CYTOTOXICITY OF SPERGUALIN AND AMINE OXIDASE ACTIVITY IN MEDIUM

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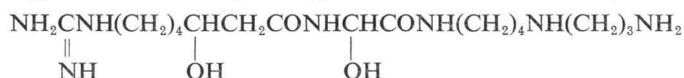
The cytotoxicity of spergualin on cultured L5178Y cells is dependent on the kind of serum contained in culture media. Spergualin has stronger cytotoxicity to L5178Y cells in calf serum ($IC_{50}=2$ μg/ml) than in horse serum ($IC_{50}=60$ μg/ml). This was thought to be caused by amine oxidase in sera. Because calf serum was rich in amine oxidase and horse serum was very poor. Spergualin was found to be oxidized by either calf serum or amine oxidase purified from beef plasma. Aminoguanidine, an amine oxidase inhibitor suppressed the spergualin effect to inhibit the growth of L5178Y cells in calf serum to the level in horse serum. On the other hand, in horse serum the spergualin cytotoxicity was enhanced by addition of amine oxidase. These results suggested to us that spergualin might be inactive in itself and that the amine oxidase-oxidized product might play an essential role in inhibiting the growth of cells.

An antitumor antibiotic, spergualin was discovered by Umezawa and coworkers in 1981[1]. As shown by the following, it is an interesting new type of antitumor antibiotic[2];



It significantly prolongs the life span of mice transplanted with leukemic cells such as myeloid leukemia C1498, masto-cytoma P815, thymoma EL-4, lymphocytic leukemia P388 and L1210.

In this paper we report that spergualin is inert in a culture medium in which the content of amino oxidase is poor. The cytotoxic effect of spergualin may be due to the amine oxidase-oxidized product.

Materials and Methods

Materials

Calf and horse sera were purchased from Flow Laboratories Inc., U.S.A. and Grand Island Biological Co., U.S.A., respectively. Aminoguanidine sulfate was purchased from Tokyo Kasei Kogyo Co., Ltd., Japan. Amine oxidase from beef plasma (18.96 units/g material, 23.55 units/g protein) was purchased from Miles Laboratories (PTY) Ltd., South Africa. Its certificate of analysis indicated that this was though to be classified as diamine oxidase EC 1.4.3.6 (pyridoxal-containing). Guaiacol (*O*-methoxyphenol) and spermidine phosphate were purchased from Wako Pure Chemical Industries, Ltd., Japan.

Culture of L5178Y Cells

Mouse lymphoblastoma L5178Y cells were cultured in RPMI1640 medium supplemented with 10% inactivated calf or horse serum. Cell growth was determined by counting cell number using a Coulter Counter.

Assay of Amine Oxidase

Amine oxidase activity was assayed by determination of produced hydrogen peroxide[3,4], this

Fig. 1. Effect of spergualin on cell growth of L5178Y.

L5178Y cells (5×10^4/ml) were incubated in RPMI1640 medium supplemented with 10% calf (○) or horse (●) serum for 2 days.

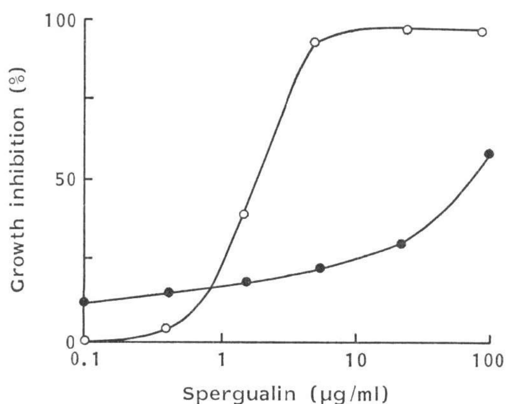
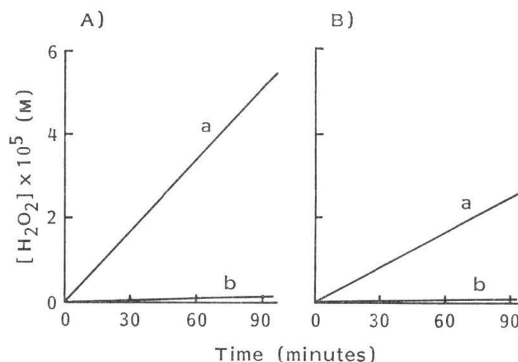


Fig. 2. Amine oxidase activity in sera.

A) Amine oxidase activity in 13.3% calf (a) or horse (b) serum was determined by using spermidine as the substrate.

B) Oxidation of spergualin by calf (a) or horse (b) serum was tested by using spergualin as the substrate.



was coupled with peroxidase and the generated hydrogen was trapped by guaiacol as a chromogenic donor. Incubation mixture (1.5 ml) contained 25 μl of 10 mM substrate, 25 μl of 20 mM guaiacol, 50 μl of 40 μg of horseradish peroxidase/ml, 1 ml of 0.1 M sodium phosphate buffer pH 7.2 and 0.4 ml of water or enzyme solution. Incubation was started by addition of substrate and color formation was monitored continuously by a Beckman DU-8 spectrophotometer at 436 nm at 37°C. Hydrogen peroxide concentration was calculated, assuming that the molar extinction coefficient (ε_{436}) of the pigment is 2.55×10^4 $M^{-1}\cdot cm^{-1}$ and one mol of pigment is produced by consumption of 4 mol of hydrogen peroxide.

Results

Cytotoxicity of Spergualin in Media Containing Different Sera

As shown in Fig. 1 the effect of spergualin on cell growth of L5178Y cells was different depending on different sera contained in culture media. In media containing calf serum spergualin had stronger cytotoxicity than in horse serum media. IC_{50} (50% inhibition concentration against cell growth) was 2 μg/ml in calf serum and 60 μg/ml in horse serum. A similar result was found in L1210 cell proliferation; IC_{50} was 8 μg/ml in calf serum medium and 100 μg/ml in horse serum medium. Similar results have been reported for the cytotoxic effect of spermine and spermidine[5,6]. Polyamine oxidase contained in serum was suggested to be responsible for *in vitro* cytotoxicity of these polyamines. Therefore, as described below we tested the relation between spergualin's activity and amine oxidase activity.

Amine Oxidase Activity in Sera

Amine oxidase activity in sera was determined using spermidine as a substrate. As shown in Fig. 2A, calf serum treated at 60°C for 30 minutes was rich in amine oxidase and horse serum contained only just detectable amounts of amine oxidase. Spergualin was oxidized easily in calf serum and hardly in horse serum as shown in Fig. 2B. This suggested that spergualin became a cytotoxic substance after oxidation by amine oxidase.

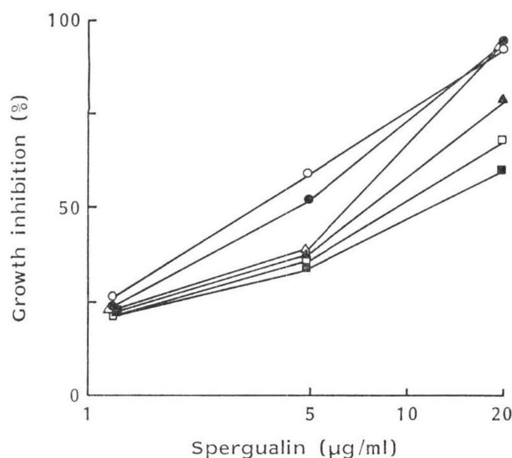
Table 1. Inhibition of enzymatic oxidation of spergualin by calf serum by aminoguanidine.

Aminoguanidine (μg/ml)	Oxidative activity	
	ΔE_{436}/ 40 minutes	Inhibition (%)
—	0.1342	
3.33	0.0734	45.3
6.66	0.0389	71.0
10	0.0261	80.5
13.3	0.0130	90.3
20	0.0076	94.3

Incubation mixture contained 20% calf serum, 167 μM spergualin, various concentration of aminoguanidine and others described in Materials and Methods.

Fig. 3. Effect of aminoguanidine on spergualin's cytotoxic activity in calf serum.

L5178Y cells (5×10^4/ml) were grown in the presence or absence (○) of various amounts of aminoguanidine in the medium with 10% calf serum. Aminoguanidine concentration was as follows: 5 (●), 10 (△), 20 (▲), 40 (□) and 80 (■) μg/ml. Growth inhibition by aminoguanidine was less than 2%.



Suppression of Spergualin's Cytotoxic Activity in Calf Serum by Addition of an Amine Oxidase Inhibitor

The effect of aminoguanidine, an amine oxidase inhibitor[6] on oxidation of spergualin by calf serum was tested and shown in Table 1. Aminoguanidine inhibited amine oxidase activity in calf serum. Aminoguanidine was added to medium supplemented with calf serum and spergualin's activity against L5178Y cell growth was tested. As shown in Fig. 3 aminoguanidine suppressed spergualin's action in concentration-dependent mode.

Fig. 4. Oxidation of spermidine and spergualin by amine oxidase from beef plasma.

The activity of amine oxidase from beef plasma (133 μg/ml) was shown using spermidine (a) and spergualin (b~e) as substrates. Further horse serum was added in the concentration of 6.6% (c) and 20% (d). Oxidative activity of 20% horse serum against spergualin was shown by (e).

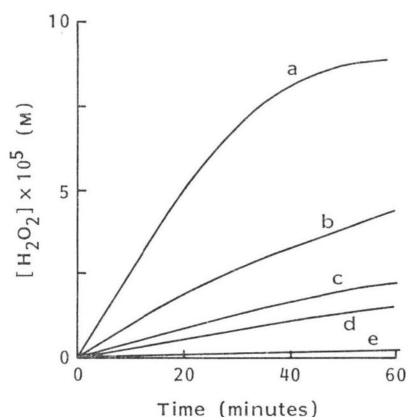
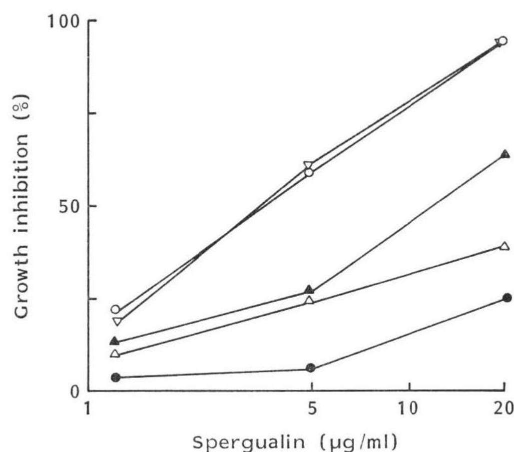


Fig. 5. Effect of amine oxidase on spergualin's cytotoxic activity in horse serum.

L5178Y cells (5×10^4/ml) were grown in the medium containing 10% horse serum with or without (●) amine oxidase. Amine oxidase concentration was 50 (△), 100 (▲) and 200 (▽) μg/ml. Growth inhibition by amine oxidase was less than 6%. Spergualin's cytotoxicity in calf serum was shown by (○).



Enhancement of Spergualin Cytotoxicity in Horse Serum by Addition of Amine Oxidase

As shown in Fig. 4 the amine oxidase prepared from beef plasma had the same substrate specificity as the calf serum enzyme, and it was inhibited by addition of horse serum. Horse serum may contain any inhibitor of amine oxidase. Addition of amine oxidase enhanced cytotoxic activity of spergualin in horse serum to the level in calf serum as shown in Fig. 5. It is further evidence that spergualin's cytotoxicity depends on amine oxidase activity in culture medium.

Discussion

From the unique structure, a unique mechanism of action was expected for spergualin. Actually in our studies nucleic acids and protein synthesis in intact cells were not inhibited at 100 μg/ml of spergualin. When the effect on rate-limiting enzymes of polyamine biosynthesis was tested, rat liver ornithine decarboxylase was not inhibited at 1.5 mg/ml and *S*-adenosylmethionine decarboxylase was inhibited so weakly as 50% at 1 mg/ml and 83% at 10 mg/ml. Therefore we thought there was another mechanism for the action of spergualin. The results described above suggest that spergualin's cytotoxicity *in vitro* is exerted after activation by amine oxidase. We have studied the *in vitro* cytotoxicity of about 100 derivatives (or analogues) of spergualin and their *in vivo* effect in prolonging the survival period of mice bearing L1210. Then, we found that the cytotoxicity *in vitro* does not require the whole structure except for the terminal polyamine. The compounds containing the following amines inhibited the growth of L1210 cells in calf serum medium as well as spergualin; putrescine, norspermidine, homospermidine and spermine. Contrary to the action *in vitro*, only few of derivatives or analogues prolonged the survival period of mice inoculated with L1210 cells. A guanidine moiety was present in all such compounds exhibiting antitumor effect *in vivo*. However, it should be considered that even in *in vivo* experiments, spergualin might be oxidized by amine oxidase in tumor cells and the cells might be damaged, because in our experiments acylation and alkylation of spermidine's terminal amine destroyed any activity *in vivo* and *in vitro*. Mouse serum is poor in amine oxidase, therefore the antitumor effect of spergualin is not due to the oxidation of spergualin in blood. If oxidation of spergualin is necessary for antitumor activity, it may occur near or in tumor cells. The product of oxidation of spergualin by amine oxidase *in vitro* is very unstable and we were not successful in its isolation. Meanwhile this oxidation may be one of pathways for biological degradation of spergualin.

As reported elsewhere[7], we found that (±)-15-deoxyspergualin has stronger antitumor effect than spergualin. Thus, this derivative is being studied for the purpose of the development of an anticancer drug. We continue the studies on the molecular mechanism of action of spergualin and its effective derivatives and analogues.

Acknowledgments

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