IN VITRO IMMUNOSUPPRESSIVE PROPERTIES OF SPERGUALINS TO MURINE T CELL RESPONSE

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(Received for publication September 12, 1988)

Deoxyspergualin has strong immunosuppressive activity in animals. However, it shows less *in vitro* immunosuppressive activity at the therapeutic concentration used for *in vivo* administration. Recently, we reported that there are some technical problems with *in vitro* experiments. In this report, the effects of spergualins were examined under *in vitro* conditions which excluded these problems, and compared with cyclosporine A (CYA). Spergualins have suppressive effects on mixed lymphocyte response (MLR) and cytotoxic T lymphocyte induction. Furthermore, interleukin-2 (IL-2) induced proliferation of concanavalin A blasts and CTLL-2 were inhibited at low concentration. However, spergualins have little effect in the early stage of MLR or the mitogen response. These results suggest that spergualins act on the proliferation and differentiation of T cells which respond to growth factors, such as IL-2.

Deoxyspergualin (DSG, NKT-01), an analog of spergualin[1], has antitumor activity[2] and immunosuppressive activity[3]. As reported previously, DSG acts to prolong allogeneic[4~8] and xenogeneic graft[9] survival in various experimental transplantation models and shows therapeutic effect on various autoimmune disease models[10]. We have been investigating the mode of action of DSG on immunosuppressive activity, but it shows little *in vitro* activity at the therapeutic concentration used for *in vivo* administration. Recently we reported that there are some technical problems with *in vitro* experiments, that is, stability of DSG in culture medium[11] and oxidation by amine oxidase in fetal calf serum (FCS)[12]. In this report, the *in vitro* effect of spergualins was examined using a stable analogue, deoxymethylspergualin (MeDSG), and a culture medium which has practically no amine oxidase activity. The immunosuppressive effect of spergualins on murine lymphocytes were demonstrated *in vitro* and the immunosuppressive properties were compared with cyclosporine A (CYA).

Materials and Methods

Animals

BALB/c and C3H/HeN mice in specific disease free condition were obtained from Charles River Japan Inc., (Kanagawa, Japan).

Culture Medium and Agents

RPMI-1640 and minimum essential medium (MEM) were obtained from Gibco Laboratories Inc. (Grand Island, NY, U.S.A.). Serum from BALB/c mice was collected 2 hours after bleeding *via* cardiac puncture. CLICK's medium was prepared as described by CLICK *et al.*[13]. DSG (1-amino-19-guanidino-11-hydroxy-4,9,12-triazanonadecane-10,13-dione) and MeDSG (1-amino-19-guanidino-11-methoxy-4,9,12-triazanonadecane-10,13-dione) were provided by Takara Shuzo Co., Ltd., (Kyoto,

Japan). Spergualins were dissolved in physiological saline, sterilized by Millipore filtration and diluted to the appropriate concentration with the indicated medium. CYA was kindly supplied by Sandoz Ltd., (Basel, Switzerland) *via* the Late Prof. H. UMEZAWA, dissolved in ethanol and diluted in the indicated medium. Recombinant human interleukin-2 (r-IL-2) was purchased from Shionogi & Co., Ltd., (Osaka, Japan). Concanavalin A (Con A) and methyl α-D-mannopyranoside (α-MM) were purchased from Pharmacia Fine Chemicals Inc., (Uppsala, Sweden) and Sigma Chemical Company (St. Louis, MO, U.S.A.), respectively. [^{3}H]Thymidine ([^{3}H]TdR, 15.5 Ci/mmol), and $Na_2{}^{51}CrO_4$ were obtained from New England Nuclear (Boston, MA, U.S.A.).

Allogeneic Mixed Lymphocyte Response (MLR)

MLR's were performed as described previously[11]. Briefly, responder spleen cells (5×10^5) from BALB/c mice (H-2^d) were admixed with mitomycin C treated stimulator spleen cells (5×10^5) from C3H/HeN (H-2^k), and plated in triplicate wells on 96-well round-bottomed microtest plates (Coster, Cambridge, England) in culture medium. Culture medium used for testing the MLR was RPMI-1640 supplemented with 0.5% murine serum, 1 mM sodium pyruvate and 25 μM of 2-mercaptoethanol (completed RPMI-1640). Sixteen hours before harvesting, 1 μCi of [^{3}H]TdR was added to each well.

Alloreactive Cytotoxic T Lymphocyte (CTL) Assay

Alloreactive CTL induction was carried out using a modification of the method of PECK and BACH[14]. Briefly, responder spleen cells (2.5×10^6/ml) from C3H/HeN were admixed with X-ray-irradiated (2,000 rad) spleen cells (2.5×10^6/ml) from BALB/c mice, and plated on 24-well tissue culture plates (Coster) in CLICK's medium supplemented with 0.5% murine serum and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). After 5 days incubation, the cells were harvested and 3×10^5 viable cells were reincubated for 4 hours with 1×10^4 of ^{51}Cr labeled P815 target cells in MEM on 96-well round-bottom microtest plates (Linbro Chemical., New Haven, CT, U.S.A.). The percentage of specific ^{51}Cr release was calculated according to

$$\%\ \text{specific}\ {}^{51}\text{Cr release} = \frac{{}^{51}\text{Cr release experiment's} - {}^{51}\text{Cr release spontaneous}}{{}^{51}\text{Cr release freeze and thaw} - {}^{51}\text{Cr release spontaneous}} \times 100$$

Mitogenic Response

Mitogenic response was measured by incubating murine spleen cells (2×10^5) with 0.5 μg/ml of Con A in complete RPMI-1640. Four hours before harvesting, 1 μCi of [^{3}H]TdR was added to each well.

Response to IL-2-dependent Proliferation of Con A-stimulated Cells and CTLL-2

IL-2-dependent proliferation of Con A-stimulated cells and CTLL-2 were carried out according to the modification of the methods of YUKIOKA *et al.*[15]. Con A-stimulated cells were prepared from spleen cells and incubated in complete RPMI-1640 medium with 0.5 μg/ml of Con A. After 45 hours, cells were washed with RPMI-1640 supplemented with 10 mg/ml of α-MM 1×10^4 cells were plated on 96-well plates and cultured in 200 μl of RPMI-1640 supplemented with 5 mg/ml of α-MM and r-IL-2 (100 U/ml) for 72 hours. Eight hours before harvesting, 1 μCi of [^{3}H]TdR was added to each well. CTLL-2 (1×10^4) were plated on 24-well plates in 1 ml complete RPMI-1640 and r-IL-2 (100 U/ml) in the presence or absence of agents. Four hours before harvesting, 200 μl of cell suspension were dispersed from the 24-well plate to a 96-well microtest plate well, and 1 μCi of [^{3}H]TdR was added to each well.

Results

Effect on MLR and CTL Induction

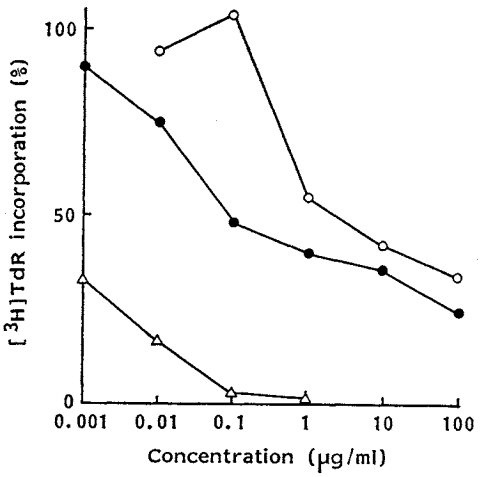
The *in vitro* effect of DSG, MeDSG and CYA on allogeneic MLR was assessed. These compounds inhibited the MLR dose-dependently when added to the MLR at the initiation of culture (Fig. 1). DSG and MeDSG markedly inhibited the MLR from 1 μg/ml, and 0.01 μg/ml, respectively.

The concentration value of DSG and MeDSG for 50% inhibition (IC_{50}) was 2.8 μg/ml and 0.08 μg/ml, respectively. CYA exhibited complete suppression of the MLR from 0.1 μg/ml and the IC_{50} was less than 0.001 μg/ml.

The effect of DSG, MeDSG and CYA on CTL induction was examined next. When these compounds were added at the initiation of culture, marked inhibition of alloreactive CTL induction was observed (Fig. 2). The IC_{50} values of DSG, MeDSG and CYA were 0.5 μg/ml, 0.1 μg/ml and 0.05 μg/ml, respectively. Thus, these compounds suppressed both growth and the functional killing of alloreactive T lymphocytes.

Fig. 1. Effect of DSG, MeDSG and CYA on MLR.

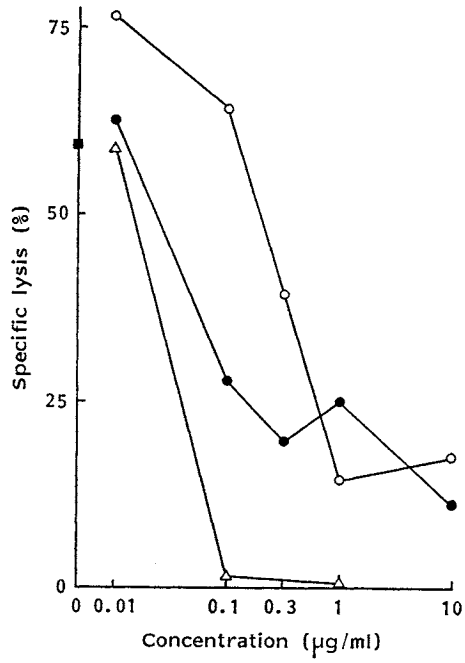
DSG (○), MeDSG (●) and CYA (△) were added at initiation of culture.



BALB/c responder cells (5×10^5) were cultured with C3H/HeN stimulator cells (5×10^5) for 90 hours. The data are presented as percentage of the control. Mean control dpm of [^{3}H]TdR uptake for MLR was 170,194±19,715.

Fig. 2. Effect of DSG, MeDSG and CYA on CTL induction.

DSG (○), MeDSG (●) and CYA (△) were added at initiation of culture, specific lysis of control (■).



CTL were induced in one way MLR for 5 days culture containing C3H/HeN responder and BALB/c stimulator spleen cells. Cytolytic activity was assessed in a 4-hour ^{51}Cr release assay using P815 target cells (E:T=30). Spontaneous ^{51}Cr release and maximal cpm are 829.7 cpm and 6,970.8 cpm, respectively.

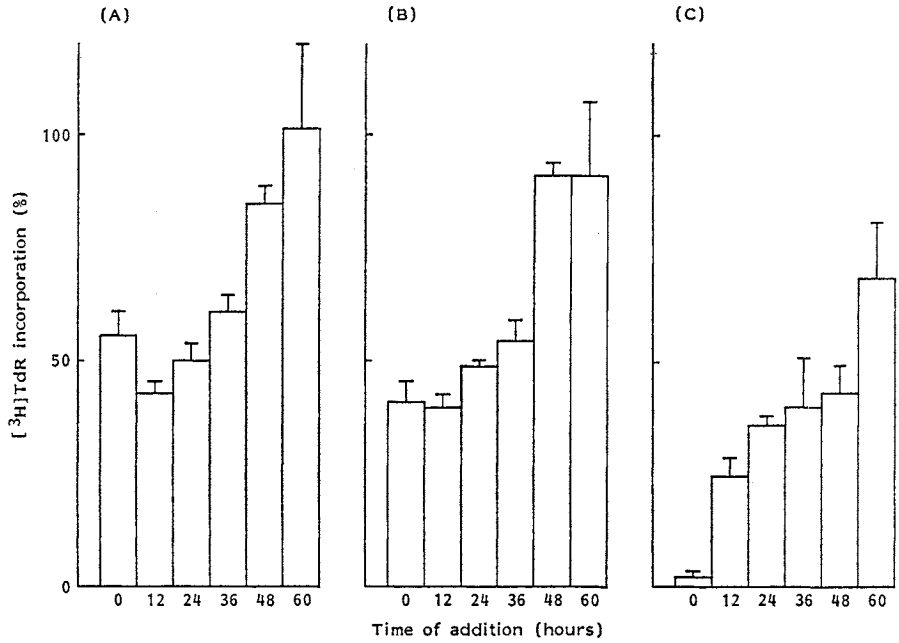
Table 1. The effect of MeDSG and CYA on the time course of MLR.

Time (hours)	[^{3}H]TdR incorporation (dpm)		
	Control	MeDSG (1 μg/ml)	CYA (0.1 μg/ml)
42	20,973±2,388	25,895±1,488*	3,107±961**
66	48,130±5,823	50,111±3,855	3,809±763**
90	67,643±11,544	35,160±85**	3,294±227**
114	31,200±2,268	20,153±657**	2,067±636**

Significantly different from control by t-test, * $P<0.05$, ** $P<0.01$.

BALB/c responder cells (5×10^5) were cultured with C3H/HeN stimulator cells (5×10^5) for various hours.

Fig. 3. Effect of DSG, MeDSG and CYA on the stage of the MLR.



MLR were carried out as described in Fig. 1. DSG 1 μg/ml (A), MeDSG 1 μg/ml (B) and CYA 0.1 μg/ml (C) were added at 0, 12, 24, 36, 48, 60 hours and incorporation of [^{3}H]TdR were measured at 90 hours after initiation of culture. The data are presented as percentage of control. Mean control dpm of [^{3}H]TdR uptake for MLR was 213,877$\pm$10,439 dpm.

Kinetic Studies of MLR

The effect of MeDSG and CYA on the time course of MLR was examined. These compounds were added at the initiation of MLR and the incorporation of [^{3}H]TdR was measured at 42, 66 and 90 hours after initiation of MLR. CYA at 0.1 μg/ml strongly inhibited the MLR at every measurement point. In contrast, TdR incorporation was not inhibited at 42, 66 hours by 1 μg/ml of MeDSG. However, MeDSG significantly inhibited MLR after 90 hours measurement (Table 1). Thus, this effect of MeDSG is time dependent.

The suppressive activity of these compounds at different stage of the MLR was investigated at 1 μg/ml of DSG and MeDSG, and 0.1 μg/ml of CYA. These compounds were added at varying times to culture after initiation of MLR. Both DSG and MeDSG exhibited a significant inhibition up to 36 hours after MLR initiation (Fig. 3). In contrast, CYA exhibited potent inhibitory effect when it was added at initiation. Therefore, CYA inhibited the initiation stage of MLR whereas MeDSG rather inhibited the stage of proliferation which induced after recognition of *allo* antigen than the initiation stage of MLR.

Effect on Lymphocyte Blastogenesis and IL-2-dependent Proliferation of Both Con A-stimulated and CTLL-2 Cells

To confirm whether the effect of spergualins is dependent on incubation period or on stage of lymphocyte activation, the effect of MeDSG and CYA on lymphocyte blastogenesis induced by Con A and IL-2-dependent proliferation of Con A-stimulated cells was compared (Table 2). Both experi-

Table 2. Effect of MeDSG and CYA on Con A-induced lymphocyte blastogenesis and IL-2-dependent proliferation of Con A-stimulated cells.

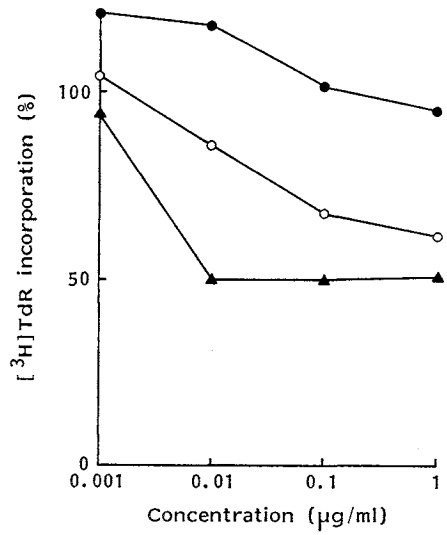
Agents	Dose (μg/ml)	[³H]TdR incorporation			
		Con A-induced lymphocyte blastogenesis		IL-2-dependent proliferation	
		dpm	%	dpm	%
Control		644,806	100	228,606	100
MeDSG	10	533,995**	82.8	132,473*	57.9
	1	616,725	95.6	135,638*	59.3
	0.1	629,587	97.6	127,427*	55.7
CYA	1	4,328**	0.7	25,192**	11.0
	0.1	14,946**	2.3	206,584	90.4
	0.01	266,635**	41.4	193,180	84.5

The SD value does not exceed 20%.
Significant difference from control by t-test, * $P<0.05$, ** $P<0.01$.

ments were performed after 72 hours incubation. MeDSG exhibited little effect on mitogenic response, however, it inhibited IL-2-dependent proliferation at concentrations greater than 0.01 μg/ml. In contrast, CYA inhibited the Con A-induced lymphocyte blastogenesis dose-dependently and IC_{50} was less than 0.01 μg/ml. However, CYA didn't exhibit a marked effect on IL-2-induced proliferation at such a low concentration. This was inhibited only at 1 μg/ml. These results indicate that spergualins act on the proliferative stage of lymphocytes. The effect of MeDSG and CYA on IL-2-induced proliferation of CTLL-2 which is a IL-2-dependent murine T cell line was investigated (Fig. 4). Incubation with MeDSG for 24 hours had little effect, however the 48 and 72 hours incubation exhibited marked inhibition of proliferation. CYA inhibited proliferation only at a high concentration (data not shown). Thus, spergualins inhibited IL-2-induced proliferation in a manner dependent on incubation period.

Fig. 4. Effect of MeDSG on IL-2-dependent proliferation of CTLL-2 line.

Cells of the murine T line CTLL-2 (1×10^4/ml) were incubated for 24 (●), 48 (○), 72 (▲) hours.



The data are presented as the percentage of control. Mean control dpm of [³H]TdR uptake following 24, 48 and 72 hours incubation were 38,402.7 dpm, 165,103.5 dpm, 424,988.3 dpm, respectively, and cell numbers were 1.5×10^4/ml, 6.2×10^4/ml, 40.8×10^4/ml, respectively.

Discussion

We previously reported that DSG has immunosuppressive activity in animals and acts to prolong allograft survival in many experimental transplantation models[4~9]. However, investigating the mode of action of spergualins *in vitro* is difficult because: 1) DSG is hydrolyzed in culture medium during incubation causing loss of activity; 2) DSG is oxidized by amine oxidase contained in fetal calf serum and the oxidized products have cytotoxic effects on lymphocytes and leukemia cells, but no relation

to *in vivo* activity.

In this paper, we investigated the effect of spergualins using a stable analogue of spergualin and murine serum. The serum has practically no amine oxidase activity.

Under these conditions, MeDSG has suppressive effects on MLR and CTL induction at 0.1 μg/ml. This concentration is comparable to the therapeutic concentration of DSG shown by *in vivo* administration. The effect of spergualins and CYA on the time course of proliferation of MLR showed that CYA inhibited the [^{3}H]TdR incorporation of MLR when it was added at the stage of initiation. In contrast, DSG and MeDSG inhibited the MLR at a relatively later stage. The kinetic study of MeDSG and CYA addition to culture of MLR revealed that CYA has strong suppressive effects on the early stage of MLR, however DSG affects the relatively later stage of the response.

Spergualins did not exhibit a marked effect on mitogenic response. However, spergualins at low concentration did suppress the IL-2-dependent cell proliferation of both Con A-stimulated and CTLL-2 cells. The emergence of this effect is time dependent. In contrast, CYA exhibited a marked effect on mitogenic response, but little effect on IL-2-induced proliferation.

These results suggest that the mode of action of spergualin is a suppressive effect on the proliferation and differentiation of T cells. These are able to respond to growth factors such as IL-2. The fact that spergualins have less effect on the early stage of MLR or the mitogen response suggests that spergualins might not affect $G_0 \rightarrow G_1$ conversion.

SUZUKI *et al.*[6] reported that DSG acts to prolong the allogeneic rat heart transplantation when its administration commenced at the onset of rejection. In contrast, CYA shows little effect in such a case. This supports our suggestion that DSG affects those T cells and effects were observed at later stage than those of CYA.

KRÖNKE *et al.*[16] reported that CYA affected the pretranscriptional level of mRNA encoding for IL-2. In contrast, spergualins have little effect on the early stage of the MLR and mitogenic response. Therefore, they should have little effect on the cascade. Instead, they may affect the proliferative stage after IL-2 and IL-2 receptor binding assembly.

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