

## STUDIES OF AN IMMUNOMODULATOR, SWAINSONINE

I. ENHANCEMENT OF IMMUNE RESPONSE  
BY SWAINSONINE *IN VITRO*

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Swainsonine isolated from *Metarhizium* sp., was found to enhance the activities of the mouse immune system *in vitro*. Concanavalin A stimulated lymphocyte proliferation and proliferative response in mixed lymphocytes culture, which were suppressed by immunosuppressive factor obtained from serum of sarcoma 180 tumor bearing mouse, were restored to normal levels by the addition of swainsonine. Furthermore, the concanavalin A induced incorporation of [<sup>3</sup>H]thymidine into mouse spleen cells was remarkably increased by treatment with swainsonine over a wide dose range. From studies using fluorescence activated cell sorting, swainsonine was shown to enhance the expression of concanavalin A receptors of spleen cells.

Some degree of immunosuppression commonly is associated with the infection and tumor disease process<sup>1-3</sup>. The cause of this is not fully understood. It has been suggested that immunosuppressive humoral factors in normal or malignant serum may play a role<sup>4,5</sup>. Some investigators have reported that tumor extracts and sera of tumor bearing hosts contain immunosuppressive factors<sup>4-7</sup>.

We have partially purified the immunosuppressive factor from serum of tumor bearing mice. This factor was found to suppress both concanavalin A (ConA)-induced lymphocyte proliferation and mixed lymphocyte reaction *in vitro*.

In the process of searching for new types of immunoactive substances from microorganisms, we have found that swainsonine<sup>8</sup> isolated from the fungus *Metarhizium* sp. exhibits a competitive action against immunosuppressive factors produced in the serum of tumor bearing mice and has the capacity to restore the depression of lymphocytes.

In this paper we describe the isolation of swainsonine from the fungus and its mode of action in the immunosuppressive experimental system *in vitro*.

### Materials and Methods

#### Culture and Medium Conditions

The microorganism used in this study, *Metarhizium anisopliae* F-3622 was obtained from a soil.

The seed medium contained corn starch 1%, soluble starch 1%, glucose 1%, corn steep liquor 0.5%, dried yeast 0.5%, cotton seed meal 0.5% and CaCO<sub>3</sub> 0.2%, pH 6.2. The production medium contained soluble starch 2%, glucose 1%, cotton seed meal 0.2%, gluten meal 0.4%, peanut powder 0.4%, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.05%, CoCl<sub>3</sub>·6H<sub>2</sub>O 0.0004%, CaCO<sub>3</sub> 0.2% and Adekanol (antiform) 0.025%, pH 6.0.

A loopful of slant culture of *M. anisopliae* F-3622 was inoculated to each of ten 500-ml Erlenmeyer

flasks containing 100 ml of the seed medium and cultured at 25°C for 96 hours on a rotary shaker with 7.6-cm throw at 200 rpm. The resultant culture was inoculated into the same seed medium (80 liters) in a 200-liter jar fermentor, which had been sterilized at 120°C for 30 minutes, and cultured at 25°C for 48 hours under aeration of 80 liters/minute and agitation of 265 rpm. Thirty five liters of the culture were inoculated to the production medium (1,760 liters) in a 2,000-liter stainless steel fermentor, which had been sterilized at 120°C for 30 minutes, and cultured at 25°C for 72 hours under aeration of 1,760 liters/minute and agitation of 180 rpm.

#### Animals

Female ICR/JCL, C57BL/6, and BALB/c (8 weeks of age) were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animal (Hamamatsu, Japan).

#### Preparation of Immunosuppressive Factor from Tumor-bearing Mouse Serum

Sarcoma 180 (S-180) cells maintained *in vivo* in ICR/JCL mouse in ascites form, were used.

ICR mice were subcutaneously inoculated with 0.2 ml of S-180 suspension ( $5 \times 10^6$  cells/ml). S-180 bearing mice were bled from the heart under light ether anesthesia between 7 and 9 days after transplantation and serum was collected.

Immunosuppressive factor was partially purified from the S-180 tumor bearing mice serum according to the method described by OH and MOOLTEN<sup>7)</sup>. Briefly, the serum (50 ml) was delipidated with 1/10 volume of 4% phosphotungstic acid and 1/40 volume of 2 M magnesium chloride and centrifuged at  $6,000 \times g$  for 10 minutes. Excess phosphotungstic acid and magnesium ion were removed by dialysis against phosphate-buffered saline (PBS: 0.15 M sodium chloride and 0.01 M phosphate buffer, pH 7.4). Then, partially delipidated serum was precipitated with ammonium sulfate at 50% saturation. The supernatant was removed by centrifugation at  $20,000 \times g$  for 30 minutes. The precipitates were redissolved in a small volume of H<sub>2</sub>O and dialyzed against PBS overnight at 4°C. The dialyzed solution was chromatographed on Sephadex G-200 column in PBS. Each eluate (1.5 ml) was assayed for immunosuppressive activity.

#### Assay of Mitogen Induced Mouse Spleen Cell Proliferation

Female BALB/c strain (8 weeks of age) was used.

The tissue culture medium employed was a complete medium designated Roswell Park Memorial Institute (RPMI)-1640. All media employed contained 100 units/ml of benzylpenicillin and 100  $\mu$ g/ml of streptomycin sulfate and 5% fetal calf serum.

Spleens were removed under sterile conditions and washed with Hanks solution and then teased in the tissue culture medium. The cells were suspended in the tissue culture medium to contain  $5 \times 10^5$  cells/ml. Into each hole of a microtitration plate (Falcon No. 3040) was poured 0.1 ml of the above cell suspension and 0.1 ml of the prescribed concentrate of the compound and/or immunosuppressive factor described above. ConA was utilized for cell stimulation at a final concentration of 1  $\mu$ g/ml. The culture was incubated in triplicate at 37°C in a humidified atmosphere (95% air, 5% CO<sub>2</sub>) for 48 hours.

Mitogen induced mouse spleen cell proliferation was assayed for tritiated thymidine (<sup>3</sup>H-thymidine) incorporation. In all tests, 20  $\mu$ l of 10  $\mu$ Ci/ml of [<sup>3</sup>H]thymidine was added to each hole for the 48 hours of culture. After a further 24-hour incubation, the resultant cells were filtered with filter paper, Whatman GF83 and washed successively with saline and with 5% trichloroacetic acid. The filter paper was dried and placed in a scintillator (toluene 1 liter containing 0.1 g of *p*-bis-(5-phenyl-oxazole)benzene and 4 g of 2,5-diphenyloxazole), and [<sup>3</sup>H]thymidine incorporated into DNA was measured.

#### Assay of Mixed Lymphocyte Reaction

Female C57BL/6 mice, 8 weeks in age, and female BALB/c mice, 8 weeks in age were sacrificed. Then spleens were aseptically removed and single cell suspensions were prepared as sources of stimulating and responding cells, respectively. Prior to culture, the stimulator cells were treated with 200  $\mu$ g/ml of mitomycin C for 45 minutes at 37°C, followed by three washes with PBS.

They were established at a final concentration of  $0.2 \times 10^6$  cells/0.2 ml of reaction mixture and

cultured in microtitration plate (Falcon, No. 3040) using RPMI 1640 medium supplemented with 10% fetal bovine serum and  $5 \times 10^{-5}$  M 2-mercaptoethanol. Furthermore, into each hole of the plate was poured 0.1 ml of the above cell suspension and 0.1 ml of the prescribed concentrate of the compound and/or immunosuppressive factor described before. The culture was continued for 120 hours and pulse-labeled with [ $^3$ H]thymidine for 24 hours before the termination of incubation. The cells were collected and radioactivity was counted.

#### ConA Binding Assay with Fluorescence Activated Cell Sorter (FACS)

Spleen cells ( $5 \times 10^6$ ) suspended in 2 ml of RPMI 1640 medium supplemented with heat inactivated fetal calf serum (FCS), 2 mM glutamine,  $2 \times 10^{-5}$  M  $\beta$ -mercaptoethanol, and antibiotics, were placed in tissue culture multi-well plate (24 flat bottom well, Linbro) containing 20, 2 or 0.2  $\mu$ g of swainsonine. The culture was incubated at 37°C in a humidified atmosphere (95% air, 5% CO<sub>2</sub>) for 4 or 48 hours. After incubation, spleen cells treated with swainsonine were harvested and washed three times with RPMI 1640 medium containing 5% FCS to remove swainsonine.

The cells ( $1 \times 10^6$ ) were pelleted by centrifugation and incubated for 30 minutes at 4°C in the presence of fluorescein-conjugated ConA (ConA-FITC, 2  $\mu$ g/0.05 ml RPMI 1640 medium containing 5% FCS and 0.02% sodium azide). After the incubation, the cells were washed three times and then suspended in RPMI 1640 medium containing 5% FCS and 0.02% sodium azide.

The cells stained with ConA-FITC were subjected to analysis on a FACS (FACS IV, Becton Dickinson and Co., Mountain View, CA). Analysis of the fluorescence profile of stained cells was performed according to the method described by HERZENBERG *et al.*<sup>9)</sup>. The analytical pattern was recorded by direct photography.

## Results

### Identification and Characterization of the Producing Strain

The strain F-3622 which produced swainsonine, was originally isolated from a soil sample collected at Izumi City, Osaka Prefecture. Its mycological characteristics are as follows.

On various culture media, the conidiomata consisting of sporodochial conidiophores and tall green columns of conidia are abundantly produced. Conidiogenesis was enteroblastic; phialidic. The conidiophores bear an aggregation of closely interwoven hyphae as a superficial fertile layer. They are hyaline, smooth, 20~45  $\mu$ m long and 2~5  $\mu$ m thick, and composed of closely penicillate branches. At the tip of each branch, one to five phialides, which are cylindrical to lecythiform, 6~13  $\mu$ m long and 1.5~4  $\mu$ m thick, are formed. The conidia are pale green, smooth, one-celled, oblong, truncate at both ends, 4~8 (~10.5)  $\times$  1.5~3  $\mu$ m in size. They are aggregated into columnar masses, which are dark olive green, 50~500  $\mu$ m diameter and 200~800  $\mu$ m high. The vegetative hyphae are septate, hyaline and branch, and hyphal cells are 1.5~17  $\mu$ m thick. Chlamydospores are absent.

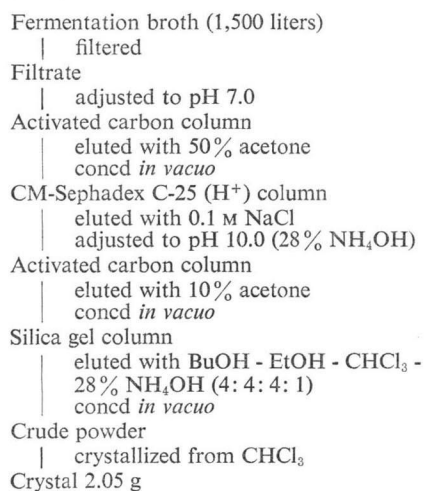
Colonies on malt extract agar grow restrictedly, attaining 3.0 cm in diameter after 2 weeks at 25°C. The colony surface is raised, floccose, white and dark olive green. The sporodochia are formed as green dots in or on white aerial hyphae. The reverse is uncolored.

From the morphological characteristics, the strain F-3622 was considered to be *M. anisopliae* (Metschnikoff) Sorokin<sup>10,11,12)</sup>. And above-mentioned characteristics corresponded with the description with a few exceptions; colony color *etc.*<sup>13)</sup>. Consequently, we identified strain F-3622 as one strain of *Metarhizium anisopliae*.

### Isolation and Physico-chemical Properties of Swainsonine

The procedure for the isolation is summarized in Fig. 1. The culture broth obtained was filtered with an aid of diatomaceous earth (5 kg). The filtrate obtained (1,200 liters) was adjusted to pH 7.0

Fig. 1. The procedure for the isolation of swainsonine from fermentation broth of *M. anisopliae* F-3622.



column (4 liters). The carbon column was washed with water (8 liters) and eluted with 10% acetone (10 liters). The active fractions were concentrated *in vacuo* to a volume of 2 liters and adjusted to pH 3.0 with 6 N HCl, and applied to a column of activated carbon (1 liter). The active principle was then eluted with 4 liters of water. The active fractions were combined and adjusted to pH 10 with NH<sub>4</sub>OH and passed through an activated carbon column (400 ml). The carbon column was washed with water (800 ml) and eluted with 10% acetone (1 liter). The active fractions were concentrated *in vacuo* to dryness (8.1 g). The crude sample was dissolved in 20 ml of water and subjected to chromatography on a silica gel column (600 ml) developed with a mixture of BuOH - EtOH - CHCl<sub>3</sub> - NH<sub>4</sub>OH (4: 4: 4: 1). The active fractions eluted were combined and concentrated *in vacuo* to dryness. The crude sample was dissolved in 200 ml of water and passed through a column of carbon (50 ml). After washing with water, the column was eluted with 10% acetone (200 ml). The active fraction was evaporated to dryness under reduced pressure to yield purified active materials as free base (2.6 g). Colorless crystals were obtained from chloroform (needles, 2.05 g).

Its purity was confirmed by <sup>1</sup>H NMR (Fig. 2) and mass spectrum. The molecular weight of this crystalline material is 173 with a molecular formula of C<sub>8</sub>H<sub>15</sub>NO<sub>3</sub>. Its IR spectrum had absorption bands at 3430 and 3350 cm<sup>-1</sup>, and its <sup>1</sup>H NMR spectrum (D<sub>2</sub>O) had signals at 4.03~4.37 (2H, m), 3.57~4.03 (1H, m), 2.77~3.10 (2H, m), 2.23~2.63 (1H, m), 1.00~2.23 (6H, m) (Fig. 2). These results of the material were almost in accord with those of swainsonine<sup>8)</sup>. Furthermore, analytical data of triacetate of the material was superimposable with the resonance data of the literature<sup>8)</sup>. The NMR spectrum (CD<sub>3</sub>OD) had signals at  $\delta$  5.54 (1H, dd,  $J=6.2, 4$  Hz), 5.23 (1H, ddd,  $J=6.2, 8.2$  Hz), 4.97 (1H, ddd,  $J=10, 10, 8$  Hz), 3.18 (1H, dd,  $J=11.8$  Hz), 2.57 (1H, dd,  $J=11.8$  Hz), 2.09 (3H, s), 2.05 (3H, s), 1.99 (3H, s), 1.55~2.20 (6H, m). From these experimental results, it has been confirmed that the material was identical to swainsonine.

Swainsonine was purified from the culture broth of *M. anisopliae* F-3622 by monitoring the competitive action against the inhibitory activity of proliferative responses of mouse spleen cells to ConA, which induced by adding the immunosuppressive factor to the assay mixture.

with 6 N NaOH and passed through an activated carbon column (350 liters). The column was washed with water (600 liters) and eluted with 50% acetone (700 liters). The active fraction was concentrated *in vacuo* to a volume of 42 liters and 80 liters of methanol were added. The resultant precipitate was removed by filtration and the filtrate was concentrated *in vacuo* to a volume of 30 liters, and neutralized with 6 N NaOH. The neutralized solution was charged to CM-Sephadex C-25 (H<sup>+</sup> form, 10 liters). The column of CM-Sephadex was washed with deionized water (20 liters) and then developed with 0.1 M NaCl (40 liters). The active fractions were combined and adjusted to pH 10 with NH<sub>4</sub>OH and passed through an activated carbon

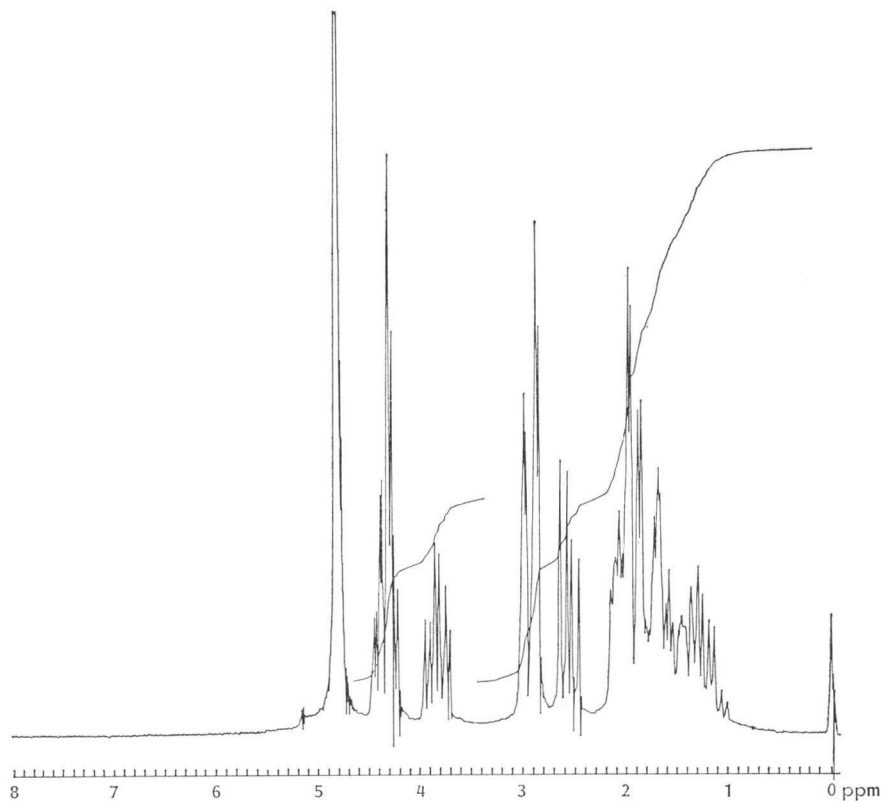
Fig. 2.  $^1\text{H}$  NMR spectrum of swainsonine isolated from *M. anisopliae* F-3622 ( $\text{D}_2\text{O}$ ).

Table 1. Purification of immunosuppressive factor.

Fraction	Protein <sup>a</sup> (mg)	SD <sub>50</sub> <sup>b</sup>	Recovery (%)
Tumor bearing mouse serum	5,380	3.90	100
Delipidated serum	1,900	1.80	75.3
50% saturated $(\text{NH}_4)_2\text{SO}_4$ precipitates	980	1.63	42.9
Sephadex G-200 peak fraction	16	0.05	22.8

<sup>a</sup> Protein concentration of the test sample was determined by the method of Lowry *et al.*<sup>15)</sup> with a bovine serum albumin standard.

<sup>b</sup> Defined as the protein concentration (mg/ml) that produces 50% suppression of the control ConA response<sup>7)</sup>.

#### Partial Purification of Immunosuppressive Factor

A summary of the purification procedure of immunosuppressive factor from tumor bearing mouse serum is shown in Table 1.

The ammonium sulfate precipitated material was separated on a Sephadex G-200 column. Most of the suppressive activity was associated with a peak fraction which eluted in the void volume of the column (data not shown). The Sephadex G-200 peak fraction represents a 78-fold purification. Approximately 23% of the total protein is recovered. This eluted fraction was used as the immunosuppressive factor in the following experiments.

From further experimental results, it has been demonstrated that the lyophilized Sephadex

Table 2. Suppression of ConA induced mouse spleen cell proliferation by immunosuppressive factor and its restoration by swainsonine.

Sample No.	Treatment of spleen cell added	[ <sup>3</sup> H]Thymidine uptake (cpm)*	
1	Non treated control	1,184±53	
2	ConA 3 µg/well	1	124,267±3,459
		0.3	60,400±2,552
		0.03	13,298±1,122
3	Immunosuppressive factor 8 µl/well	6	354±34
		4	333±3
		2	419±47
		0.2	1,310±68
4	Swainsonine 20 µg/well	2	1,263±50
		0.2	1,751±50
		0.02	1,668±26
		0.002	1,688±119
		0.0002	1,497±114
		0.00002	1,318±7
5	ConA 1 µg/well + immunosuppressive factor 8 µl/well	6	1,571±234
		4	2,652±114
		2	28,154±7,838
		0.2	59,123±3,078
6	ConA 0.3 µg/well + swainsonine 20 µg/well	2	69,986±7,994
		0.2	123,043±14,646
		0.02	109,027±4,540
		0.002	115,976±5,242
		0.0002	102,540±9,404
		0.00002	66,764±1,504
7	ConA 1 µg/well + immunosuppressive factor 6 µl/well + swainsonine 20 µg/well	2	38,576±857
		0.2	52,048±1,588
		0.02	72,370±1,752
		0.002	66,857±7,804
		0.0002	69,313±965

\* Mean±S.E. (n=4).

G-200 peak fraction was extracted with 0.2 N acetic acid and the all of recovered activity was associated with molecular weights of smaller than 160,000 daltons by gel filtration. The details will be the subject of a future report.

#### Competitive Effect of Swainsonine against the Immunosuppressive Factor Obtained from Tumor Bearing Mice Serum

The suppressive effect of immunosuppressive factor on mitogenic activities of spleen cells and its restoration by swainsonine were shown in Table 2.

Immunosuppressive factor from the tumor bearing mice serum profoundly suppressed the mitogen induced stimulation of [<sup>3</sup>H]thymidine incorporation by mouse spleen cells (Table 2). This suppression was dose-dependent.

The addition of swainsonine to the culture containing immunosuppressive factor prevented the suppression. This result indicates that swainsonine has the capacity to restore the depression of mitogenic responses of mouse spleen cells by immunosuppressive factor.

Furthermore, the addition of swainsonine to mouse spleen cell culture did not affect mitogenic activity of mouse spleen cell or spleen cell viability.

Table 3. Inhibition of MLC stimulated spleen cell [<sup>3</sup>H]thymidine uptake by immunosuppressive factor and its restoration by swainsonine.

Experiment No.	Reaction	[ <sup>3</sup> H]Thymidine uptake (cpm)*
1	Unstimulated spleen cell (responder alone)	4,359 ± 430
2	MLC without immuno- suppressive factor	18,603 ± 224
3	MLC with immunosuppressive factor	3,303 ± 519
	3 μg/well	2,350 ± 90
4	MLC with immunosuppressive factor (3 μl)	25,287 ± 1,246
	2 μg/well	27,087 ± 860
	+swainsonine	31,046 ± 1,723
	0.2	24,789 ± 679
	0.002	13,310 ± 816
	0.0002	10,777 ± 1,670
5	MLC with immunosuppressive factor (5 μl)	6,658 ± 111
	2 μl/well	7,618 ± 501
	+swainsonine	7,562 ± 153
	0.02	11,347 ± 906
	0.002	6,650 ± 626
	0.0002	4,009 ± 390
6	MLC with swainsonine	34,289 ± 1,446
	2 μg/well	36,800 ± 1,185
	0.2	34,331 ± 782
	0.02	29,446 ± 264
	0.002	21,250 ± 509
	0.0002	22,662 ± 1,318

\* Mean ± S.E. (n=4).

When ConA and swainsonine were added simultaneously to culture, ConA induced stimulation of [<sup>3</sup>H]thymidine incorporation for mouse spleen cells was increased 10 times as much as that seen with ConA only.

#### Immunosuppressive Effect of Tumor Bearing Mouse Serum on Mixed Lymphocyte Culture and Its Restoration by Swainsonine

Immunosuppressive factor from tumor bearing mouse serum inhibited [<sup>3</sup>H]thymidine uptake into mixed lymphocyte culture (MLC)-stimulated spleen cells.

When the immunosuppressive factor was incubated with swainsonine, the inhibitory activity was removed. The addition of swainsonine to MLC enhanced remarkably the MLC induced stimulation of [<sup>3</sup>H]thymidine incorporation for mouse spleen cells (Table 3).

#### Effect of Swainsonine on ConA Receptor Binding Assayed in the FACS

The fluorescence distribution of spleen cells stained with ConA-FITC for 4 and 48 hours in the presence or absence of swainsonine at the concentrations of 0.1, 1 and 10 μg/ml is shown in Fig. 3. At 4-hour incubation 20,000 (live) cells and at 48-hour 5,000 (live) cells were analyzed, respectively. There was a loss in the viability of the original number of cells in the presence and absence of swainsonine at 48-hour incubation. The fluorescence profile at both 4- and 48-hour incubation in the presence of swainsonine demonstrated a significant increase in the brightly stained spleen cell population.

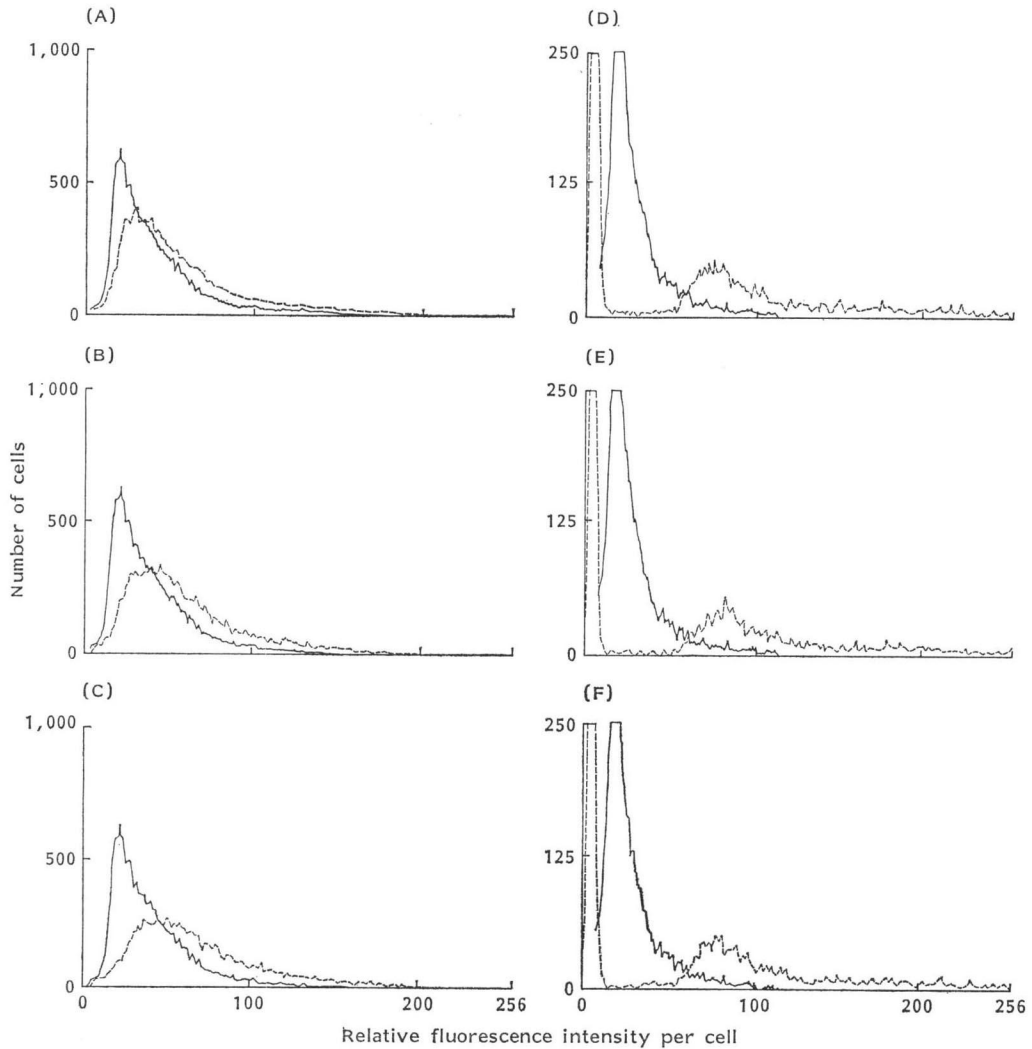
Furthermore, the fluorescence shift of the ConA-FITC stained spleen cells at 48-hour incubation

Fig. 3. Analysis of ConA receptor of spleen cells treated with by the FACS.

The spleen cells were incubated with various concentrations of swainsonine for 4 hours [(A) (B) (C)] or 48 hours [(D) (E) (F)], and then incubated with ConA-FITC.

[(A), (D)]: swainsonine, 0.1  $\mu\text{g}/\text{ml}$ ; [(B), (E)]: swainsonine, 1  $\mu\text{g}/\text{ml}$ ; [(C), (F)]: swainsonine, 10  $\mu\text{g}/\text{ml}$ .

— Con A only; ----- Con A after swainsonine.



in the presence of swainsonine was much larger than that at 4-hour incubation. Similar observations were obtained from the fluorescence shift at 24-hour incubation, as compared with that at 4-hour incubation (data not shown).

These results indicate that swainsonine increases the expression of ConA receptors of spleen cells.

#### Discussion

Swainsonine has been isolated from *Swainsona* sp.<sup>8)</sup> and locoweed<sup>14)</sup>, plants that induce in live-



stock a condition resembling the lysosomal storage disease mannosidosis. Recently it was isolated from *Rhizoctonia leguminicola*, a fungus<sup>15)</sup>. We now demonstrate that *Metarhizium* sp., another fungus also produces swainsonine.

We showed that in the presence of swainsonine, ConA induced incorporation of [<sup>3</sup>H]thymidine into mouse spleen cells was increased 10 times as much as that seen with ConA only (Table 1). Furthermore, from studies with FACS at a concentration of swainsonine (0.1, 1 and 10  $\mu$ g/ml) using ConA-FITC at 40  $\mu$ g/ml, the appearance of the ConA receptors can be accelerated (Fig. 3). It seems that swainsonine will induce ConA receptor in mouse spleen cells. Similar results have been obtained by ELBEIN *et al.*, in MDCK cells, CHO cells and B16 melanoma cells<sup>16)</sup>. These cells showed a 50~100% increase in their ability to bind [<sup>3</sup>H]ConA in the presence of swainsonine and substantial decrease in the binding of <sup>3</sup>H-labeled wheat germ agglutinin. They have suggested that these alterations may be due to an increase in high mannose (or hybrid) types of oligo-saccharides of receptors and a decrease in the complex types.

The following observation may be noted. Swainsonine is a potent inhibitor of  $\alpha$ -D-mannosidase<sup>9)</sup> and blocks the synthesis of glycoprotein containing N-linked complex oligosaccharides. Hybrid glycoprotein might be produced in the presence of swainsonine by human skin fibroblast and rat liver golgi preparations<sup>17)</sup>.

In the experiments reported in this paper, ConA stimulated lymphocyte proliferation and the proliferative response in mixed lymphocytes culture were suppressed by immunosuppressive factor obtained from serum of tumor bearing mice. They were restored to normal levels by the addition of swainsonine. Although it is suggested that there is a possibility of a competitive binding of both immunosuppressive factor and swainsonine to the surface of some population of lymphocytes, the exact mechanism of action of immunosuppressive factor and swainsonine is not known at this time.

We will describe the immune responses and antitumor effect of swainsonine *in vivo* in the following paper.

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