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15-Deoxyspergualin treatment of graft rejection in man: effect on mononuclear cells

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Abstract. We studied the effects of 15-deoxyspergualin (DSG) on human mononuclear cells in blood when DSG was administered as anti-rejection treatment to kidney-transplant patients in combination with methylprednisolone (MP) in a safety study. The numbers of leucocytes, lymphocytes and monocytes, the percentages of T and B cells and the expression of the interleukin 2 receptor and the human leucocyte antigen DR locus (HLA-DR) were determined in blood from these patients and from patients treated with MP only. We found that the number of lymphocytes and monocytes decreased in all groups, as also did the CD4/CD8 ratio and the HLA-DR expression on monocytes. DSG counteracted the increase in the number of leucocytes observed in patients treated with MP only. Different effects of DSG and MP were also observed on B cells. While the percentage of CD20+ cells increased in the MP group, it remained unaltered in patients given low-dose DSG and/or was decreased in those given higher doses. Since available evidence suggests an effect of DSG on B-cell reactivity, this drug may become an important addition to the arsenal of immunosuppressive drugs in clinical transplantation.

Key words: 15-Deoxyspergualin, in kidney transplantation, in humans – Kidney transplantation, in humans, 15-deoxyspergualin – Rejection, 15-deoxyspergualin, in human kidney transplantation – Mononuclear cells, 15-deoxyspergualin

The antibiotic spergualin, which was first isolated from a soil sample at Ohira-san, Japan, is produced by *Bacillus laterosporus* [37]. Spergualin has been found to inhibit antibody production and delayed-type hypersensitivity responses to sheep red blood cells in mice and to prolong skin allograft survival in rats [39]. Among many derivatives synthesized from spergualin, 15-deoxyspergualin (DSG) was selected because it was most effective in prolonging the lives of mice inoculated with mouse leukaemia L-1210 cells [38]. DSG has by now been extensively explored in various experimental organ transplant models. In the rat model, prolonged allograft survival has been shown for skin [4, 6], kidney [4, 41], liver [7, 8], heart [4, 28], islet cells [4], and pancreas [3, 32]. Heart allografts in mice [3] and skin [6] and heart allografts [3] in monkeys can be protected. DSG has been found to reverse ongoing acute rejection of kidney grafts in dogs [12]. In addition, the drug can prevent and reverse acute graft-versus-host disease in mice [24]. Xenograft survival can also be promoted [3, 29, 40], and this effect is more pronounced when DSG is used than when FK506 or cyclosporin A (CyA) is used [3, 29].

However, the mechanism of action of DSG is still poorly understood, and there are conflicting results regarding its immunological effects. DSG was first thought to act mainly on macrophages [4–6, 20]. It was shown to inhibit the secretion of hydrolytic enzymes and the generation of oxygen radicals [4] and to reduce the percentage of splenic macrophages expressing class II antigens [5]. More recently, it has been argued that any suppression of macrophage function may depend on an inhibitory effect of DSG on the production of a T-cell-derived factor which activates macrophages, and that DSG thus acts mainly on T cells [43]. However, the results regarding the effect of DSG on lymphocyte reactivity are also contradictory, ranging from reports demonstrating a lack of effect on lymphocyte proliferation in response to alloantigens [7, 29] to those arguing that DSG affects the response to alloantigens but not to the mitogen concanavalin A (ConA) [9, 41], or that DSG inhibits proliferative responses induced by both allogeneic cells and mitogens (ConA, phytohaemagglutinin, pokeweed mitogen) [14]. Inhibition of delayed-type hypersensitivity to sheep red blood cells in mice has been reported [21, 39], but results showing a lack of effect on this response have also been demonstrated [4]. There seems to be general agreement, however, that DSG does not inhibit de novo interleukin 2 (IL-2) production [18, 22, 43], and this clearly distinguishes DSG from CyA and FK506, the main mechanism of action of which is to

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Table 1. Effect of treatment with MP and DSG on number of leucocytes and percentage of lymphocytes and monocytes in peripheral blood of kidney recipients. NS, Not significant

Parameter	Treatment	Effect[a] (change in mean value)	Significance[b]	Difference in effect between treatment with MP and MP plus DSG[c]
No. of leucocytes	MP only	Increase	$P < 0.01$ day 3; $P < 0.05$ day 4	–
	MP + 2 mg/kg DSG	No change	NS	$P < 0.05$ day 3
	MP + 4 mg/kg DSG	Decrease	$P < 0.05$ day 3	$P = 0.05$ day 3
	MP + 6 mg/kg DSG	Decrease	$P < 0.05$ day 2	$P < 0.01$ days 2, 3; $P < 0.05$ day 4
Percentage of lymphocytes	MP only	Decrease	$P < 0.01$ days 2, 3; $P < 0.05$ day 4	–
	MP + 2 mg/kg DSG	Decrease	NS	NS
	MP + 4 mg/kg DSG	Decrease	$P < 0.05$ day 3	NS
	MP + 6 mg/kg DSG	Decrease	NS	NS
Percentage of monocytes	MP only	Decrease	$P = 0.01$ day 3, $P < 0.05$ day 3	–
	MP + 2 mg/kg DSG	Decrease	NS	NS
	MP + 4 mg/kg DSG	Decrease	$P < 0.01$ day 3	NS
	MP + 6 mg/kg DSG	Decrease	NS	NS

[a] Effect of treatment is given as a decrease/increase in mean value for each group of patients, whether this is statistically significant or not
[b] Student's *t*-test for paired values was used to calculate the significance of differences in values before (day 1) and during treatment (days 2–5)
[c] Student's *t*-test for independent values was used to calculate the significance of differences between the group of patients treated with MP only and the groups also given DSG

inhibit the production of various T-cell lymphokines [13, 34]. Evidence now points towards a suppressive effect of DSG on the induction of cytotoxic T lymphocytes [9, 25, 27, 39] and on antibody production [11, 18, 23, 40], but we are still far from understanding the mechanism of action of DSG.

Recently, DSG was used in a dose-finding study in renal transplant recipients at Huddinge Hospital in Stockholm and the Akademiska Hospital in Uppsala [30]. In this study, patients with acute rejection were treated with methylprednisolone (MP) plus DSG given at three different dosages. The study enabled us to evaluate the effect of DSG on human mononuclear cells. Peripheral blood samples were taken from the patients, and lymphocytes and monocytes were studied in regard to subsets and expression of different surface antigens, including CD2, CD3, CD20, CD4/CD8 ratio, the IL-2 receptor (IL-2R, CD25) and HLA-DR on B cells ($CD20^+$), T cells ($CD3^+$) and monocytes ($CD14^+$). The results suggest that the in vivo effects of DSG and MP are different, insofar as the number of leucocytes and the percentage of B cells are increased during treatment with MP alone; whereas they remain unaltered or are reduced by treatment with MP in combination with DSG.

Materials and methods

Kidney transplant recipients were given DSG in conjunction with MP as treatment for acute rejection [30]. The dose of MP was 0.5 g on day 1 and then 0.25 g on each of the following 3 days. DSG was given at three different dosages. The first patient group received DSG 2 mg/kg day i. v. for 5 days, the second patient group 4 mg/kg day and the third patient group 6 mg/kg day. Blood samples were collected in EDTA-treated tubes every day or every other day for 7 days from three patients given 2 mg/kg, five patients given 4 mg/kg and five given 6 mg/kg, the first sample being taken just before the first infusion of DSG (= day 1). In some cases samples were also taken on days 9 and 12. Six patients given only MP (standard treatment for rejection) were also included in the study, to distinguish the effect of DSG from that of MP.

Monocytes and lymphocytes were analysed using different surface markers to identify different cell populations and cell subsets. A total of 100 µl EDTA-treated whole blood was added to tubes containing 5–20 µl FITC- or phycoerythrin-conjugated monoclonal antibodies (mAbs) diluted with 100 µl phosphate buffered saline (PBS), and incubated in the dark at 4 °C for 20 min. Red cells were then lysed by the addition of an ammonium chloride solution (0.154 *M* NH_4Cl, 1 m*M* $KHCO_3$, 0.09 m*M* EDTA, pH 7.3). The tubes were kept at room temperature for 5 min before the non-lysed cells (granulocytes, monocytes and lymphocytes) were spun down, the lysing buffer was aspirated and the cells were resuspended in PBS. These cells were analysed by a fluorescence-activated cell sorter (FACScan, Becton-Dickinson, Mountain View, Calif.; USA). MAbs used were: positive control for the gating procedure CD45/CD14 (LEUCO-gate), negative control IgG_1IgG_2, and mAbs against CD2, CD3, CD4, CD8, CD20, CD14, IL-2R (CD25), and HLA-DR. The mAb against HLA-DR was used in combination with mAbs against a T-cell marker (CD3) a B-cell marker (CD20) and a monocyte marker (CD14; all mAbs were obtained from Becton-Dickinson, San Jose, California, USA except CD2-PE, which came from Dakopatts A/S, Glostrup, Denmark). The number of leucocytes in the blood of the patients was determined by the hospital's chemistry laboratory. Student's *t*-test was used for the statistical analysis.

Results

Effect on numbers of total leucocytes, lymphocytes and monocytes

Table 1 summarizes the effects of the rejection treatment on the number of leucocytes and the fraction of leucocytes that are lymphocytes or monocytes in the peripheral blood. In patients treated with MP only, the number of leucocytes in the blood rose during treatment, but returned to normal immediately after the last dose of MP. DSG seemed to eliminate this effect of MP, and a signifi-

Table 2. Effect of treatment with MP and DSG on T cells in peripheral blood of kidney recipients. NS, Not significant

Parameter	Treatment	Effect[a] (change in mean value)	Significance[b]	Difference in effect between treatment with MP and MP plus DSG[c]
CD2	MP only	No change	NS	–
	MP plus DSG	No change	NS	NS
CD3	MP only	No change	NS	–
	MP plus DSG	No change	NS	NS
CD4/CD8 ratio	MP only	Decrease	NS	–
	MP + 2 mg/kg DSG	No change	NS	NS
	MP + 4 mg/kg DSG	Decrease	$P < 0.05$ day 3	NS
	MP + 6 mg/kg DSG	Decrease	$P < 0.05$ days 2, 3	NS
IL-2R expression	MP only	No change	NS	–
	MP plus DSG	No change	NS	NS
HLA-DR expression	MP only	No change	NS	–
	MP plus DSG	No change	NS	NS

[a] Effect of treatment is given as a decrease/increase in mean value for each group of patients, whether this is statistically significant or not

[b] Student's *t*-test for paired values was used to calculate the significance of differences in values before (day 1) and during treatment (days 2–5)

[c] Student's *t*-test for independent values was used to calculate the significance of differences between the group of patients treated with MP only and the groups also given DSG

cant decrease was seen during the first days of treatment when 4 or 6 mg/kg DSG was given. Thus, there was a significant difference in effect between MP alone and MP plus DSG.

The numbers of lymphocytes and monocytes were calculated from the number of leucocytes and the percentages of lymphocytes and monocytes received from the FACS analysis. Following the drug treatment, a decrease in both the fraction (Table 1) and the total number of lymphocytes and monocytes was seen in all groups, except the group given the lowest DSG dose, 2 mg/kg. The reduction, however, was significant only in the MP only and the MP plus DSG 4 mg/kg groups.

Effect on T cells

The influence of the drug treatment on T cells is summarized in Table 2. The percentage of lymphocytes expressing T cell markers (CD2 and CD3) was not notably altered following treatment with MP alone or MP in combination with DSG (Fig. 1). A decrease in the CD4/CD8 ratio occurred in the majority of patients, regardless of treatment schedule. Since all patients received MP, the change was probably caused by this drug. However, the reduction in the ratio was statistically significant only in the 4 and 6 mg/kg DSG groups. One patient in the MP only group had to be excluded from this analysis since she had too few $CD8^+$ cells, and this made an accurate calculation of the ratio impossible.

Activated T lymphocytes usually express IL-2R and HLA class II antigens. In the peripheral blood from our patients, very few T cells ($CD3^+$) expressed either of these surface molecules. DSG or MP treatment caused no change in the expression of such activation markers. However, we found that the expression of IL-2R on $CD3^-$ cells, i.e. non-T cells, was decreased in these patients, in whom the inital value was high (not shown).

Effect on B cells

In the MP only group, an initial small reduction in the percentage of $CD20^+$ cells was followed by a rise. In the DSG groups, however, the percentage of B cells remained unaltered. In the highest-dose DSG group, two patients had a distinct decrease in the number of $CD20^+$ cells (Fig. 2). Consequently, the difference in effects between treat-

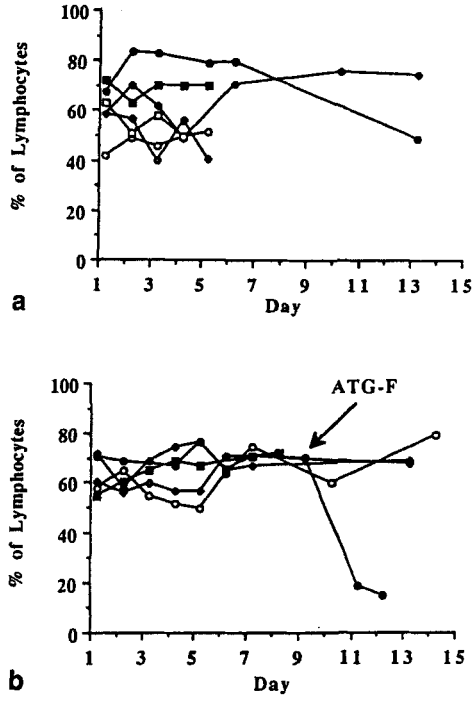

Fig. 1a, b. Effect of treatment of kidney recipients **a** with methylprednisolone (MP) only ($n = 6$) or **b** MP plus 15-deoxyspergualin (DSG) 6 mg/kg day ($n = 5$) on the percentage of T cells in peripheral blood, as assessed with a monoclonal antibody (mAb) against CD3. Each patient is represented by a separate line. Antithymocyte globulin-F (ATG-F) was given to one patient on day 9 in the DSG group

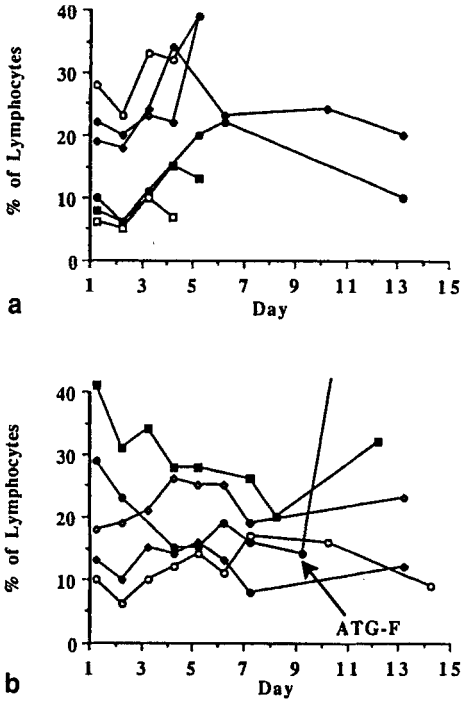

Fig. 2 a, b. Effect of treatment of kidney recipients **a** with MP only ($n = 6$) or **b** MP plus DSG 6 mg/kg day ($n = 5$) on the percentage of B cells in peripheral blood, as assessed with a mAb against CD20. Each patient is represented by a separate line. ATG-F was given to one patient on day 9 in the DSG group

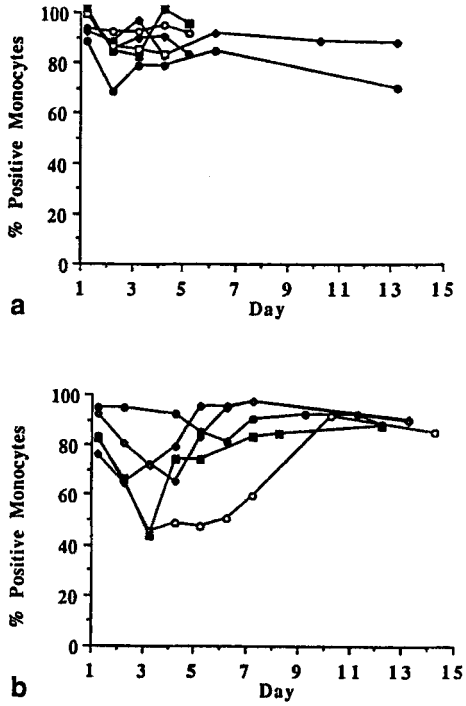

Fig. 3 a, b. Effect of treatment of kidney recipients **a** with MP only ($n = 6$) or **b** MP plus DSG 6 mg/kg day ($n = 5$) on percentage of monocytes expressing HLA-DR. Cells were stained with a fluorescein isothiocyanate-conjugated mAb against CD14 and a phycocrythrin conjugated mAb against HLA-DR and analysed by flow cytometry. Each patient is represented by a separate line

ment with MP alone and treatment with MP plus 4 or 6 mg/kg DSG was significant on days 3 and 5, respectively (Table 3).

No effect of the drug was seen on HLA-DR expression in the B cells (Table 3).

Effect on monocytes

As mentioned above, both the fraction and the total number of monocytes in the peripheral blood were reduced following treatment with MP or MP plus DSG (Table 1).

During treatment with MP, the percentage of monocytes expressing HLA-DR was reduced (Fig. 3), but the mean difference did not exceed 10%. Treatment with MP in combination with DSG also had a suppressive effect on HLA-DR expression (Fig. 3). There was no significant difference between the effects of MP alone and MP plus DSG (Table 4).

Discussion

Although clinical trials have now begun with DSG [1, 2, 15, 31, 36], the mechanism of action of the drug remains unclear, partly because DSG is unstable under culture conditions and therefore difficult to study in vitro [10].

In monitoring our patients we found no effect of DSG on the percentage of T cells or the percentage of activated T cells, as assessed by the expression of IL-2R and HLA-DR antigens. There was a reduction of the CD4/CD8 ratio in T cells and of IL-2R expression in $CD3^-$ cells (which would be mainly B cells) in all treatment groups, and thus these effects may be caused by MP. Corticosteroids are known to inhibit IL-1 production [16], and since IL-1 is a co-stimulator for $CD4^+$ T cells [17] and also induces IL-2R expression [35], the reduction in the CD4/CD8 ratio and IL-2R expression could be an indirect consequence of this inhibitory effect. Hence, there was no evidence that the T cells were affected by the administration of DSG.

The percentage of monocytes expressing HLA-DR was decreased whether MP only was given or MP plus DSG. In some early studies on DSG, a suppressive effect on HLA-DR expression was thought to be an important mechanism of action for the drug. However, in this pilot study of DSG in humans, all patients received MP, and DSG did not significantly enhance the effect produced by MP on the monocytes. Therefore, no conclusion can be drawn regarding the influence of treatment with DSG alone. Indeed, DSG may actually potentiate the suppressive effect of MP on the percentage of monocytes expressing HLA-DR. More data are clearly needed concerning this matter.

The administration of DSG counteracted the increase in the total number of leucocytes and the percentage of B cells observed following treatment with MP alone. A leucopenic effect of DSG has also been observed in the various clinical studies with DSG carried out in Japan [1, 2, 15,

Table 3. Effect of treatment with MP and DSG on B cells in peripheral blood of kidney recipients. NS, Not significant

Parameter	Treatment	Effect[a] (change in mean value)	Significance[b]	Difference in effect between treatment with MP and MP plus DSG[c]
CD 20	MP only	Increase[d]	$P = 0.01$ day 3, $P < 0.05$ day 5	–
	MP + 2 mg/kg DSG	No change	NS	NS
	MP + 4 mg/kg DSG	Decrease	NS	$P < 0.05$ day 3, $P = 0.05$ day 5
	MP + 6 mg/kg DSG	Decrease	NS	$P = 0.05$ day 5
HLA-DR expression	MP only	No change	NS	–
	MP plus DSG	No change	NS	NS

[a] Effect of treatment is given as a decrease/increase in mean value for each group of patients, whether this is statistically significant or not
[b] Student's *t*-test for paired values was used to calculate the significance of differences in values before (day 1) and during treatment (days 2–5)
[c] Student's *t*-test for independent values was used to calculate the significance of differences between the group of patients treated with MP only and the groups also given DSG
[d] The day after the first dose of MP there was a significant decrease in the percentage of B cells ($P < 0.05$)

Table 4. Effect of treatment with MP and DSG on monocytes in peripheral blood of kidney recipients. NS, Not significant

Parameter	Treatment	Effect[a] (change in mean value)	Significance[b]	Difference in effect between treatment with MP and MP plus DSG[c]
HLA-DR expression	MP only	Decrease	$P = 0.01$ day 2	–
	MP + 2 mg/kg DSG	Decrease	NS	NS
	MP + 4 mg/kg DSG	Decrease	NS	NS
	MP + 6 mg/kg DSG	Decrease	$P < 0.05$ day 2, $P = 0.05$ day 3	NS

[a] Effect of treatment is given as a decrease/increase in mean value for each group of patients, whether this is statistically significant or not
[b] Student's *t*-test for paired values was used to calculate the significance of differences in values before (day 1) and during treatment (days 2–5)
[c] Student's *t*-test for independent values was used to calculate the significance of differences between the group of patients treated with MP only and the groups also given DSG

31]. The severity of leucopenia seems to be proportional to the dose and duration of treatment and may be caused by a cytostatic effect on haematopoietic stem cells, where the proliferation and differentiation of immature bone marrow cells is suppressed [26, 27]. The reduction in B-cell fraction seen in our patients is particularly interesting since it is in accordance with previous reports showing inhibitory effects on antibody responses in mice [4, 6, 18, 25] and dogs [23]. Although this study provides no information on antibody production, it indicates that human B cells are also affected. The ability of DSG to prolong the lives of mice inoculated with mouse leukaemia L-1210 cells [37, 38], in earlier studies of DSG interpreted as an anti-tumour effect, may be due to a suppressive effect of DSG on antibody production. The so-called immunological enhancement effect, where antibodies are produced against tumour-associated antigens or against major histocompatibility complex antigens on tumors, would thereby be inhibited.

Are B cells and antibodies important in acute rejection? In other words, could the decreased B-cell fraction be an important factor when it comes to the effect of the drug on rejection reactions?

Acute rejection has been thought to depend mainly on cellular mechanisms, while the humoral response has been implicated mainly in hyperacute and chronic rejection. However, a humoral response with antibodies against donor antigens is also probably involved in acute rejections. Rejection may be induced by donor-derived antigen presenting cells (passenger cells). It is also possible that antigens shed from the allograft can be picked up and presented as peptides in the context of class II on host cells inducing the host lymphoid system. Cytotoxic T cells thereby induced would not be harmful to the graft, recognizing self-HLA plus allopeptide (processed antigen) not present on the graft. B cells, however, recognize the unprocessed antigen – i.e. allo-HLA – and thus humoral-mediated injury to the graft may follow (see [19]). Circulating donor-specific HLA antibodies are often found in conjunction with acute rejection episodes, and these may cause tissue damage by direct complement-mediated cell lysis or via complement-independent pathways, such as antibody-dependent cell-mediated cytotoxicity [33, 42]. The effect of DSG on such mechanisms is still unknown, but will form the basis for further studies.

If the observed effect of DSG on the B-cell fraction is important for its immunosuppressive activity in vivo, this may contraindicate the use of DSG in combination with MP, since the two drugs have opposing effects in this respect. Amemiya et al. [2] have shown, however, that DSG therapy in humans is more effective when combined with MP. Again, further studies are clearly needed.

The difference between the mechanisms of action of DSG and those of CyA and FD506 is intriguing. The field of transplantation would obviously benefit from an arsenal of drugs affecting the immune response at different levels. It is also of interest that DSG may be useful in xenotransplantation, where different immunoregulatory mechanisms are in action. However, the immunosup-

pressive effects of DSG are still largely unknown and further studies are needed to define optimal immunosuppression for different kinds of transplants and the various rejection mechanisms.

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