INDUCTION OF ANTITUMOR RESISTANCE TO MOUSE LEUKEMIA L1210 BY SPERGUALINS

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Spergualin and its analog, 15-deoxyspergualin showed a marked antitumor effect against L1210 by intraperitoneal and oral administrations. After treatment with these substances 40- or 60-day survivors (cured mice of L1210) were resistant to reinoculation of L1210 cells. They were resistant only to L1210. The antitumor effector cells in these mice were determined to be T cells. NK activity of spleen cells was also enhanced by spergualins. The antitumor activity of 15-deoxyspergualin was markedly reduced in immuno-deficient mice. IL (interleukin)-2 production, but not IL-1, was enhanced in supernatant of mixed lymphocyte cultures by treatment with 15-deoxyspergualin. The mechanism of action of 15-deoxyspergualin on the immune system was discussed.

Spergualin is an antitumor antibiotic which we discovered. It is produced by *Bacillus laterosporus*[1], and it exhibits antitumor activity against murine leukemias such as lymphatic leukemia L1210 and P388, mastocytoma P815, thymoma EL-4, myeloid leukemia C1498, *etc.*[2].

As reported previously[3], among spergualin analogs 15-deoxyspergualin has a stronger antitumor activity than spergualin. In testing for the antitumor effect of spergualin and 15-deoxyspergualin against L1210, we found that both induced tumor resistance in mice cured of L1210. In this paper we report the antitumor effect of spergualin and 15-deoxyspergualin against L1210 and their effect in inducing tumor resistance in L1210 and in activating interleukin 2 production.

Materials and Methods

Mice

Specific pathogen-free CDF_1 (Balb/c×DBA/2), DBA/2, Balb/c, and C3H/He strains, 4 to 6 weeks of age, were purchased from Charles River Japan Inc. They were housed in plastic cages and maintained under specific pathogen-free conditions at 23±2°C in 55±5% humidity. Experiments were started when mice were 8 to 12 weeks old.

Spergualins

Spergualin and 15-deoxyspergualin were prepared by Takara Shuzo Co., Ltd., according to the methods reported previously[2,3]. They were dissolved in saline or medium.

Tumor and Cell Lines

Mouse lymphatic leukemia L1210 (IMC strain) was maintained in CDF_1 mice by weekly intraperitoneal injection. Meth A fibrosarcoma and Ehrlich carcinoma were maintained by weekly passage in ascitic form in Balb/c and ICR, respectively.

Evaluation of Antitumor Activity

Antitumor activity of spergualins against L1210 IMC strain cells was studied throughout this report. CDF_1 mice were inoculated intraperitoneally with 0.25 ml of diluted ascitic fluid containing 10^5 L1210 cells. Spergualins were dissolved in saline and injected intraperitoneally from one day after tumor inoculation, once daily for 10 days. Forty or sixty days after the first inoculation of L1210 cells, surviving mice were reinoculated with 10^5 L1210 cells intraperitoneally. The specificity of antitumor resistance was examined as follows; mice cured of L1210 were inoculated with various numbers of tumor cells such as Meth A fibrosarcoma, Ehrlich ascites tumor, and L1210, and the number of survivors was determined at 40 days after tumor inoculation.

The antitumor activity of 15-deoxyspergualin in immuno-deficient mice was also tested. CDF_1 mice were irradiated with 400 rad, after which 10^5 L1210 cells were immediately inoculated intraperitoneally. 15-Deoxyspergualin was injected one day after the tumor inoculation daily for 9 days. Antitumor activity was evaluated by the mean survival days (M.S.D.) of 5 to 10 mice and expressed in terms of T/C percentage or the number of survivors at 40 or 60 days after tumor inoculation.

Tumor Neutralization Assay

A tumor neutralization test was performed by the WINN assay[4]. Spleen cells were collected from mice which had survived longer than 40 days after inoculation of L1210 cells and fractionated by a Nylon wool column (LEUKO-PAK, Fenwal Laboratories, Illinois, U.S.A.) into nonadherent and adherent cell populations. To destroy T cells, spleen cells were incubated with diluted Anti-thy 1.2 serum (Cederlane Laboratories Ltd., Ontario, Canada) for 30 minutes at 4°C and then with complement for 60 minutes at 37°C. Each cell population (the effector) was mixed with 10^5 L1210 cells at a ratio of 200 : 1 and injected intraperitoneally into five mice. Tumor neutralization activity was evaluated in terms of M.S.D.

NK Activity

The NK activity of spleen cells of surviving CDF_1 mice treated with spergualins was determined at 14 days after transplantation of L1210 cells according to the method described by KIESSLING *et al.*[5]. YAC-1 cells, used as a target cell, were labeled with ^{51}Cr (New England Nuclear, Boston, Mass., U.S.A.), and 2×10^5 cells/ml were incubated with effector cells at ratios of 25 : 1, 50 : 1 and 100 : 1 for 4 hours. After incubation the supernatant was collected and ^{51}Cr radioactivity was counted in a gamma counter (Aloka Co., Ltd., Tokyo). The maximum counts in target cells were determined after disruption by freezing and thawing. The mean percentage of specific cytotoxicity was calculated as follows:

$$\% \text{ Cytotoxicity} = \frac{\text{Test count} - \text{Spontaneous count}}{\text{Maximum count} - \text{Spontaneous count}} \times 100$$

Mixed Lymphocyte Culture

The mixed lymphocyte culture (MLC) reaction was performed by culturing spleen cells taken from C3H/He ($H\text{-}2^k$) as a responder with stimulator spleen cells taken from Balb/c ($H\text{-}2^d$), the latter having been incubated with 50 μg/ml of mitomycin C (Kyowa Hakko Kogyo Co., Ltd., Tokyo) at 37°C for 20 minutes. Spleen cells were treated with 0.8% ammonium chloride to remove erythrocytes and resuspended at 2×10^6 cells/ml in RPMI 1640 supplemented with 5% fetal calf serum (FCS; GIBCO, Grand Island, N.Y.), 2-mercaptoethanol (2-ME, 5×10^{-5} M), nonessential amino acids, sodium pyruvate, and penicillin and streptomycin. After mixing, cells were placed in 96-well microtiter-plates and cultured at 37°C in 5% CO_2 in air for 5 days. To measure the MLC reaction, cells were pulsed with 0.1 μCi [^{3}H]thymidine (New England Nuclear, Boston, Mass., U.S.A.) for 18 hours before assay, and [^{3}H]thymidine incorporation into cultured cells was estimated in a liquid scintillation counter (Aloka Co., Ltd., Tokyo).

Cytokine Assays

Interleukin 2 (IL-2) activity in supernatants of MLC was assayed according to the method described by LARSON and COUNTINHO[6].

The supernatant was collected 24 hours after the start of MLC and dialyzed against RPMI 1640

Table 1. Antitumor effect of intraperitoneally injected spergualin and 15-deoxyspergualin on L1210.

ip (mg/kg/day)	Spergualin		15-Deoxyspergualin	
	T/C (%)*	60-Day survivors	T/C (%)*	60-Day survivors
25	250	1/5	273	0/5
12.5	359	2/5	298	0/5
6.2	274	2/5	298	1/5
3.1	160	0/5	382	2/5
1.6	113	0/5	311	3/5
0.8	96	0/5	122	3/5
0.4	96	0/5	145	3/5
0.2	82	0/5	104	0/5

* Excluding 60-day survivors.

Table 2. Antitumor effect of orally administered spergualin and 15-deoxyspergualin on L1210.

po (mg/kg/day)	Spergualin		15-Deoxyspergualin	
	T/C (%)*	60-Day survivors	T/C (%)*	60-Day survivors
400	509	0/5	313	0/5
200	>705	5/5	324	0/5
100	115	0/5	>705	5/5
50	100	0/5	464	4/5
25	91	0/5	122	0/5

* Excluding 60-day survivors.

for 24 hours; the non-dialyzable fraction was used for the determination of IL-2 activity.

The effect of spergualins in inducing the production of interleukin 1 (IL-1) was examined. $P388D_1$ cells (1×10^6 cells/ml) were incubated with or without 10 μg/ml of lipopolysaccharide (LPS) at 37°C for 4 hours and then washed with medium three times. After washing, cells were resuspended in medium at 5×10^5 cells/ml and cultured with various concentrations of 15-deoxyspergualin for 24 hours. The culture supernatant was obtained and dialyzed against RPMI 1640 without FCS and 2-ME for 20 hours, and the non-dialyzable fraction was used for the determination of IL-1. IL-1 activity was assayed according to the method of OPPENHEIM *et al.*[7].

Results

Antitumor Activity against L1210 and Induction of Tumor Resistance

As shown in Table 1, intraperitoneal injection of spergualin at 3.1~25 mg/kg body weight or 15-deoxyspergualin at 0.4~25 mg/kg prolonged the survival time of L1210-bearing mice. Oral administration of these compounds also had an antitumor effect at doses of 200~400 mg/kg for spergualin or 50~400 mg/kg for 15-deoxyspergualin (Table 2). The number of 60-day survivors at higher doses was less than that at lower doses by both routes. In terms of antitumor effect, 15-deoxyspergualin was superior to spergualin. The number of mice surviving at 60 days after tumor inoculation was increased by administration of 15-deoxyspergualin. Sixty-day survivors, designated as "cured" mice, were obtained from several experiments and reinoculated with the same number of L1210 cells as used for the first inoculation. The number of tumor-free mice 40 days after the second inoculation was determined. As shown in Table 3, all cured mice survived 40 days after the second tumor inoculation.

Table 3. Antitumor effect of spergualin (SG) and 15-deoxyspergualin (15-DSG) on L1210 and rejection of L1210 in cured mice[a].

1st Inoculation				2nd Inoculation	
Treatment		60-Day survivors[b]		40-Day survivors[c]	
mg/kg	Route	SG	15-DSG	SG	15-DSG
25	ip	1/6	—	1/1	—
12.5	ip	9/17	3/8	9/9	3/3
6.2	ip	6/11	2/8	6/6	2/2
3.1	ip	6/11	4/9	6/6	4/4
1.6	ip	—	10/13	—	10/10
0.8	ip	—	9/13	—	9/9
0.4	ip	—	8/13	—	8/8
400	po	3/6	—	3/3	—
200	po	8/10	5/6	8/8	4/5
100	po	—	7/10	—	7/7
50	po	—	8/10	—	8/8

[a] CDF_1 mice were inoculated with 10^5 L1210 cells intraperitoneally (the 1st inoculation) and treated with SG or 15-DSG daily for 10 days from 1 day after the inoculation of L1210 cells. Sixty days thereafter, the number of survivors (cured mice) were assessed and reinoculated with 10^5 L1210 cells intraperitoneally (the 2nd inoculation). After the 2nd inoculation, mice were not treated with SG or 15-DSG and the number of 40-day survivors were assessed.

[b] No. of cured mice/total No. of mice treated.

[c] No. of tumor-free mice/total No. of cured mice.

Table 4. Specificity of antitumor resistance in cured mice after the therapy by spergualin (SG) or 15-deoxyspergualin (15-DSG)[a].

No. of tumor cells inoculated	No. of tumor-free mice/Total No. of mice cured of L1210					
	L1210		Meth A		Ehrlich	
	SG	15-DSG	SG	15-DSG	SG	15-DSG
10^6	5/5	5/5	0/5	0/5	0/5	0/5
10^5	5/5	5/5	0/5	0/5	0/5	0/5
10^4	5/5	5/5	0/5	0/5	0/5	0/5

[a] Mice cured of L1210 after therapy with 6.25 mg/kg of spergualin (SG) or 3.12 mg/kg of 15-deoxyspergualin (15-DSG) were inoculated with various tumors, and the number of tumor-free mice present 40 days after tumor inoculation was assessed.

Next, the specificity of the antitumor resistance induced in the cured mice was examined. Sixty-day survivors which had been treated with 6.25 mg/kg of spergualin or 3.12 mg/kg of 15-deoxyspergualin were injected with various numbers of tumor cells such as Meth A carcinoma, Ehrlich ascites tumor and L1210, all of which are able to grow in CDF_1 mice. As shown in Table 4, the cured mice were only resistant to L1210, whereas other tumors grew.

The antitumor activity of 15-deoxyspergualin in immunodeficient CDF_1 mice (made so by X-ray-irradiation) was also tested. After 400 rad of irradiation, mice were given 10^5 L1210 cells and then injected with 6.25 mg/kg of 15-deoxyspergualin from one day after the transplantation daily for 9 days. The results (Fig. 1) indicate that the antitumor activity of 15-deoxyspergualin is markedly reduced in these mice.

Cytotoxic Effector Cells in Cured Mice

Since we showed that therapy with spergualin or 15-deoxyspergualin induced tumor resistance,

Fig. 1. Antitumor activity of 15-deoxyspergualin against L1210 in normal and irradiated mice.
■ Control, □ 0.78 mg/kg, ○ 3.12 mg/kg, ● 12.5 mg/kg.

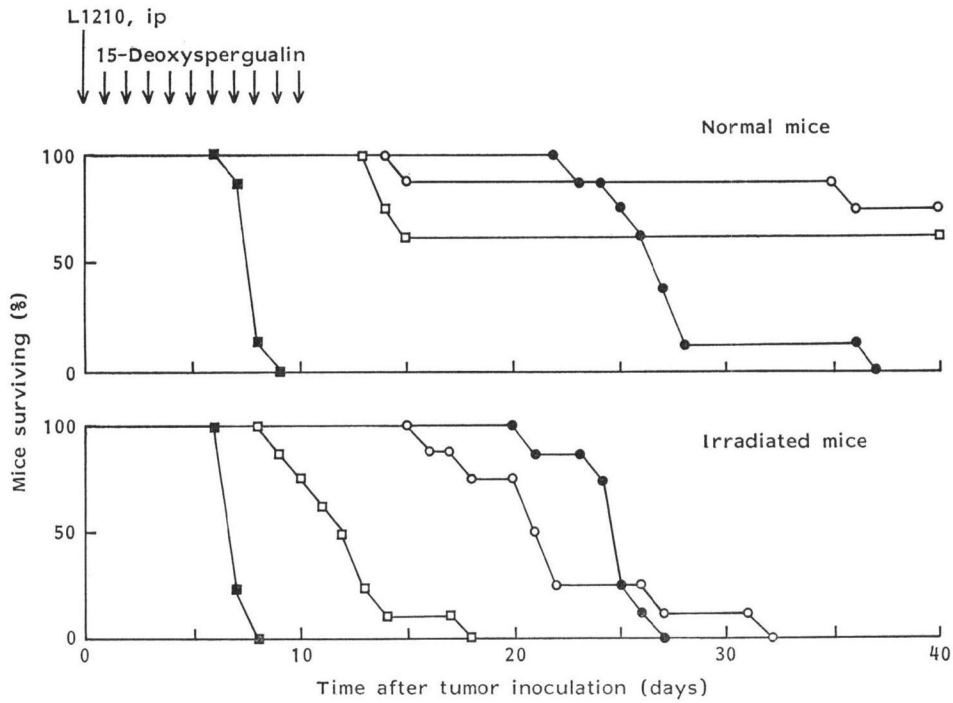


Table 5. Antitumor activity of spleen cells taken from mice cured of L1210 by 15-deoxyspergualin.

Spleen cell[a] treatment	Normal mice		Cured mice	
	M.S.D.[b]	30-Day survivors	M.S.D.[b]	30-Day survivors
None	10.6±0.9	0/5	27.8±2.9	1/5
Nylon wool column				
Non adherent	10.6±0.9	0/5	27.0	4/5
Adherent	10.6±0.5	0/5	27.4±1.5	0/5
Anti-thy 1.2 serum and C′	10.4±0.5	0/5	16.4±4.0	0/5

[a] Spleen cells were mixed with 10^5 L1210 cells at a ratio of 200 : 1 and injected intraperitoneally into CDF_1 mice.

[b] Results are expressed as mean survival days (M.S.D.) and as the number of 30-day survivors. The 30-day survivors were excluded from the M.S.D.

C′: Complement.

we examined the nature of the cytotoxic effector cells in 15-deoxyspergualin-treated mice by the WINN assay. As shown in Table 5, spleen cells prolonged survival time markedly. Among cells fractionated on a Nylon wool column, only those cells which passed through the column were effective in both prolonging the survival time and in increasing the number of 30-day survivors; adherent cells prolonged the survivial time but did not increase the number of 30-day survivors. Moreover, treatment of the spleen cells with Anti-thy 1.2 serum and complement reduced their cytotoxicity, indicating that cytotoxic T cells are induced by 15-deoxyspergualin.

NK Activity in L1210-Bearing Mice Given Spergualins

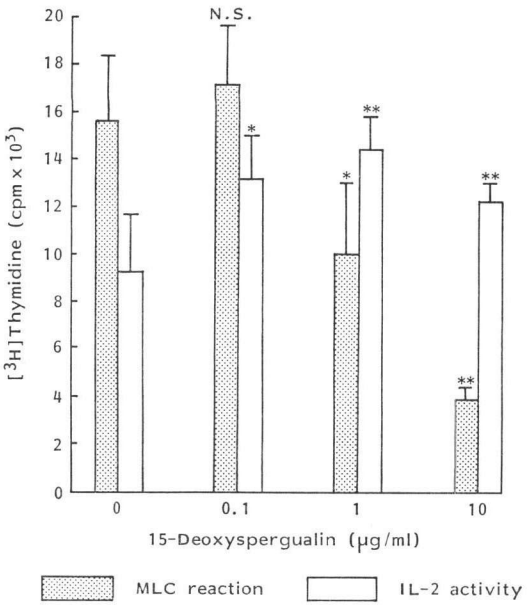
NK activity of spleen cells of survivors at 14 days after inoculation of L1210 cells was examined

Table 6. Enhancement of NK activity in spleen cells of L1210-bearing mice after treatment with spergualin (SG) or 15-deoxyspergualin (15-DSG) against YAC-1 cells.

Mice taken spleen cells	Dose (mg/kg/day)	Effector/Target ratio		
		25	50	100
		% Cytotoxicity (±SD)[b]		
Expt 1				
Normal	—	9.2±1.1	12.5±0.7	15.8±2.1
	SG			
L1210-bearing[a]	6.25	10.7±0.4	17.6±0.8***	19.7±1.0*
	12.5	10.4±0.3	16.1±2.8*	18.1±1.3
	25.0	16.9±1.4***	22.3±1.4***	26.9±1.0***
Expt 2				
Normal	—	2.4±0.8	4.0±0.6	Not tested
	15-DSG			
L1210-bearing	1.25	5.7±0.5***	10.5±0.3***	Not tested
	2.5	4.2±0.9*	6.8±0.7**	Not tested
	5.0	3.2±0.5	4.9±0.7	Not tested

[a] Non-treated mice were dead within 8 to 9 days after inoculation with L1210 cells in both experiments.
[b] *P*-value against % cytotoxicity of normal spleen cells; * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Fig. 2. Effect of 15-deoxyspergualin on MLC reaction and IL-2 production.
* $P<0.05$, ** $P<0.01$. N.S.: Not significant.



against YAC-1 cells. As shown in Table 6, daily injection of 6.25 to 25 mg/kg of spergualin or 1.25 to 2.5 mg/kg of 15-deoxyspergualin for 9 days from 1 day after L1210 inoculation enhanced NK activity in spleen cells of 14-day survivors significantly. It was stronger than that of normal mice, although the daily injection of 5 mg/kg of 15-deoxyspergualin was less effective.

Influence on Cytokine Production

Influence of 15-deoxyspergualin on production of cytokines participating in the generation of cytotoxic T cells and/or NK cells was tested. The addition of 15-deoxyspergualin at 0.1 to 10 μg/ml with or without 10 μg/ml of LPS to P388D$_1$ cell cultures did not show any effect on IL-1 production although LPS enhanced IL-1 production (data not shown).

As shown in Fig. 2, the addition of 15-deoxyspergualin to MLC showed dose-dependent inhibition of the response at doses of 1 and 10 μg/ml. Influence of the drug on IL-2 production by MLC was examined by determining the cytokine content of supernatants obtained from 24 hour-cultures. IL-2 activity in supernatants of MLC incubated with 15-deoxyspergualin was enhanced significantly over a wide dose range, even at 10 μg/ml (Fig. 2). This effect was also shown by spergualin. The response of MLC in which the responder cells had been incubated with 15-deoxyspergualin for 4 to 8 hours prior to being mixed

with stimulator cells was not inhibited even at 100 μg/ml and IL-2 production in the supernatant was also enhanced markedly.

Effect of 15-Deoxyspergualin-treated L1210 Cells on Induction of Antitumor Resistance

In order to clarify whether 15-deoxyspergualin-treated L1210 cells can induce antitumor resistance, L1210 cells were incubated with 100 μg/ml of 15-deoxyspergualin for 24 hours and washed. Various numbers of cells (10^3 to 10^7 cells/mouse) were then inoculated intraperitoneally and the survival time of the mice was examined. Mice inoculated with 10^3 to 10^4 cells survived 40 days after the inoculation although mice inoculated with 10^5 to 10^7 cells died within 8 to 10 days, the same survival time as mice inoculated with non-treated L1210 cells. The surviving mice were inoculated with 10^3 to 10^5 L1210 cells, but these mice died within the same period as controls.

Discussion

Spergualin and its analog, 15-deoxyspergualin, showed a marked antitumor action against L1210. We found that mice cured of L1210 by treatment with these substances were resistant to a second inoculation of L1210 cells. As reported previously[3], the antitumor activity of 15-deoxyspergualin against L1210 is stronger than that of spergualin. This was also confirmed in the present experiments by both intraperitoneal injection and oral administration.

After therapy, 60-day survivors were reinoculated with L1210 cells, and we found that the growth of L1210 was inhibited in most of the mice treated with either substance by either route of administration (only one mouse died, this was treated by oral administration at a high dose of 15-deoxyspergualin). Specificity of the antitumor resistance was examined by inoculation of syngeneic and allogeneic tumors into cured mice. They were resistant only to L1210, indicating that treatment with these substances induced antitumor resistance specific for L1210 in these mice. The effector cells were determined to be T cells by the WINN assay, although adherent cells showed slight antitumor activity. It will be reported elsewhere[8] that 15-deoxyspergualin has no marked effects on macrophage functions such as phagocytosis, lysosomal enzyme release, and generation of superoxide anion. NK activity of spleen cells of 14-day survivors was also enhanced by spergualins.

The antitumor activity of 15-deoxyspergualin was markedly reduced in immuno-deficient mice. As reported previously, cytotoxicity of spergualin *in vitro* is due to acrolein which is produced by amine oxidase in sera[9]. However, mouse serum has low amine oxidase activity; therefore, most of the antitumor activity of spergualins may be due to activation of the immune system.

The mechanism of action of 15-deoxyspergualin on the immune system was studied by testing its effect of the production of IL-1 and -2, cytokines which participate in activation of T cells[10~13]. Results indicated that IL-2, but not IL-1, production is enhanced in supernatants of MLC. This enhancing effect was seen even though the MLC response was suppressed. Moreover, as will be reported, we have found that daily injection of 15-deoxyspergualin into mice augmented the response of their lymphocytes to IL-2[8]. Therefore, the mechanism of induction of antitumor resistance to L1210 by spergualin and 15-deoxyspergualin may be due to stimulation of IL-2 production and induction of IL-2 responsiveness in lymphoid cells.

To rule out the possibility of a direct effect of 15-deoxyspergualin on the antigenicity of cells, L1210 cells incubated with 15-deoxyspergualin and then washed were injected into mice and the survival was examined. The treated L1210 cells failed to establish antitumor resistance in mice just like the non-treated cells.

On the other hand, we have reported on an immuno-suppressive effect of spergualin[14] and 15-deoxyspergualin[8], and this effect was dose-dependent. Since higher doses of spergualins produced fewer 60-day survivors of L1210 inoculation than lower doses in these experiments, immuno-suppression may account for this phenomenon.

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