a humidified atmosphere of 5 % CO_2. A fixed volume of supernatant was collected from each well after centrifugation at 1500 *g* for 10 min. The ^{51}Cr release was counted in a gamma-counter (Aloka, JDC-752). The percentage of cytotoxicity was calculated according to the following formula:

$$\%\text{Cytotoxicity} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100$$

Suppressor cell reactivity. Suppressor cell reactivity in the MLR was also assayed by adding 0.5×10^4, 1.0×10^4, and 2.0×10^4 spleen cells from DSG-treated LEW rats with surviving ACI grafts to the MLR when responder cells from normal LEW rats were exposed to irradiated ACI or Wistar (third party) stimulator cells. In the control experiment, spleen cells from normal LEW rats were added.

Adoptive transfer by spleen cell. LEW rats were given 250 rads of whole body irradiation and, on the following day, grafted with ACI or Wistar (third party) hearts. On day 1 of grafting, they received an intravenous (i.v.) injection of 2.0×10^8 spleen cells. The spleen cells were obtained from DSG-treated LEW rats with surviving ACI grafts or normal LEW rats. Survival of the graft was the endpoint of this experiment.

Experimental design. Heart allografts were transplanted from ACI to LEW rats following 10 days of 5 mg/kg daily DSG treatment (from day 4 to day 13 after transplantation). On day 20 the spleen cells of LEW recipients with surviving ACI heart grafts, were used for testing activation by the following assays: (1) lymphocyte proliferative response, (2) cytotoxic T cell activity, (3) suppressor cell reactivity in the MLR, (4) adoptive transfer assay.

Statistical analysis: The statistical significance of the results was assessed by Student's *t*-test.

Results

Untreated allografted hearts (LEW/AIC, $n = 6$) were all rejected, with a mean survival time (MST) of 6.0 ± 0.7 days. However, the survival of ACI heart allografts in LEW recipients treated with 5 mg/kg daily of DSG for 10 days (from day 4 to day 13 postoperatively) was markedly prolonged, with graft MST of 29.8 ± 3.0 days (Table 1). The histopathological findings also demonstrated that severe hemorrhage, edema, and necrosis of myocardial muscle cells were present in the control, whereas rats treated with DSG showed only focal cellular infiltration among the myocytes on day 20 after grafting.

Mixed lymphocyte reaction

To test the proliferative response of spleen cells from DSG-treated LEW rats with surviving ACI heart grafts, spleen cells from DSG-treated recipients or normal LEW rats were used as responder cells, and normal ACI spleen cells were used as stimulator cells. The results are shown in Table 2. Responder cells from DSG-treated LEW recipients showed almost no proliferative response against ACI rat stimulator cells as compared with normal LEW responder cells.

CML activity

The cytotoxic activity of spleen cells from DSG-treated LEW rats with surviving ACI heart grafts on the donor strain target cell is shown in Table 2. The cytotoxic activity of spleen cells from LEW rats with rejected ACI heart grafts was taken as the control. The results showed that the mean cytotoxic activity was 20.20 ± 1.8 % in spleen cells from untreated LEW rats with rejected allograft but was 5.70 ± 2.8 % in spleen cells from the DSG-treated LEW recipients.

Table 3. Inhibition of MLR by adding spleen cells from DSG-treated rat with surviving ACI heart graft or normal LEW rat

MLR responder[a]/stimulator[b]	Cells added[c] (normal LEW rat) (n)	Inhibition (%)	Cells added[d] (DSG-treated LEW recipient rat) (n)	Inhibition (%)
LEW/ACI	0.5×10^4	-83.4 ± 28.0	0.5×10^4	34.5 ± 17.4
LEW/ACI	1.0×10^4	-101.0 ± 35.6	1.0×10^4	58.9 ± 16.8
LEW/ACI	2.0×10^4	-112.2 ± 64.3	2.0×10^4	86.2 ± 13.0
LEW/Wistar	0.5×10^4	-18.9 ± 32.0	0.5×10^4	22.8 ± 10.0
LEW/Wistar	1.0×10^4	-8.8 ± 17.6	1.0×10^4	50.2 ± 19.4
LEW/Wistar	2.0×10^4	-25.5 ± 28.1	2.0×10^4	76.0 ± 14.6

[a] Normal LEW spleen cells served as responder cells
[b] ACI or Wistar splenic stimulator cells
[c] Various concentrations of spleen cells obtained from normal LEW rat as control were added to MLR
[d] Various concentrations of spleen cells obtained from DSG-treated LEW rat with surviving ACI heart graft were added to MLR

Table 4. Survival of ACI and Wistar heart allografts in irradiated LEW rats after adoptive transfer of spleen cells from normal or DSG-treated LEW rats with surviving ACI heart grafts

Heart donor[a]	Cell transfer[b]		Graft survival (days [n])	Mean ± SD	P value
	Lymphocyte donor	No. of cells			
ACI	–	–	5, 6 (3), 7 (2)	6.1 ± 0.7	
ACI	Normal LEW	2.0×10^8	5, 6 (2), 7, 8	6.4 ± 1.1	NS
ACI	DSG-treated LEW with surviving ACI graft	2.0×10^8	5, 6, 7, 8, 10	7.2 ± 1.9	NS
Wistar	Normal LEW	2.0×10^8	14, 15 (2), 16, 17	15.4 ± 1.1	
Wistar	DSG-treated LEW with surviving ACI graft	2.0×10^8	14, 15 (2), 17, 18	15.8 ± 1.6	NS

[a] ACI or Wistar hearts heterotopically transplanted to 250-rad-X-irradiated LEW rats
[b] Spleen cells from normal or DSG-treated LEW rat with surviving ACI heart graft transferred shortly to the unmodified LEW rat with ACI or Wistar heart graft on day 1 of grafting

Suppression of MLR

Studies were performed to assess the capacity of spleen cells obtained from DSG-treated recipients with surviving heart allografts to inhibit the MLR response. Spleen cells from normal LEW rats were used as responder cells and normal ACI or Wistar (third party) spleen cells were the stimulator cells. The results of experiments in which 0.5×10^4, 1.0×10^4, 2×10^4 of spleen cells obtained from DSG-treated LEW rats with surviving ACI heart grafts or from normal LEW rats were added to the MLR at the initiation of cultures are illustrated in Table 3. Spleen cells from DSG-treated rats markedly suppressed the MLR when the responder cells from normal LEW rats were exposed to irradiated ACI or Wistar (third party) stimulator cells. The addition of diluted cells to the MLR also resulted in cell-dose-dependent suppression. There were no significant differences in the suppressive rate between the donor strain and third party MLR. In contrast, when cells from the normal LEW rat were added, no inhibition was observed, in either the donor strain or the third party MLR response.

Effect of splee cell transfer

The alloreactivity of the spleen cells from DSG-treated LEW rats with surviving ACI heart grafts was further analyzed by adoptive cell transfer experiments. Control LEW rats undergoing irradiation alone or irradiation plus transfer of normal LEW spleen cells rejected their ACI grafts: the graft MST was 6.1 ± 2.1 days and 7.0 ± 2.1 days, respectively and in the case of Wistar grafts (third party), the graft MST was 6.1 ± 1.1 days and 6.3 ± 1.2 days, respectively. The transfer of 2×10^8 spleen cells fro DSG-treated LEW rats with a surviving ACI graft did not significantly prolong the graft MST of the ACI or Wistar (third party) heart transplanted into irradiated LEW rats, although in 1 rt the graft survival was prolonged to 10 days after grafting (Table 4).

Discussion

It was confirmed in our experiments that a short course of DSG treatment at a dosage of 5 mg/kg daily from day 4 to day 14 posttransplantation, could inhibit the capacity of LEW rats to reject ACI heart grafts. This result strongly supports the clinical study result in renal transplantation by Amemiya et al. [2] that DSG is most effective in rescuing ongoing rejection.

With regard to the mechanism, we examined the MLR response and cytotoxic activity of spleen cells from DSG-treated LEW rats with surviving ACI heart grafts. The results showed that both the MLR response and CML activity were significantly decreased with spleen cells from the DSG-treated recipients. This effect strongly suggests that DSG could inhibit the lymphocyte response and cytotoxic T cell activity in the spleen in the rats with allografts and consequently allow the prolongation of allograft survival. These results coincide with our previous results in the human in vitro study of deoxymethylspergualin, which showed a suppression in the MLR and CML [10]. Moreover, our data also demonstrated that the CML response

in the spleen cells of DSG-treated rats with surviving allografts is lower than in the spleen cells of rats with rejected allografts towards donor strain target cells. The findings indicate that the cytotoxic activity was most closely associated with allograft survival in the rat [9].

The mechanism of inducing allograft survival after a short course of DSG treatment was further studied by adding spleen cells to the MLR assay. The results show that the MLR in LEW spleen cells to donor party (ACI) and third party (Wistar) stimulator spleen cells were inhibited in a cell-dose-dependent manner. As various mechanisms of graft survival, such as the modulation of graft antigen expression [6, 14], depletion of clonally active cells following immunosuppression and exposure to the graft [7, 8], antigen/antibody blockade of effector cells [15, 16], activation of suppressor cells [3, 13], and production of suppressor humoral factor(s), have been postulated. Our results strongly suggest that DSG may fail to affect suppressor cells, and these cells may progressively develop and play a partial role in continuing allograft survival states. In addition, the inhibition of the MLR response between the donor and third party stimulator cells showed no significant difference on adding spleen cells from DSG-treated recipients. This indicates that the property of inhibition, on day 20 postgrafting, was a nonspecific suppression.

Studies to determine whether the spleen cells taken from DSG-treated rats with surviving allografts would be able to prolong the survival of the donor strain (ACI) or third party (Wistar) heart grafted into irradiated LEW rats showed that the spleen cells did not significantly prolong the graft MST of LEW recipients, either in the donor strain group or in the third party group, compared with the control group. The reasons for these results are not yet understood. We believe that the suppressive effect of spleen cells from DSG-treated rats with surviving allografts in the early phase may not be sufficient to control graft rejection.

In conclusion, the cellular mechanism of inducing heart allograft survival by DSG may mainly include (1) a decrease in lymphocyte response, (2) an inhibition of cytotoxic T cell acitivty, and (3) an induction of suppressor cells. Our next question is what is the role of humoral immunity in this rat model with DSG treatment.

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