# SERUM EFFECT ON CELLULAR UPTAKE OF SPERMIDINE, SPERGUALIN, 15-DEOXYSPERGUALIN, AND THEIR METABOLITES BY L5178Y CELLS

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Spergualin (SG) and 15-deoxyspergualin (DSG) were more slowly incorporated into L5178Y cells than spermidine. SG and DSG inhibited carrier-mediated transport of [$^3$H]-spermidine competitively with inhibition constants of 0.67 mM and 0.45 mM, respectively. Addition of calf serum stimulated uptake of [$^3$H]spermidine into the cells in a serum concentration-dependent manner. The effect was not observed when horse serum was used in place of calf serum. Preincubation of spermidine in calf serum for 1 hour before addition to cells remarkably decreased cellular incorporation of tritium. Three amine oxidase inhibitors, aminoguanidine, 3-hydroxybenzyloxyamine, and semicarbazide, inhibited stimulation of uptake of [$^3$H]spermidine by calf serum and the decrease of it by preincubation in calf serum. So we propose that cellular incorporation or binding of products generated by oxidation of spermidine by amine oxidase in calf serum was much faster than that of spermidine itself and they were unstable and transformed quickly to unincorporable or non-binding substances if cellular targets were not present.

Effect of amine oxidase inhibitors on cytotoxic activity of SG and DSG were determined in low and high concentrations of calf serum. In the presence of 10% calf serum in the basal medium, cytotoxicity to L5178Y cells by SG and DSG was suppressed at high drug concentrations (above 10 $\mu$g/ml) and enhanced at low drug concentrations (below 2.5 $\mu$g/ml) by amine oxidase inhibitors. In the presence of 0.5% calf serum suppression of cytotoxicity at high drug concentrations by amine oxidase inhibitors was also observed, but enhancement at low drug concentrations was obscure. These data may suggest the existence of two kinds of cytotoxic mechanism of SG and DSG, one dependent on and one independent of amine oxidase in serum.

Spergualin (SG) is an antitumor antibiotic produced by a strain of *Bacillus laterosporus*[1] and its structure has been determined to be (−)-(15*S*)-1-amino-19-guanidino-11,15-dihydroxy-4,9,12-triazanonadecane-10,13-dione.[2,3] It exhibits antitumor activity against various leukemias such as L1210, EL-4, P388, C1498, and P815.[1,4] 15-Deoxyspergualin (DSG) is a more active derirative[5~7] than SG and is under clinical investigation in Japan and the U.S.A. Its structure is (±)-1-amino-19-guanidino-11-hydroxy-4,9,12-triazanonadecane-10,13-dione.[5,6] They are interleukin 2 inducers[8] and are thought to exhibit antitumor activity at least in part through activation of the immune system.[8,9] But the biochemical mechanisms are not known yet. We have been studying the mechanism of their cytocidal activity,[10,11] which we think is important as the first reaction after drug administration and a trigger of activation of the immune system.

In this report we describe the mechanism of uptake of SG and DSG by leukemia cells and the existence of two kinds of cell-kill mechanisms, one dependent on and one independent of amine oxidase activity in serum.

## Materials and Methods

### Materials

Materials used and their sources were as follows: Calf serum, from Flow Laboratories Inc., U.S.A.; IBL medium (a mixture of DULBECCO's MEM and F-12 HAM supplemented with insulin, transferrin, Hepes, and sodium selenite), from Immuno Biological Laboratories, Japan; RPMI1640 media, from Nissui Pharmaceutical Co., Ltd., Japan; aminoguanidine sulfate (AG), from Tokyo Kasei Kogyo Co., Ltd., Japan; 3-hydroxybenzyloxyamine dihydrogenphosphate (3HBA) and semicarbazide hydrochloride (SC), from Nakarai Chemicals Ltd., Japan; spermidine phosphate, guaiacol, liquid paraffin, and peroxidase from horseradish, from Wako Pure Chemical Industries, Ltd., Japan. Toray silicone SH550, from Toray Silicone Co., Japan; [$^3$H]spermidine·3HCl[terminal methylenes-$^3$H(N)] (44.5 Ci/mmol), from New England Nuclear, U.S.A. [$^3$H]SG was prepared with biosynthetically by us using [$^3$H]spermidine. Its specific activity was 0.514 Ci/mol. [$^{14}$C]15-DSG (27 Ci/mol) was provided by Nippon Kayaku Co., Ltd., Japan. PSC scintillation cocktail was purchased from Amersham Co., U.S.A. Hepes was purchased from Sigma Chemical Company, U.S.A. SG and DSG were prepared by Central Research Laboratory, Takara Shuzo Co., Ltd., Japan.

### Culture of L5178Y Cells

Mouse lymphoblastoma L5178Y cells were cultured in IBL medium supplemented with calf serum. Cell growth was determined by counting cell number with a Coulter Counter.

### Drug Uptake

A cell suspension containing 5 to $10\times10^6$ cells/ml was prepared in RPMI1640-20 mM Hepes, pH 7.2. One hundred µl of radioactive drug solution was mixed with 200 µl of cell suspension. After the desired incubation, 250 µl of the aliquot was layered on a 0.5-ml mixture of silicone oil and liquid paraffin (84:16, by weight). After centrifugation in an Eppendorf microcentrifuge and washing of the aqueous layer two times with PBS, the oil was discarded by suction. The cell pellet was solubilized by treatment with 0.3 ml of 0.5 N KOH at 60°C for 20 minutes and mixed with 1.5 ml of PCS scintillation cocktail. Radioactivity was determined by a Beckman LS9800 series liquid scintillation system.

### Assay of Amine Oxidase

Amine oxidase activity in calf serum was assayed by determination of produced hydrogen peroxide as previously described.[10] Incubation mixture (1.5 ml) contained 2.5 µl of 10 mM spermidine or SG, 25 µl of 20 mM guaiacol, 50 µl of 40 µg horseradish peroxidase/ml, 1.2 ml of IBL medium, 50 µl of inhibitor solution and 0.15 ml of calf serum.

## Results

Drug uptake by L5178Y cells was determined for [$^3$H]spermidine, [$^3$H]SG, and [$^{14}$C]DSG. The uptake of each was dependent on time and temperature. As shown in Table 1, though the drug concentration of spermidine was two-three orders of magnitude lower than that of SG and DSG, the

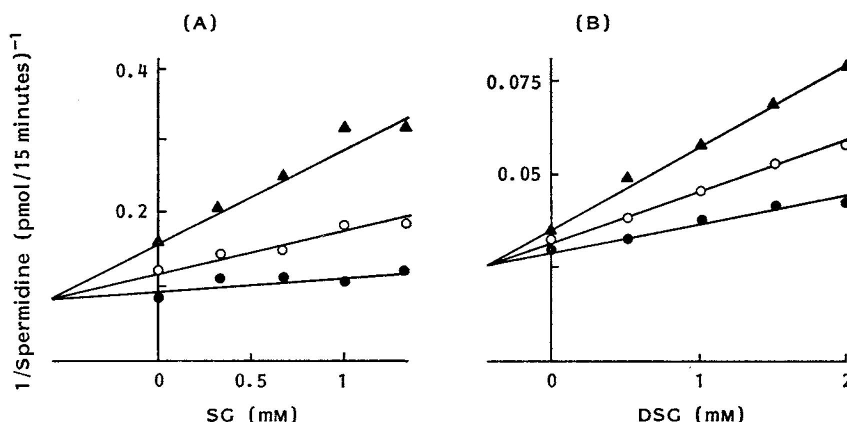
Table 1. Drug uptake by L5178Y cells.

| | Experimental | | Drug incorporated into the cells | |
|---|---|---|---|---|
| | Drug | Cell number | | |
| Spermidine | 1.67 µM | $1.2\times10^6$ | 58.5 pmol | 14.1% |
| Spergualin | 1.22 mM | $2.1\times10^6$ | 1.07 nmol | 0.35% |
| 15-Deoxyspergualin | 11.8 mM | $2.5\times10^6$ | 2.27 nmol | 0.07% |

Incubation mixture contained radioactive drug and L5178Y cells in 0.25 ml of medium without serum. After incubation for 30 minutes at 37°C drug incorporated into cells was determined and shown as a percentage of added drug.

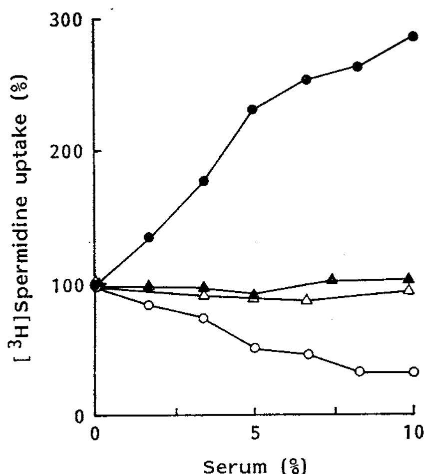
Fig. 1. Kinetic analysis of the inhibition by SG and DSG of [$^{3}$H]spermidine uptake by L5178Y cells.

Spermidine concentrations were 1 $\mu$M (▲), 1.67 $\mu$M (○), and 3.33 $\mu$M (●).




L5178Y cells were suspended in RPMI1640-20 mM Hepes, pH 7.2, without serum. Cell density in (A) and (B) were $4.8\times10^{6}$/ml and $7.34\times10^{6}$/ml, respectively. Each mixture, which contained [$^{3}$H]spermidine (50 $\mu$M, 9.8 $\mu$Ci/ml) and SG (A) or DSG (B) at various concentrations in a total volume of 100 $\mu$l of RPMI 1640-20 mM Hepes, was added to 200 $\mu$l of L5178Y cell suspension. After incubation for 15 minutes at 37°C, a 250-$\mu$l volume was processed to determine radioactivity incorporated into cells as described in Materials and Methods. The results were expressed at Dixon plots.

Fig. 2. Effect of serum on [$^{3}$H]spermidine uptake by L5178Y cells.




Incubation mixtures (100 $\mu$l) contained 10 $\mu$l of [$^{3}$H]spermidine (50 $\mu$M, 9.8 $\mu$Ci/ml), 0 to 30 $\mu$l of calf (○ and ●) or horse (△ and ▲) serum, and RPMI1640-20 mM Hepes, pH 7.2. They were prepared in an ice bath and added to 200 $\mu$l of L5178Y cell suspension ($6.4\times10^{6}$/ml) after preincubation (○ and △) for 60 minutes at 37°C or without preincubation (● and ▲). After 16 minutes at 37°C a 250-$\mu$l volume of each sample was processed as described in Materials and Methods. The data are shown as percentages of [$^{3}$H]-spermidine uptake without serum.

Table 2. Effect of amine oxidase inhibitors on [$^{3}$H]spermidine uptake in the presence of calf serum by L5178Y cells.

| Inhibitors | Calf serum | Preincubation | |
|---|---|---|---|
| | | Without (pmol) | With (pmol) |
| None | − | 37.0 | 29.0 |
| None | + | 105 | 8.9 |
| AG (160 $\mu$g/ml) | + | 35.1 | 26.9 |
| 3HBA (5 $\mu$g/ml) | + | 36.8 | 30.3 |
| SC (40 $\mu$g/ml) | + | 31.0 | 23.4 |

Experimental conditions were the same as given in Fig. 2.

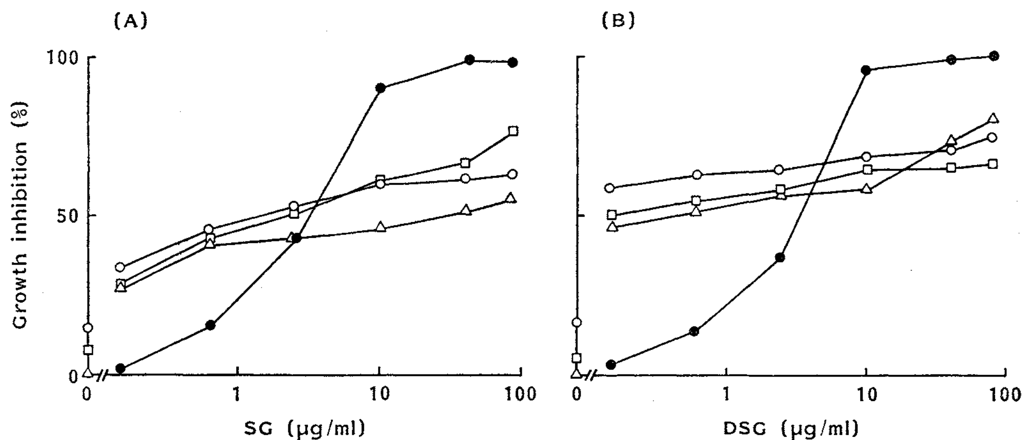
incorporation rate of spermidine was very high and the rates of SG and DSG were very low. Because of the low specific activity of radioactive SG and DSG and low incorportaion by L5178Y cells, uptake characteristics of those drugs were studied by the competition method for [$^{3}$H]spermidine uptake.

Spermidine uptake by L5178Y cells followed saturation kinetics. *Km* and Vmax were calculated as 1.1 $\mu$M and 23.8 pmol/15 minutes/$10^{6}$ cells, respectively. As shown in Fig. 1, SG and DSG linear-competitively inhibited the uptake of

spermidine, with inhibition constants of 0.67 mM and 0.45 mM, respectively. DSG is a mixture of epimers at C-11 but in this study we supposed them to have the same properties in terms of uptake, because DSG epimers had the same inhibitory activity to [$^{3}$H]spermidine uptake. From these results it appeared that spermidine was incorporated into L5178Y cells by a carrier-mediated mechanism and SG and DSG were transported by the carrier of spermidine. Addition of calf serum during the course of [$^{3}$H]spermidine uptake by L5178Y cells gave a different effect dependent on the presence or absence of preincubation with calf serum and spermidine. As shown in Fig. 2, when calf serum and [$^{3}$H]-spermidine were preincubated for 1 hour at 37°C before L5178Y cells were added the uptake of radioactivity decreased to 30% in a serum concentration-dependent manner. Without preincubation it was enhanced 3-fold, again in proportion to the serum concentration. The effect was not seen when horse serum was used instead of calf serum. As shown in Table 2 the combination of calf serum and any of three amine oxidase inhibitors (AG, 3HBA, or SC) cancelled the calf serum effect on [$^{3}$H]spermidine uptake. Spermidine is oxidized by amine oxidase in calf serum, and this enzyme is known to be in low concentration in horse serum.[10,12] So it appears that oxidation of spermidine by amine oxidase in calf serum resulted in products which were more rapidly incorporated into or bound to cells than spermidine or quickly changed to unreactive substances in the absence of cells.

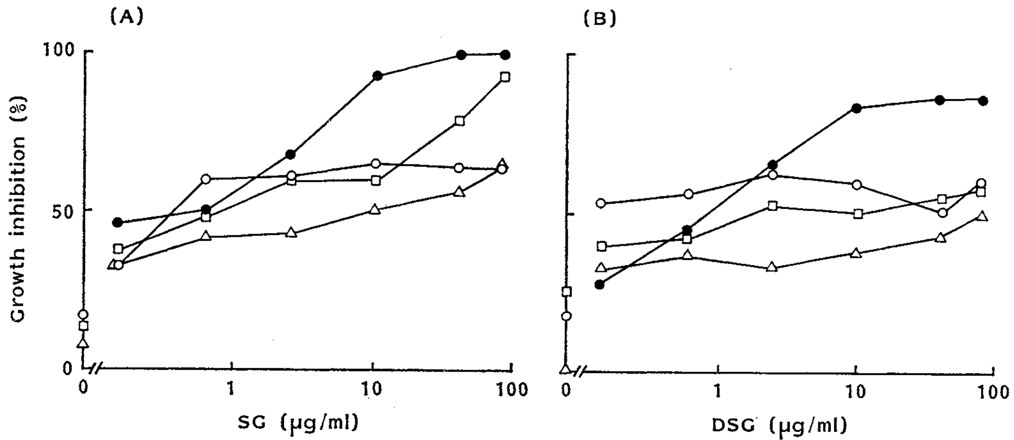
Effect of amine oxidase inhibitors on cytotoxic activity of SG and DSG were determined in high and low concentrations of calf serum (Figs. 3 and 4). IBL medium which was supplemented with a hormone and growth factors was used as a basal medium. This medium was completely free from amine oxidase activity and gave almost the same growth rate of L5178Y cells as when the cells were in the presence of serum at concentrations of 0.5% or 10%. In the presence of 10% calf serum, cytotoxicity by SG and DSG was suppressed at high drug concentrations (above 10 μg/ml) but enhanced at low drug concentrations (below 2.5 μg/ml) by amine oxidase inhibitors. When the calf serum concentration was low (0.5%), cytotoxicity below 2.5 μg/ml of SG and DSG, without inhibitor, was strong like in the case of 10% calf serum with amine oxidase inhibitors. Suppression of cytotoxicity at high drug concentrations by amine oxidase inhibitors was also observed but enhancement at low drug con-

Fig. 3. Effect of amine oxidase inhibitors on cytotoxic activity of SG and DSG in the presence of 10% calf serum.



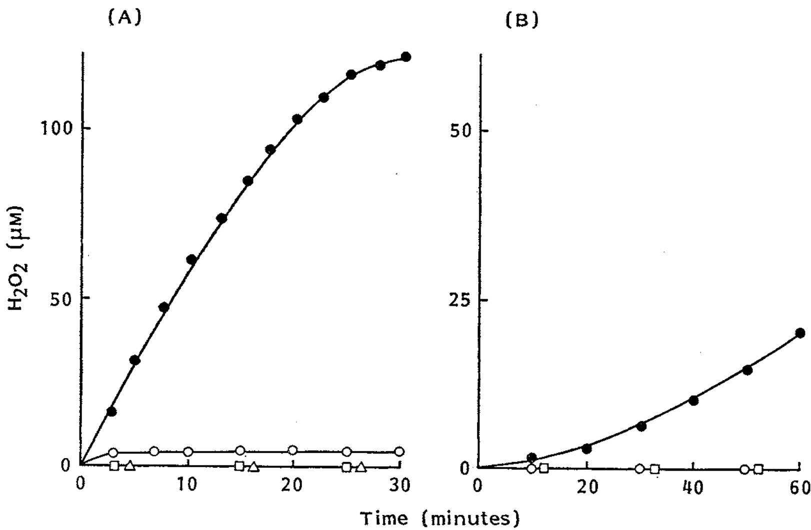

L5178Y cells ($5\times10^{4}$/ml) were grown in IBL medium supplemented with 10% calf serum for 48 hours at 37°C in the absence or presence of SG (A) or DSG (B) and amine oxidase inhibitor AG 320 μg/ml (○), 3HBA 10 μg/ml (□), or SC 40 μg/ml (△) or without inhibitor (●).

Fig. 4. Effect of amine oxidase inhibitors on cytotoxic activity of SG and DSG in the presence of 0.5% calf serum.




All the experimental conditions were the same as in Fig. 3 except that the calf serum concentration was 0.5%.

Fig. 5. Effect of amine oxidase inhibitors on oxidation of spermidine and SG by calf serum.




Spermidine (A) or SG (B) was oxidized by 10% calf serum in IBL medium with amine oxidase inhibitor, AG 320 $\mu$g/ml (○), 3HBA 10 $\mu$g/ml (□), or SC 40 $\mu$g/ml (△) or without inhibitor (●).

centrations was not found because of the considerable cytotoxicity without inhibitors. As shown in Fig. 5, 167 $\mu$M of spermidine (116 $\mu$g/ml) and SG (91.3 $\mu$g/ml) were oxidized by 10% calf serum in IBL medium. The three kinds of amine oxidase inhibitor suppressed oxidation of spermidine and SG completely. DSG was not tested concerning effect of amine oxidase inhibitor because it was very slowly oxidized at a rate about 15% of that of SG, though the data is not shown.

## Discussion

Polyamines are known to be taken up into L1210 cells by a common transport system[13] and

various $N^4$- and $N^1$,$N^8$-spermidine derivatives are competitive inhibitors of spermidine uptake.[14) Because the structures of SG and DSG are kinds of $N^8$-acyl derivatives, their uptake was studied by the competition method for [$^3$H]spermidine uptake by L5178Y cells. SG and DSG inhibited spermidine uptake competitively. Their respective affinity to the carrier was 610- and 250-fold, smaller than that of spermidine based on their *Ki* and *Km* of spermidine. Thus we propose, that SG and DSG are incorporated into cells by a spermidine-carrier, but with low efficiency. As previously reported,[11) spermidine is cytotoxic only in the presence of calf serum, but SG and DSG showed an antiproliferative effect also in the presence of human serum which contained amine oxidase activity less than that found in 0.1% calf serum. In this paper we described that spermidine was incorporated as itself and as oxidation products generated by amine oxidase in calf serum, products which had greater affinity for the cells than spermidine. The products were thought to be unstable, because the uptake of $^3$H would be enhanced by preincubation with calf serum if they were stable. From the results of Figs. 3 and 4, SG and DSG appear to exert their cytotoxic activity through two routes. One occurs by oxidation products from SG and DSG by extracellular amine oxidase in serum and this pathway is inhibitable by amine oxidase inhibitors. The other is an intracellular phenomenon independent of amine oxidase in serum and is enhanced by amine oxidase inhibitors because extracellular amine oxidase would be unavailable to trap SG and DSG and thus their cellular incorporation would increase. As reported elsewhere,[15) SG and DSG have higher affinity for amine oxidase from bovine plasma than for the spermidine-carrier. SG is a substrate of amine oxidase (*Km*: 71 $\mu$M), but DSG is very slowly oxidized in spite of its high affinity and might safely be said to be a kind of inhibitor (*Ki*: 7.8 $\mu$M). *Km* of SG and *Ki* of DSG are much smaller than their *Ki* for the spermidine-carrier, so intracellular uptake of SG and DSG was prevented in the presence of calf serum and the cytotoxicity seen is supposedly mainly caused by aldehydes produced by extracellular amine oxidase.

When amine oxidase activity in serum was inhibited by inhibitors, cytotoxicity as an intracellular event became demonstrable. This type of cytotoxicity was not caused by spermidine and other polyamines.[14) The mechanism of the latter cytotoxicity is unclear, but there is a possibility of involvement of aldehyde formation by intracellular aminoguanidine-insensitive amine oxidase, *e.g.*, polyamine oxidase. We are now studying the role of metabolism of SG and DSG in their antitumor activity.

Acknowledgments

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