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Synergistic effect of donor-specific blood transfusion and a short course of deoxyspergualin in rat kidney transplantation

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Abstract Deoxyspergualin (DSG), an analogue of spergualin produced by *B. laterosporus*, has a strong immunosuppressive effect in various transplantation models. We have investigated the mechanism of donor-specific prolongation of survival time in rat kidney grafting by donor-specific blood transfusion (DST) and a short course of DSG. Lewis (LEW) kidney allografts were transplanted into fully allogeneic BN rats. Fresh, whole LEW blood 1.0 ml, was injected i.v. into BN rats 2 days prior to transplantation. Then, DSG, 6 mg/kg per day, was administered by i.m. injection on days 0, 1, and 2 after transplantation. The recipients were divided into five groups: group 1 ($n = 6$) no treatment: group 2 ($n = 6$) DST only; group 3 ($n = 7$) DSG only; group 4 ($n = 7$) DST and DSG; and group 5 ($n = 6$), third party (ACI rats) blood transfusion and DSG. Lymphocytes (cervical lymph nodes) and serum were harvested from BN recipients on day 7 postgrafting. For suppressor cell assays, lymphocytes from BN recipients in each group were added as a third cell to the mixed lymphocyte reaction (MLC) between nontransplanted BN lymphocytes (responder) and LEW or other third party (PVGC, ACI, WKA rats) lymphocytes (stimulator). Antidonor lymphocytotoxic antibody (ADLA) was checked by microcytotoxicity assays. Median survival times (MST) for each group were: group 1, 10 days; group 2, 10 days; group 3, 13 days; group 4, 75 days; and group 5, 13 days. Remarkable prolongation of MST was only noted in group 4. In the suppressor cell assay, group 4 showed significant suppression (40 %; $P < 0.05$); the other groups did not show any suppression. This suppressive activity in group 4 was effective only during the MLC between BN and LEW, not during the MLC of third party-BN combinations. Thus, suppressor cells from DST/DSG-treated BN recipients appear to be donor-specific. In the microcytotoxicity assay, the only group that showed any ADLA was group 2, which was not treated with DSG. These results clearly show that both induction of donor-specific suppressor cells and inhibition of ADLA production are associated with the remarkable donor-specific prolongation of kidney allograft survival in DST/DSG-treated recipients.

Key words Donor-specific transfusion, rat, kidney · Kidney, rat, donor-specific transfusion · Deoxyspergualin, kidney, rat · Rat, kiney, deoxyspergualin

Introduction

Deoxyspergualin (DSG), an analogue of spergualin, is produced as a metabolite by *Bacillus laterosporus* [18] and has been confirmed to have a strong immunosuppressive effect in various transplantation models including mice [15], rats [23], dogs [1], monkeys [4], and humans [2]. The mechanism of action of DSG has not been clarified yet, but some reports have shown that DSG has a totally different mechanism of action [5] than newly developed, potent immunosuppressive drugs such as cyclosporin (CyA) [7], FK 506 [20], or OKT3 monoclonal antibody [3]. One of the most interesting mechanisms of action is that DSG shows a strong immunosuppressive effect in the late phase of mixed lymphocyte culture (MLC), whereas CyA affects only the early phase of MLC [5, 6]. Also, in vivo, greater immunological unresponsiveness has been achieved in allografted rats by initiating the administration of DSG from the onset of acute rejection rather than on the day of grafting [17]. Suzuki et al. also showed that the induction of suppressor cells by DSG plays an important role in the maintenance of immunological unresponsiveness [17]. Furthermore, it is reported that DSG has a direct immunosuppressive effect on activated B cells but not on resting B cells, suggesting that DSG blocks B-cell maturation [6, 12]. These experimental findings suggest that the mechanisms of its action might include the specific inhibition of expanding lymphocyte clones [6, 12, 17] and the preservation of suppressor cells [17].

Given the above-mentioned results, this study was intended to induce donor-specific immunological unresponsiveness. In order to sensitize the recipients (BN rats), donor-specific blood transfusion (DST) from Lewis to BN rats was employed. After DST, renal transplantation was done and DSG was administered at the time of transplantation in order to inhibit the second phase of the immune response, namely, expansion of donor-specific lymphocytes.

Materials and methods

Animals

Inbred male rats weighing 150–250 g were maintained by ad libitum feedings of laboratory chow. Lewis (LEW, $RT1^l$) rats were obtained from Charles River Japan (Kanagawa, Japan). Brown-Norway (BN, $RT\text{-}1^n$) and PVGC ($RT\text{-}1^c$) rats were obtained from Seiwa Experimental Animal Laboratory (Fukuoka, Japan). ACI ($RT\text{-}1^a$) and WKA ($RT\text{-}1^k$) rats were purchased from the Hoshino Experimental Animal Laboratory (Saitama, Japan) and Shizuoka Agricultural Cooperative for Experimental Animals (Hamamatsu, Japan), respectively. "Principles of laboratory animal care" (NIH publication No. 86–23, revised 1985) were followed throughout the experiment.

Renal transplantation

Orthotopic renal transplantation was performed from LEW to BN rats using a microsurgical technique [8]. Briefly, under light ether anesthesia, the donor renal artery attached to the aorta and the donor renal vein were anastomosed end-to-side to the recipient abdominal aorta and inferior vena cava, respectively, using a microsurgical technique. Ureters were anastomosed end-to-end over a fine, 5-mm-long, polyethylene internal stent. The average total ischemic time was less than 30 min. All recipients were bilaterally nephrectomized at the time of transplantation. Graft function was assessed by recipient survival and confirmed by histopathological examination. If the autopsy disclosed abnormal findings such as hydronephrosis and infection, the animal was excluded from the experimental group.

Blood transfusion

Blood was obtained from a group of three of more LEW (donor-specific) or ACI (third party) rats via aortic puncture. Freshly pooled, whole heparinized blood was injected i.v. into BN recipients at a dose of 1.0 ml 2 days prior to transplantation.

Deoxyspergualin (DSG)

The deoxyspergualin, kindly donated by Nippon Kayaku (Tokyo, Japan) as a powder, was dissolved in saline at a concentration of 5 mg/ml, and 6 mg/kg per day of DSG was administered i.m. on days 0, 1, and 2 after transplantation.

Experimental groups

The animals were divided into five groups. Group 1 ($n = 6$) was the control group; rats in this group were not treated with DST or DSG. Group 2 ($n = 6$) was the DST group; only DST, no DSG, was administered. Group 3 ($n = 7$), the DSG group, was treated with DSG only; no DST was administered. Group 4 ($n = 7$), the DST and DSG group, was treated with both DST and DSG. Finally, group 5 ($n = 6$), the third party (ACI rats) blood transfusion and DSG group, was administered blood from third party rats and treated with DSG.

Lymphocyte harvesting

Lymphocytes were harvested from the cervical lymph nodes of BN recipients on day 7 postgrafting. Removed lymph nodes were minced on a stainless-steel mesh and resuspended in 10 ml of RPMI-1640 medium; then, 2 ml of TRIS-NH_4Cl was added to lyse red blood cells. After being washed three times with RPMI-1640, the lymphocytes were used for the following experiments.

Mixed lymphocyte culture (MLC) and suppressor cell and humoral factor assay

Cervical lymph node cells and serum were harvested from BN recipients on day 7 postgrafting. The stimulator cells from nontransplanted LEW rats were incubated with mitomycin C at a final concentration of 15 μg/ml for 30 min at 37 °C. After incubation, the stimulator cells were washed with RPMI-1640 three times. Then, 1×10^6 responder cells from each group and 2×10^6 stimulator cells

that had been treated with mitomycin C were added to flat-bottomed, 96-well microculture plates. For suppressor cell assays, lymphocytes from BN recipients from each group were added at the beginning of each reaction as a third cell to the MLC between nontransplanted BN lymphocytes (responder) and LEW or other third party (PVGC, ACI, or WKA rats) stimulator lymphocytes (responder: additional cells ratio, 5:1). In order to detect the presence of suppressive humoral factors, serum from BN recipients (group 4) was added to the MLC between nontransplanted BN and LEW. After the cultures had been incubated for 5 days at 37 °C in a humidified atmosphere of 5 % CO_2 in air, 0.5 mCi of 3H-thymidine was added for 3 h. Cells were harvested and counted in a liquid scintillation apparatus. The results were expressed as mean counts per minute (cpm) of triplicate cultures. The percentage of suppression was calculated using the formula:

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

Preparation of T lymphocytes

Cervical lymph node cells from BN recipients were passed through nylon-wool columns. In brief, after sterilization the column was rinsed with Hanks' balanced salt solution (HBSS) containing 10 % fetal calf serum (FCS) at 37 °C. The column was drained of excess medium and placed in an incubator at 37 °C for 1 h before loading the cells, and then lymphocytes were applied to the column. The column was left to stand for 60 min at 37 °C, lymphocytes were subsequently eluted with warm HBSS to obtain nylon-wool-nonadherent T cells.

Radiosensitivity of suppressor T cells

To determine the radiosensitivity of the suppressor T cells induced in this system, cervical lymph node cells from BN recipients were irradiated with serial doses of 200, 400, 800, and 1500 rad prior to addition to the MLC.

Antidonor lymphocytotoxic antibody (ADLA)

Five milliliters of serum was obtained from nontransplanted BN, both before and 7 days after DST treatment. Serum was also obtained from transplanted BN recipients just prior to pre-DST treatment, on the day of transplantation (2 days after DST treatment), and on day 7 postgrafting (9 days after DST treatment). Microcytotoxicity assays were performed as follows. Briefly, a 1.0-μl sample of serum from nontransplanted BN, DST-administered BN, or transplanted BN recipients was added to each well of a Terasaki HLA plate (Sumitomo, Japan). Donor LEW lymphocytes (2×10^3/well) from cervical lymph nodes were then added to each well and covered with liquid paraffin to prevent evaporation and incubated for 40 min at room temparature. After incubation, 5 μl of low-toxic guinea pig complement (Cedarlane Laboratories, USA) was added and incubated for 90 min at room temperature. After incubation, lymphocytes were stained with eosin Y solution and fixed with neutral formalin, and the numbers of dead cells were determined semiquantitatively under the microscope. When viability was less than 95 %, ADLA was considered to be positive.

Table 1 Graft survival time (*3rd BTF* third party blood transfusion)

Group	Survival time (days)	Median survival time
1 (untreated)	6, 8, 9, 10, 10, 10	10
2 (DST alone)[a]	6, 8, 9, 10, 12, 12	10*
3 (DSG alone)[b]	10, 11, 12, 13, 14, 14, 30	13**
4 (DST+DSG)	43, 53, 75, 75, 78, 153, 200 <	75***
5 (3rdBTF+DSG)[c]	10, 12, 13, 14, 14, 13	13**

* P = NS vs group 1; ** $P < 0.01$ vs group 1; *** $P < 0.005$ vs group 1

[a] Fresh 1.0 ml whole LEW blood was injected i. v. into BN rats 2 days prior to transplantation

[b] 6 mg/kg per day DSG was administered i. m. on days 0, 1, and 2 after transplantation

[c] Third party ACI rat blood served as a specificity control

Statistics

The Mann-Whitney U-test was employed for statistical analysis of survival time and percent suppression of the MLC reaction.

Results

Graft survival time

The median survival time of each group is presented in Table 1. Untreated BN recipients (group 1) rejected LEW grafts at 10 days. Administration of DST did not alter graft survival (group 2, median survival time 10 days). DSG given alone at a dose of 6 mg/kg per day (group 3) caused a slight, but significant, prolongation of graft survival time to 13 days compared with the untreated group (group 1; $P < 0.01$). Combined administration of DST and DSG (group 4) markedly prolonged graft survival to 75 days ($P < 0.005$). The group receiving third party blood transfusion showed a slight, but significant, prolongation of graft survival time to 13 days compared with group 1 ($P < 0.01$).

Suppressor activity in transplanted BN rats

In order to detect suppressor cell activity, BN recipient lymphocytes from each group were added as additional cells to one-way MLC reactions between responder, normal, BN lymphocytes and stimulator, mitomycin C-treated, LEW lymphocytes. Figure 1 shows the percent suppression of the MLC reaction between nontransplanted normal LEW (stimulator) and BN (responder) rats. Cervical lymph node cells from BN recipients treated with the combination of DST and DSG significantly suppressed the MLC reaction (83279 ± 14999 cpm for control group vs 58225 ± 16340 cpm for group 4, 40 % suppression; $P < 0.05$). In order to characterize the suppressor cell, cervical lymph node cells were fractionated

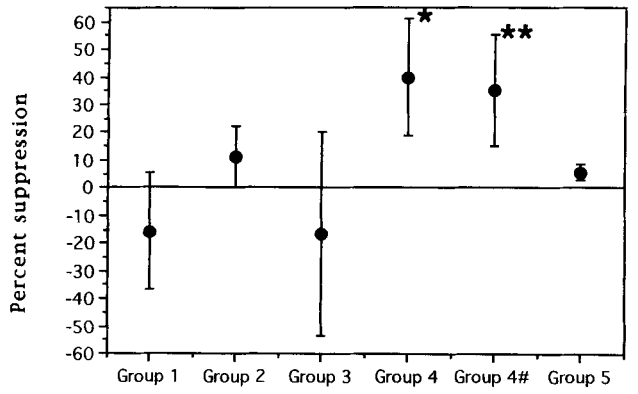

Fig. 1 Percent suppression of MLC reactivity. Group 1 ($n = 6$) no treatment; group 2 ($n = 6$) DST only; group 3 ($n = 7$) DSG only; group 4 ($n = 7$) DST and DSG; group 4# ($n = 5$) DST and DSG, assayed by T cells; group 5 ($n = 6$), third party (ACI rats) blood transfusion and DSG. * $P < 0.005$ for group 1 vs group 4; ** $P < 0.05$ for group 1 vs group 4#; P = NS for group 1 vs groups 2, 3, and 5

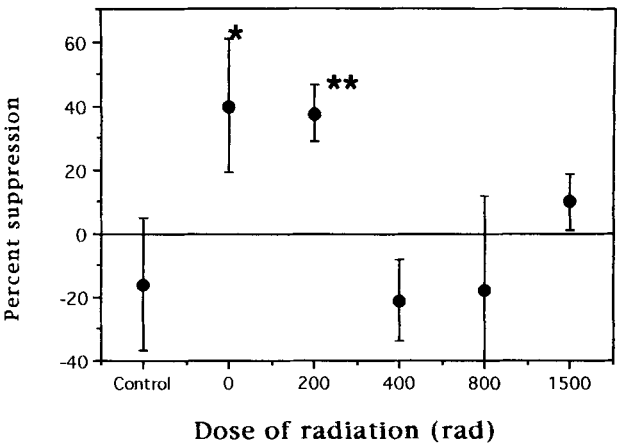

Fig. 2 Radiosensitivity of suppressor cells in DST/DSG-treated BN recipients. * $P < 0.005$ for controls (untreated recipients, group 1) vs no radiation group; ** $P < 0.05$ for controls vs 200 rad irradiated group; P = NS for controls vs 400, 800, and 1500 rad irradiated groups

on nylon-wool columns. Nylon-wool column-nonadherent cells (> 95 % T lymphocytes, data not shown) displayed 35 % suppression (70729 ± 1460 cpm for controls vs 51211 ± 8466 cpm for group 4#; $P < 0.05$). On the other hand, lymphocytes from untreated BN recipients and DST alone BN recipients (group 2) failed to suppress the MLC reaction (76109 ± 8382 cpm for controls vs 72182 ± 14573 cpm for group 2; P = NS). Although groups 3 (DSG-treated rats) and 5 (third party blood transfusion/DSG-treated rats) showed a slight, but significant, prolongation of survival time, suppressor activity of lymphocytes was not observed (52696 ± 39502 cpm for controls vs 38713 ± 29885 cpm for group 3; 82013 ± 9696 cpm for controls vs 83796 ± 6177 cpm for group 5; P = NS). Untreated rats (group 1) also did not show any suppressive activity (56241 ± 16611 cpm for controls vs 60596 ± 24589 cpm for group 1; P = NS). Since lymphocytes from group 4 showed significant MLC suppression, serum from BN recipients (group 4) was also added to the MLC between nontransplanted BN and LEW rats to detect the presence of suppressor humoral factors. Serum from group 4 did not show any inhibition of the MLC reaction (55732 ± 17321 cpm for controls vs 72452 ± 35621 cpm for the experimental group, percent suppression –30 %).

Radiosensitivity of suppressor T cells

Since suppressor cells are known to be sensitive to irradiation, the radiosensitivity of the suppressor T cells induced in group 4 was examined. Cervical lymph node cells from DST/DSG-treated BN rats were irradiated with serial doses prior to addition to MLC. Figure 2 shows that the suppressor T cells were resistant up to 200 rad, i.e., 37.6 % suppression (83279 ± 14999 cpm for controls vs 58225 ± 16340 cpm for nonirradiated rats; 69471 ± 3899 cpm for controls vs 46958 ± 5575 cpm for 200 rad irradiated rats; $P < 0.05$), but sensitive to 400 rad or more (44607 ± 6315 cpm for control vs 60002 ± 5037 cpm for 400 rad irradiated rats; 47428 ± 9872 cpm for controls vs 56394 ± 17467 cpm for 800 rad irradiated rats; 51992 ± 4122 cpm for controls vs 47269 ± 3202 cpm for 1500 rad irradiated rats).

Donor specificity of suppressor cells

In order to examine the donor specificity of suppressor cells, cervical lymph node cells from DST/DSG-treated BN recipients were added as a third cell to the MLC between non-transplanted BN (responder) and LEW or the third party (PVGC, ACI, or WKA rats) stimulator lymphocytes. Lymphocytes from DST/DSG-treated rats suppressed the MLC proliferative response of BN responder cells to donor LEW by 40 % (67869 ± 2102 cpm for controls vs 40287 ± 11534 cpm for LEW; $P < 0.05$), but not to PVGC (57504 ± 1405 cpm for controls vs 58257 ± 9977 cpm for LEW; –3 % suppression), ACI (57925 ± 11531 cpm for controls vs 49002 ± 7489 cpm for ACI; –5 % suppression), or WKA stimulators (7457 ± 2654 cpm for controls vs 12603 ± 1491 cpm for WKA; –69 % suppression; Fig. 3).

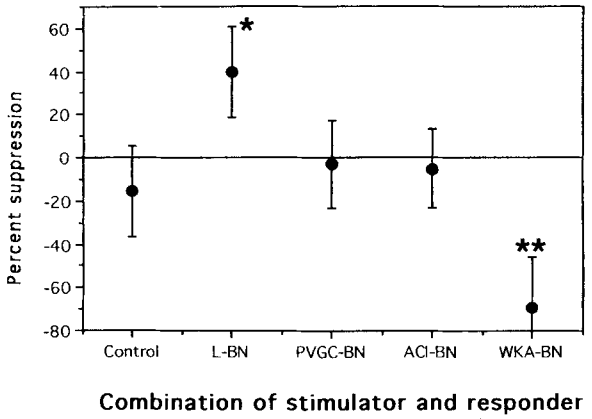

Fig.3 Specificity of suppressor cells. * $P < 0.005$ for controls (untreated recipients, group 1) vs LEW-BN; ** $P < 0.01$ for controls vs WKA-BN; P = NS for controls vs PVGC-BN and ACI-BN

Table 2 Antidonor lymphocytotoxic antibody *(ADLA)*. When the number of dead cells was more than 5 %, ADLA was considered to be positive *(P)*; when the number of dead cells was less than 5 %, ADLA was considered to be negative *(N)* (*3rd BTF*, third party blood transfusion)

Group	Pre-tx	Day of tx	Day 7 post-tx
1 (untreated)	N	N	N
2 (DST alone)	N	N	P (70 %)*
3 (DSG alone)	N	N	N
4 (DST + DSG)	N	N	N
5 (3rd BTF + DSG)	N	N	N
6 (DST but no tx)	N	–	P (80 %)*[a]

[a] Microcytotoxicity assays were done 7 days after DST
* Percentage of dead cells in microcytotoxicity

Anti donor lymphocytotoxic antibody (ADLA) in transplanted BN rats

Since blood transfusion from LEW to BN rats is known to produce anti-LEW antibody [10], we examined the presence of ADLA before DST, on the day of transplantation, and on day 7 postgrafting. The outcome of ADLA production in each group is summarized in Table 2. No ADLA was detected before DST or on the day of transplantation (i.e., 2 days after DST), but after DST, ADLA was detected in nontransplanted BN rats on day 7. On day 7 postgrafting, only group 2 (DST only administered BN recipients) produced ADLA; none of the other DSG-administered recipients (groups 3–5) or non-DST recipients (group 1) showed any production of ADLA.

Discussion

In this experiment, we clearly showed that both induction of donor-specific suppressor cells and inhibition of ADLA production were associated with a remarkable donor-specific prolongation of kidney allograft survival in DST/DSG-treated recipients.

DSG shows a strong immunosuppressive effect in the late phase of MLC, whereas CyA affects only the early phase of MLC [6]. In addition, greater immunological unresponsiveness is achieved in allografted rats by initiating the administration of DSG from the onset of acute rejection rather than on the day of grafting [17]. Furthermore, it has been suggested that DSG blocks B-cell maturation [6].

Given the above-mentioned results, this study was intended to induce donor-specific immunological unresponsiveness. Since it is known that transfusion from LEW rats to BN rats causes production of anti-LEW alloantibodies [14], we employed DST in order to sensitize BN rats by LEW. In this experiment, only combined administration of DST and DSG (group 4) markedly prolonged graft survival to 79.5 ± 35.3 days; this did not occur in the other groups (Table 1). Also, in the suppressor cell assay, group 4 showed significant suppression (40 %) while the other groups did not (Fig. 1). Moreover, suppressive humoral factors were not detected in the serum of group 4.

These findings seem to be compatible with those in other reports. Suzuki et al. demonstrated the induction of suppressor cells following 15-day DSG treatment from the onset of rejection in a rat heart allograft model [17]. They also suggested that suppressor cells might be spared by DSG, and that their activity might result in the maintenance of immunological unresponsiveness [17]. Valdivia et al. also demonstrated that combined treatment with DST and DSG prolonged graft survival time in hamster-to-rat xenotransplantation, and they suggested that suppressor cells and/or some degree of clonal deletion, but not humoral factors, participate in the phenomenon [22].

Furthermore, in our experiment, DSG inhibited the production of antidonor lymphocytotoxic antibody. To our knowledge, this is the first report of DSG inhibition of antidonor lymphocytotoxic antibody production. In xenotransplantation models, there are some reports regarding the inhibition of xenoantibody production [9, 11, 13, 21]. Nakajima et al. reported that DSG showed a significant prolongation of survival time for hamster-rat islet xenotransplantation and complete suppression of anti-hamster antibody production [13]. Valdivia et al. reported similar findings that DSG with splenectomy showed suppression of antidonor antibody production in hamster-rat cardiac xenotransplantation [21]. Recently, Marchmen et al. reported that in hamster-to-rat cardiac transplantation, combined treatment of DSG and total lymphoid irradiation lowered the rat anti-hamster lymphocytotoxic antibody titer, resulting in less humoral rejection histologically [11]. Our data seem to be compatible with these findings in xenotransplantation models.

Clinically, our model seems to be relevant to the transplantation of immunologically high responders. Recipients who have a high PRA may be good candidates for DSG prophylactic administration. Okazaki et al. reported that prophylactic administration of DSG remarkably reduced the incidence of accelerated acute rejection occurring within 5 days after transplantation in donor-specific buffy coat transfusion patients [16]. We also reported that DSG reduced the degree of vascular rejection histologically [9]. These findings suggest that DSG may be effective in sensitized recipients.

Overall, we clearly showed that both the induction of donor-specific suppressor cells and the inhibition of ADLA production were associated with a remarkable donor-specific prolongation of kidney allograft survival in DST/DSG-treated recipients. These findings suggest that DSG may be useful in the transplantation of immunologically high responders.

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