IMMUNOSUPPRESSIVE ACTIVITY OF 15-DEOXYSPERGUALIN AND ITS EFFECT ON SKIN ALLOGRAFTS IN RATS

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The immunosuppressive activity of spergualin analogues, 15-deoxyspergualin and N-30, is presented. These compounds suppressed antibody formation and establishment of delayed-type hypersensitivity to sheep red blood cells (SRBC) in mice. Among spergualin, 15-deoxyspergualin, and N-30, 15-deoxyspergualin was most effective in suppressing immune responses. It suppressed antibody formation against SRBC by spleen cell cultures in a dose-dependent fashion without reducing cell viability. The spergualins also suppressed the mixed lymphocyte culture reaction. Peritoneal exudate cells taken from mice given immunosuppressive doses of 15-deoxyspergualin were not reduced in their functions measured: Release of lysosomal enzymes and production of superoxide anions. 15-Deoxyspergualin and N-30 were markedly effective in prolonging skin graft in rats.

In a previous paper, we reported that spergualin, an antitumor antibiotic, had immunosuppressive activity and was effective in prolonging skin grafts in rats[1]. From a study on spergualin analogues, 15-deoxyspergualin and N-30 were found to have stronger antitumor activity than spergualin itself[2,3]. We report in this paper the immunosuppressive effects of 15-deoxyspergualin and N-30 in mice and their effect on skin allografts in rats.

Materials and Methods

Animals

C3H/He, C57BL/6, BALB/c, and CDF_1 (BALB/c×DBA/2) female mice, 6 to 8 weeks old, were purchased from Charles River Japan Inc., Japan. Males or females of the F344 and SHR strains of rats (8 weeks old) were also purchased from Charles River Japan Inc., Japan. They were maintained under special pathogen-free conditions at 23±1°C and 55±5% humidity.

Spergualins

Spergualin ((−)-(15*S*)-1-amino-19-guanidino-11,15-dihydroxy-4,9,12-triazanonadecane-10,13-dione), 15-deoxyspergualin ((±)-1-amino-19-guanidino-11-hydroxy-4,9,12-triazanonadecane-10,13-dione), and N-30 (10-{*N*-[4-(4-guanidinophenyl)butanoyl]-L-seryl}-1,5,10-triazadecane) were prepared by Takara Shuzo Co., Ltd., according to the methods described previously[2].

Immune Responses

Antibody formation and delayed-type hypersensitivity (DTH) to sheep red blood cells (SRBC) in mice were examined by methods reported previously[4,5]. 15-Deoxyspergualin and other spergualins

† Deceased.

were injected intraperitoneally on the day of immunization or from 1 day after the immunization, daily for 3 days in experiments on antibody formation or 4 days in those on DTH. Antibody formation was assayed by counting the number of plaque-forming cells (PFC) in spleen cell populations 4 days after the immunization. Secondary response of antibody formation was examined by the method described earlier[5]. Mice were immunized with 10^5 SRBC and given 6.25 mg/kg of 15-deoxyspergualin from 1 day after the immunization, daily for 9 days. Twenty-one days after the primary immunization, a second immunization was performed by iv injection of 10^8 SRBC, and 4 days thereafter the number of PFC in the spleen was determined.

The eliciting injection for the DTH responses was performed 4 days after the immunization, and the response was determined by measuring footpad thickness 24 hours later.

Antibody formation to SRBC in spleen cell cultures was performed according to a modification of the method reported previously[5]. Spleen cells (1×10^7 cells/ml) taken from normal CDF_1 mice were cultured in Nunclon multi dish 24 dishes (Nunc. Nihon Inter Med. Co., Ltd., Tokyo) with or without various concentrations of 15-deoxyspergualin in medium containing SRBC, and 4 days thereafter antibody formation in the cultures was determined by counting the number of PFC.

Mixed Lymphocyte Culture (MLC) Reaction

MLC reaction was tested by a commonly used method[8]. Spleen cells taken from C3H/He mice ($H\text{-}2^k$) used as the responder were mixed with stimulator spleen cells taken from BALB/c ($H\text{-}2^d$); the stimulator cells had been previously incubated with 50 μg/ml of mitomycin C (MMC) (Kyowa Hakko Kogyo Co., Ltd., Tokyo) at 37°C for 20 minutes. The mixed spleen cells were cultured with or without drugs in medium containing 10% fetal calf serum (FCS) (GIBCO, Grand Island, N.Y.) at 37°C for 5 days in 5% CO_2 in air and [^{3}H]thymidine was added 16 hours before the assay. MLC reaction was determined by measuring the incorporation of [^{3}H]thymidine into the cultured cells.

Macrophage Functions

Influence of 15-deoxyspergualin on the release of phorbol myristate acetate (PMA)-induced superoxide anion from peritoneal exudate cells was examined according to the method reported by KITAGAWA and JOHNSTON[6]. Mice were injected with 15-deoxyspergualin daily for 3 days and peritoneal exudate cells (PEC) were then taken from these mice. PEC were incubated with PMA for 1 hour and the superoxide anion content of the supernatant was determined by measuring reduction of cytochrome C. The influence of 15-deoxyspergualin on release of lysosomal enzymes from macrophages was examined according to the method reported by HASHIMOTO *et al.*[7]. PEC were taken from mice given 15-deoxyspergualin and incubated with *p*-nitrophenyl-β-D-glucuronide for determination of β-glucuronidase or *p*-nitrophenyl phosphate disodium for that of acid phosphatase. The activity of these enzymes was determined by measuring OD at 420 nm and was expressed the difference of OD ($\times10^3$). To test the influence of 15-deoxyspergualin on activated macrophages, zymosan was used for activation of macrophages. Mice were injected with 2 mg of zymosan 4 days before the assay, and 1 day thereafter were given 15-deoxyspergualin daily for 3 days; then activities of PEC were examined.

Statistical Analysis

Statistical significance was determined by Student's t-test analysis.

Results

Suppression of Antibody Formation and of DTH Responses to SRBC in Mice

Spergualin and its synthetic analogues 15-deoxyspergualin and N-30 were injected into mice from the day of immunization daily for 4 days. As shown in Fig. 1, each compound had a suppressive effect on both humoral and cellular immune responses in a dose-dependent manner.

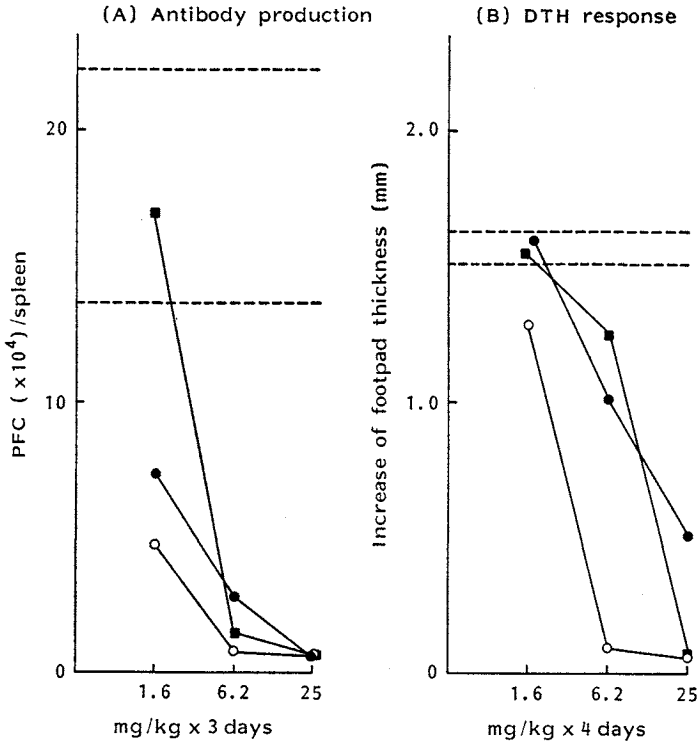
Since among these spergualins, 15-deoxyspergualin was the most immunosuppressive, thereafter,

Fig. 1. Immunosuppressive effects of spergualins.

● Spergualin, ○ 15-deoxyspergualin, ■ N-30.

(A) CDF_1 mice (5 mice/group) were immunized with 10^8 SRBC by iv and 1 day thereafter, each substance was given ip daily for 3 days. Results were determined 4 days after the immunization and was the mean of 5 mice. Statistical analysis was performed by Student's t-test.

(B) CDF_1 mice (5 mice/group) were immunized with 10^5 SRBC by iv and 1 day thereafter, each substance was given ip daily for 4 days. Four days after immunization, 10^8 SRBC was injected sc to left hind footpad, 24 hours thereafter, resulting edema was measured. Results are the mean of 5 mice. Statistical analysis was performed by Student's t-test.



the effect of 15-deoxyspergualin on antibody production and DTH response was examined. As shown in Fig. 2, the daily injection of 15-deoxyspergualin at 0.4 to 25 mg/kg after immunization suppressed antibody production significantly (Fig. 2). The injection of 15-deoxyspergualin after primary immunization suppressed the secondary antibody production (Table 1). In this experiment, the daily injection of 15-deoxyspergualin did not affect spleen weight of mice in any group.

The suppressive effect of 15-deoxyspergualin against DTH is shown in Fig. 3. The injection of 15-deoxyspergualin into mice at 1.6 to 25 mg/kg/day for 4 consecutive days from 1 day after the immunization suppressed the DTH response markedly. The response was enhanced slightly by the injection of 0.1 mg/kg/day, whereas doses lower than 0.1 mg/kg did not show any effect.

Suppression of Primary Antibody Formation to SRBC by 15-Deoxyspergualin *In Vitro*

Antibody formation against SRBC by spleen cell cultures in medium containing 10% FCS was suppressed by the addition of 15-deoxyspergualin to the cultures. As shown in Fig. 4, the addition of 15-deoxyspergualin at the start of cultures reduced the number of PFC dose-dependently. Antibody

Fig. 2. Effect of 15-deoxyspergualin on antibody production.

This experiment was done by same method as described in Fig. 1 (A).

* $P<0.001$.

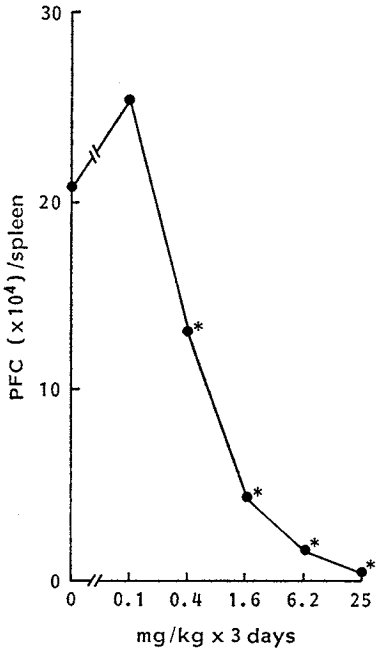


Fig. 3. Effect of 15-deoxyspergualin on DTH response.

This experiment was performed by same method as described in Fig. 1 (B).

* $P<0.01$, ** $P<0.001$.

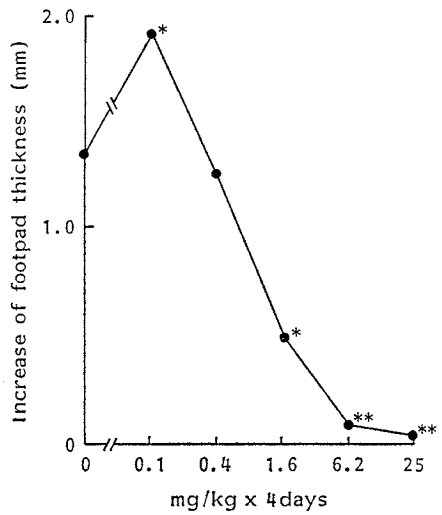


Table 1. Inhibition of secondary antibody production by 15-deoxyspergualin (15-DSG).

	First immunization (day 0)	Treatment[a] (days 1 to 9)	Second immunization (day 21)	Spleen tumor weight (mg)	PFC/spleen ($\times 10^3$)
A	10^5 SRBC iv	—	—	91.2±5.8	0.49±0.19
B	10^5 SRBC iv	—	10^8 SRBC iv	214.8±30.0	1,029.1 ±277.0[b]
C	10^5 SRBC iv	15-DSG	10^8 SRBC iv	172.8±9.4	422.4 ±195.3[c]
D	—	—	10^8 SRBC iv	125.3±7.7	382.7 ±102.4
E	—	15-DSG	10^8 SRBC iv	124.8±8.7	256.5 ±117.3[d]

[a] Mice were given 6.25 mg/kg/day of 15-DSG intraperitoneally. Results are mean value (±SD) of 5 mice.
[b] D against B; $P<0.01$. [c] C against B; $P<0.01$. [d] E against D; $P<0.01$.

formation *in vitro* was suppressed significantly at more than 0.8 μg/ml. It should be noted that in this experiment, the addition of 15-deoxyspergualin, even at 10 μg/ml, did not affect cell viability.

Suppression of MLC Reaction

The influence of spergualins on the allogeneic MLC reaction was tested. As shown in Fig. 5, each spergualins inhibited the MLC reaction in a dose-dependent manner, and N-30 and 15-deoxyspergualin showed stronger inhibitory activity than spergualin.

Influence of 15-Deoxyspergualin on PEC

Among these spergualins, because of its strongest immunosuppressive activity, we examined the action of 15-deoxyspergualin on macrophages using PEC taken from mice given immunosuppressive

Fig. 4. Inhibition of antibody formation *in vitro* by 15-deoxyspergualin.

Spleen cells (1.0×10^7 cells/ml) taken from CDF_1 mice in medium containing were cultured with 15-deoxyspergualin for 4 days. Antibody formation was determined by counting PFC. Results are the mean (±SD) of triplicate cultures.

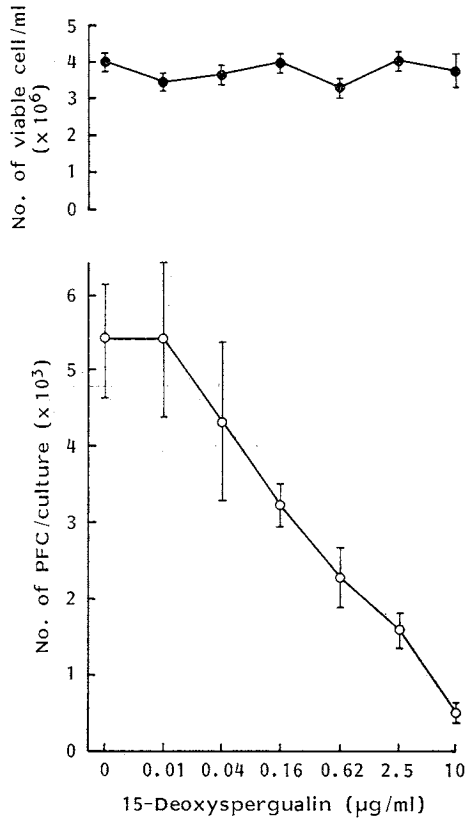


Fig. 5. Inhibitory effect of spergualins on mixed lymphocyte culture reaction.

Spleen cells taken from C3H/He mice as the responder were mixed with MMC-treated spleen cells of BALB/c mice as the stimulator and cultured with or without 15-deoxyspergualin for 5 days. [^{3}H]Thymidine (10 μl of 0.1 μCi/ml) was added 16 hours before the assay. Results was the mean cpm of triplicate cultures and expressed % of the incorporation into cultured cells. Control was 15,672±463 cpm.

● Spergualin, ○ 15-deoxyspergualin, ■ N-30.
* $P<0.01$.

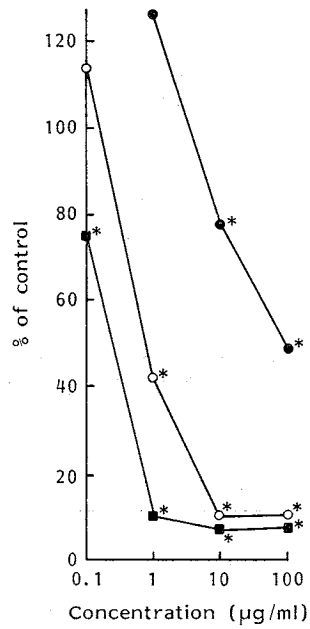


Table 2. Influence of 15-deoxyspergualin (15-DSG) on zymosan-activated macrophages.

Treatment	Schedule (days before assay)	β-Glucuronidase ΔOD_{420} (×10³)	Acid phosphatase ΔOD_{420} (×10³)	PMA-induced O_2^- nmol/mg protein
Saline	—	147±5	134±4	30.8±4.3
15-DSG, 6.25 mg/kg	−3, −2, −1	146±5	136±8	55.8±7.4
Zymosan, 100 mg/kg	−4	189±7	287±6	143.4±14.3
Zymosan+15-DSG	—	235±22	376±6	123.8±14.5

Results are mean value (±SD) of 5 mice.

doses of 15-deoxyspergualin. A single injection of 15-deoxyspergualin at 1.6~25 mg/kg did not result in any changes in the PEC functions measured, *i.e.*, release of lysosomal enzymes and PMA-induced superoxide anion formation (data not shown). The influence of 15-deoxyspergualin on zymosan-activated macrophages was examined. Mice were given zymosan once, 4 days before the assay, and given 15-deoxyspergualin daily for 3 days after the injection of zymosan. As shown in Table 2, the daily injection of 15-deoxyspergualin neither suppressed nor enhanced functions of

Table 3. Effect of spergualin analogues on skin allografts in rats by daily injection[a].

mg/kg/day[a]	15-DSG	N-30
0	9.5±2.5[b]	9.3±1.5
3.1	13.4±4.1*	12.6±1.6*
6.25	15.8±6.4*	15.4±3.8**
12.5	17.4±4.9**	15.9±4.8**

[a] Each substance was given ip from 1 day after the transplantation, daily for 10 days. Results are the mean (±SD) of 10 rats.
[b] Mean number of days allograft survived.
15-DSG: 15-Deoxyspergualin.
* $P<0.05$, ** $P<0.001$.

Table 4. Effect of 15-deoxyspergualin (15-DSG) on skin allografts in rats by injection every other day.

mg/kg/day[a]	M.S.D.[b] (days±SD)	30-Day surviving grafts
0	8.4±3.2	0/10
3.1	9.0±2.6	0/10
6.3	11.5±4.3*	2/10
12.5	12.1±5.5*	2/10
25.0	7.8±3.4	5/10

[a] 15-DSG was given from 1 day after skin graft, every other day for a total of 10 times.
[b] Mean survival days (M.S.D.) with calculations (excluding 30-day survivor).
* $P<0.05$.

normal and zymosan-activated peritoneal exudate cells, although zymosan enhanced the release of lysosomal enzymes and of PMA-induced superoxide anions.

Effect on Skin Allograft in Rats

As we have already reported the effect of spergualin on rat skin allograft[1], in this report the effect of 15-deoxyspergualin on skin allografts in rats was examined. Tail skin taken from SHR rats was grafted onto the abdomen of F344 rats and each compound was injected intraperitoneally from 1 day after the graft daily for 10 days. As shown in Table 3, each of the spergualin analogues at 3.1 to 12.5 mg/kg/day prolonged markedly the skin graft in a dose-dependent manner, though the effect of 15-deoxyspergualin was superior to that of N-30. 15-Deoxyspergualin injected every other day was also effective in prolonging skin grafts (Table 4). By this schedule of injections skin grafts were prolonged more than 30 days at higher doses, such as 6.3 to 25 mg/kg.

Discussion

The immunosuppressive effects of spergualin analogues, 15-deoxyspergualin and N-30, were presented in this report. These compounds suppressed humoral and cell-mediated immune responses in a dose-dependent manner. Among them, 15-deoxyspergualin had the strongest immunosuppressive activity as well as strongest antitumor activity[3]. 15-Deoxyspergualin suppressed the DTH response at more than 1.6 mg/kg/day; and antibody formation, at more than 0.4 mg/kg/day. It also suppressed the secondary antibody formation by daily injection for 10 days after the primary immunization.

Antibody formation by spleen cell cultures and the MLC response were also suppressed by 15-deoxyspergualin. The suppression of antibody formation *in vitro* by 15-deoxyspergualin did not change the total number of viable cells in cultures. As mentioned above, the daily administration of 15-deoxyspergualin suppressed immune responses markedly. However, various functions of peritoneal macrophages taken from mice given the immunosuppressive dose of 15-deoxyspergualin by daily injection were not affected. Moreover, as reported previously, spergualin and 15-deoxyspergualin enhanced interleukin 2 (IL-2) production in MLC; and even at high concentrations such as 100 μg/ml, IL-2 production was not reduced[3]. The mechanism of action of 15-deoxyspergualin in suppressing immune responses is now under study.

15-Deoxyspergualin and N-30 were markedly effective in prolonging skin allografts in rats, and the effect of 15-deoxyspergualin was superior to that of N-30 and spergualin. The prolongation of allografts by 15-deoxyspergualin was dependent on dose and schedule of administration. These compounds will be useful for transplantation of organs and for treatment of various immunologic diseases.

Acknowledgments

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References

1) UMEZAWA, H.; M. ISHIZUKA, T. TAKEUCHI, F. ABE, K. NEMOTO, K. SHIBUYA & T. NAKAMURA: Suppression of tissue graft rejection by spergualin. J. Antibiotics 38: 283~284, 1985
2) UMEDA, Y.; M. MORIGUCHI, H. KURODA, T. NAKAMURA, H. IINUMA, T. TAKEUCHI & H. UMEZAWA: Synthesis and antitumor activity of spergualin analogues. I. Chemical modification of 7-guanidino-3-hydroxyacyl moiety. J. Antibiotics 38: 886~898, 1985
3) ISHIZUKA, M.; T. MASUDA, S. MIZUTANI, M. OSONO, H. KUMAGAI, T. TAKEUCHI & H. UMEZAWA: Induction of antitumor resistance to mouse leukemia L1210 by spergualins. J. Antibiotics 39: 1736~1743, 1986
4) ISHIZUKA, M.; T. MASUDA, N. KANBAYASHI, S. FUKASAWA, T. TAKEUCHI, T. AOYAGI & H. UMEZAWA: Effect of bestatin on mouse immune system and experimental murine tumors. J. Antibiotics 33: 642~652, 1980
5) ISHIZUKA, M.; H. IINUMA, T. TAKEUCHI & H. UMEZAWA: Effect of diketocoriolin B on antibody formation. J. Antibiotics 25: 320~321, 1972
6) KITAGAWA, S. & R. B. JOHNSTON, JR.: Relationship between membrane potential changes and superoxide-releasing capacity in resident and activated mouse peritoneal macrophages. J. Immunol. 135: 3417~3423, 1985
7) HASHIMOTO, K.; Y. OKAWA, K. SUZUKI, Y. OKURA, S. SUZUKI & M. SUZUKI: Antitumor activity of acidic mannan fraction from Baker's yeast. J. Pharm. Dyn. 6: 668~676, 1983