STUDIES OF AN IMMUNOMODULATOR, SWAINSONINE

II. EFFECT OF SWAINSONINE ON MOUSE IMMUNODEFICIENT SYSTEM AND EXPERIMENTAL MURINE TUMOR

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We examined the effect of swainsonine on antibody response to sheep red blood cells (SRBC) in immunodeficient mice which were treated with immunosuppressive factor or antitumor drugs, or inoculated with sarcoma 180 ascites tumor. The administration of swainsonine restored the capacities of the immunodeficient mice to produce antibody against SRBC. Furthermore, swainsonine inhibited completely the growth of sarcoma 180 ascites tumor in mice. Swainsonine also reduced lung metastases of B16 melanoma in mice. These results suggest that swainsonine has potential as an immunomodulator for the treatment of immunocompromised hosts.

As reported previously[1], swainsonine restored the depression of immune responses caused by immunosuppressive factor obtained from tumor bearing mice *in vitro*. These facts suggest that there is a need for further evaluation of swainsonine as an immunomodulator.

In this paper, we study the immunomodulating activities of swainsonine *in vivo*.

Materials and Methods

Animals

Specific-pathogen-free (SPF) ICR/SJL (female, 8 weeks old), BALB/c (female, 8 weeks old), *dd*Y (male, 5 weeks old) mice were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animal (Hamamatsu, Japan) and maintained under SPF condition in our laboratory during the experiments.

Swainsonine

Swainsonine (Fujisawa Pharmaceutical Co., Ltd.) was dissolved in physiological saline and filtered through a 0.45 μm Millipore filter. Swainsonine used in this studies did not contain endotoxin as determined by the Limulus amebocyte lysate assay (Seikagaku Kogyo Co., Ltd., Tokyo, Japan). Mice were given intraperitoneally or subcutaneously an appropriate dose of swainsonine in 0.2 ml.

Tumors

The sarcoma 180 (S-180) tumor cells were transferred at 7-day intervals in female ICR/SJL mice in ascites form.

The B16 melanoma cells, syngeneic to C57BL/6 mice, were selected for high potential for lung metastasis and established in culture as described by FIDLER[2].

Preparation of Immunosuppressive Factor

Immunosuppressive factor was partially purified from serum of the S-180 tumor bearing mice according to the method described previously[1,3].

Assay for Antibody Formation to Sheep Red Blood Cells (SRBC)

SRBC were employed as antigen. The mice were immunized by a single intravenous injection of 1×10^8 SRBC. Five days thereafter, the mice were sacrificed and antibody formation was assayed according to CUNNINGHAM's modified method[4] by counting the hemolytic plaque-forming cells (pfc) per spleen of each mouse. Five mice were employed in each group.

Immunodeficient Mouse Model *In Vivo*

The following methods were employed to produce immunodeficiency in mice.

Treatment with Immunosuppressive Factor: The immunosuppressive factor was administered intravenously daily for 4 days before immunization into ICR mice.

Tumor Induced Immunodepression: ICR mice were inoculated intraperitoneally with 1×10^6 cells of S-180 at 7 days before immunization.

Treatment with Cyclophosphamide or Mitomycin C: Cyclophosphamide (Shionogi & Co., Ltd., Osaka, Japan) and mitomycin C (Nakarai Chemical Co., Ltd., Kyoto, Japan) were dissolved in saline. Cyclophosphamide (22.5 mg/kg) was injected intraperitoneally into each mouse 4 days before immunization with SRBC to reduce antibody response. Mitomycin C (1 mg/kg) was injected intraperitoneally 4, 3 and 2 days before immunization.

Murine Transplantable Tumor

The S-180 tumor cells (1×10^6/mouse) were transferred into ICR mice in ascites form. For experiment, the tumor cells collected 7 days after inoculation, were washed and suspended at 1×10^7 cells/ml in saline. The tumor cells (1×10^6 cells) were inoculated intraperitoneally into ICR mice. Fifteen days after the tumor inoculation, mice were sacrificed and the total tumor cell packed volumes in their ascites were measured by centrifugation at 1,000 rpm for 10 minutes at 4°C.

The Experimental Pulmonary Metastasis

B16 melanoma cells in exponential growth phase were harvested from subconfluent culture *in vitro*. The cells were washed and resupended in Hanks' balanced salt solution (HBSS, Flow Laboratories, Rockville, MD). A single cell suspension was obtained by passage through a Nylon mesh. In the experiments testing the effect of swainsonine on pulmonary metastasis, tumor cells (1×10^5 cells) in 0.2 ml of HBSS were inoculated intravenously into C57BL/6 mice. Swainsonine was administered subcutaneously at 3 days and 1 day before, and 1 day after the tumor inoculation. The recipient mice were sacrificed 14 days after tumor cell inoculation, and their lungs were removed. The number of colonies on the lung was measured with a dissecting microscope.

Results

Effect of Swainsonine on Antibody Formation to SRBC in the Immunodeficient Mice

The effect of swainsonine on antibody formation to SRBC was studied in mice in which the immune system was impaired by injection of immunosuppressive factor obtained from tumor bearing mice. Female ICR mice were injected with 0.3 ml of immunosuppressive factor. Four days later, they were immunized by intravenous injection with 1×10^8 SRBC. Swainsonine at doses ranging from 3.7 mg/kg to 100 mg/kg was administered intravenously for 5 consecutive days starting from the day of immunosuppressive factor injection, and after 5 days its effect on antibody formation was determined by pfc assay. The results were shown in Table 1. Compared to non-treated mice, the antibody forming capacities against SRBC in the immunosuppressive factor treated mice were markedly depressed. The depression was restored to the normal level by the intraperitoneal administration of swainsonine ($3.7\sim100$ mg/kg). Swainsonine did not enhance the capacities of normal mice to produce antibody against SRBC.

Table 1. Depression of antibody forming capacity by immunosuppressive factor and its restoration by intraperitoneal administration of swainsonine[a].

Substance		Anti-SRBC pfc ($\times 10^4$ pfc/spleen) Mean±S.E.
Control (saline)		279±12.0
Swainsonine 100 mg/kg		240±2.0
Immunosuppressive factor (0.3 ml/mouse/day)[c]		
Saline		135±9.3
Swainsonine	3.7	302±10.9[b]
(mg/kg)	11.0	292±3.7[b]
	33.0	301±3.9[b]
	100.0	253±6.9[b]

[a] Immunosuppressive factor was injected intravenously 4 days (day 0~day 3) before immunization. Swainsonine was injected intraperitoneally daily for 5 days (day 0~day 4) starting from the day of immunosuppressive factor injection.

[b] Significantly different from immunosuppressive factor-treated control at $P<0.05$ (Student's t test), 5 mice per group.

[c] 0.3 ml of the immunosuppressive factor used, was corresponded to about 2 mg of the Sephadex G-200 peak fraction taken as protein content[1].

Table 2. Depression of antibody forming capacity in sarcoma 180-bearing mice and its restoration by intraperitoneal administration of swainsonine[a].

Substance		Anti-SRBC pfc ($\times 10^4$ pfc/spleen) Mean±S.E.
Control (saline)		138±6.0
Swainsonine 100 mg/kg		119±4.6
Tumor bearer		
Saline		48±5.8
Swainsonine	3.7	56.8±7.2
(mg/kg)	11.0	75±13.1
	33.0	126±4.9[b]
	100.0	127±6.5[b]

[a] Sarcoma 180 tumor cells (1×10^6 cells) were inoculated. Seven days thereafter, mice were immunized by intravenous injection of SRBC. Swainsonine injected daily for 5 days after tumor implantation.

[b] Significantly different from tumor-bearing control at $P<0.05$, 5 mice per group (Student's t test).

Table 3. Effect of swainsonine on antibody forming capacities in mice treated with cyclophosphamide[a].

Substance		Anti-SRBC pfc ($\times 10^4$ pfc/spleen) Mean±S.E.
Control (saline)		270±11.9
Swainsonine 100 mg/kg		250±12.8
Cyclophosphamide (22.5 mg/kg)		
Saline		128±9.4
Swainsonine	3.7	305±11.5[b]
(mg/kg)	11.0	256±12.5[b]
	33.0	249±22.6[b]
	100.0	242±10.8[b]

[a] Cyclophosphamide (22.5 mg/kg) was injected intraperitoneally 4 days before immunization (day 0). Swainsonine was injected intraperitoneally daily for 5 days starting from the day of cyclophosphamide injection (day 0~day 4).

[b] Significantly different from cyclophosphamide-treated control at $P<0.05$ (Student's t test), 5 mice per group.

Table 4. Effect of swainsonine on antibody forming capacities in mice treated with mitomycin C[a].

Substance		Anti-SRBC pfc ($\times 10^4$ pfc/spleen) Mean±S.E.
Control (saline)		288±8.0
Swainsonine 100 mg/kg		328±19.3
Mitomycin C (1.0 mg/kg)		
Saline		208±8.6
Swainsonine	3.7	312±21.0[b]
(mg/kg)	11.0	303±16.0[b]
	33.0	288±12.6[b]
	100.0	350±25.9[b]

[a] Mitomycin C (1 mg/kg) was injected intraperitoneally at 4, 3 and 2 days before immunization (day 0~day 2). Swainsonine was injected intraperitoneally daily for 5 days starting from the day of mitomycin C injection (day 0~day 4).

[b] Significantly different from mitomycin C-treated control at $P<0.05$ (Student's t test), 5 mice per group.

The S-180 tumor bearing mice showed statistically depressed antibody forming capacities, when compared to non-treated mice (Table 2). ICR mice were inoculated with 1×10^6 tumor cells intraperitoneally, and after 4 days 1×10^8 SRBC were injected intravenously. Swainsonine at doses of 3.7~100 mg/kg was administered intraperitoneally for 5 days after the inoculation of tumor

Table 5. Effect of swainsonine on murine transplantable tumors.

Dose (mg/kg/day)	Total cell packed volume (ml)[a]				
0	35,	38,	39,	45,	48
3	22,	32,	40,	40,	47
10	5,	12,	23,	33,	38
30	0,	0,	0,	0,	0
100	0,	0,	0,	0,	0

The tumor cells (1×10^6 cells) were inoculated into ICR mice. Swainsonine was injected daily for 5 days after tumor inoculation. Fifteen days after the inoculation, mice were killed and the packed volume of total tumor cells was measured.

[a] 5 mice per group.

Table 6. Effect of swainsonine on the metastatic growth of B16 melanoma cells in lungs[a].

Dose (mg/kg/day)	No. of metastatic foci	
	Mean±S.E.	(Range)
0	560.7±60.3	(374~727)
100	150.2±50.1[b]	(62~329)
300	74.3±16.1[b]	(18~115)
1,000	30.4± 7.7[b]	(8~ 53)

[a] C57BL/6 mice were given subcutaneous injection of swainsonine 3 days and 1 day before and 1 day after tumor inoculation. Mice inoculated intravenously with 1×10^5 cells of B16 melanoma, were killed 14 days after tumor inoculation and the number of the pulmonary metastases in the lungs were counted.

[b] Significant reduction of metastases ($P<0.05$) compared to untreated mice, 5 mice per group.

cells. As shown in Table 2, the administration of swainsonine (11~100 mg/kg) restored significantly the capacities of tumor bearing mice to produce antibody against SRBC.

In other experiments, cyclophosphamide (22.5 mg/kg) was injected intraperitoneally 4 days before immunization into mice. The antibody formation was markedly impaired. As shown in Table 3, the administration of swainsonine at doses of 3.7~100 mg/kg restored the capacities of mice injected cyclophosphamide to form antibody against SRBC. Furthermore, as shown in Table 4, the antibody formation to SRBC in mice treated with mitomycin C (1 mg/kg) was markedly suppressed. The administration of swainsonine (3.7~100 mg/kg) reversed the suppressed immune response.

Effect of Swainsonine on the Murine Transplantable Tumor Growth

The effect of swainsonine on the murine transplantable tumor S-180 was tested. Swainsonine at doses of 3~100 mg/kg was administered daily for 5 days after tumor inoculation. As shown in Table 5, the administration of swainsonine at doses of 30 and 100 mg/kg suppressed completely on tumor growth and at 10 mg/kg inhibited the tumor growth by 50%.

Effect of Swainsonine on Experimental Pulmonary Metastasis

As shown in Table 6, the subcutaneous administration of swainsonine at all doses tested (100~1,000 mg/kg) significantly inhibited the number of B16 pulmonary metastasis by 73~95% in comparison with that of non-treated mice.

Discussion

We reported previously the action of swainsonine in enhancing immune responses *in vitro*[1]. In these studies, at first we examined the effect of swainsonine on antibody formation to SRBC in immunodeficient mice which were treated with immunosuppressive factor obtained from tumor bearing mice *in vivo*. The administration of swainsonine restored the immune suppression caused by immunosuppressive factor. In addition, swainsonine also reversed the depressed immune responses in S-180 tumor bearing mice. Furthermore, other investigators have shown that antitumor drugs such as cyclophosphamide, mitomycin C or 5-fluorouracil induced the immunodeficiency including humoral and cellular immunity in rodents[5]. In these animal models, azimexon, krestin and bestatin were also reported to restore the host resistance and immune response[6~8]. For our studies, we have chosen

cyclophosphamide and mitomycin C and then examined the effect of swainsonine on antibody formation in the drug induced immunodeficient mice. The administration of swainsonine restored to normal level the capacities of the mice injected drugs to produce antibody against SRBC. Restoration in immune deficient hosts, especially in cancer patients, is an important target. Our results suggest that the enhancing effect of swainsonine on the immune responses can be used in combination with cancer chemotherapeutic agents to prevent microbial infection or to improve immunodeficiency in cancer patients.

The effect of swainsonine on the murine S-180 transplantable tumor was examined. When swainsonine was administered intraperitoneally, there was a complete inhibition of tumor growth at high doses of swainsonine (30~100 mg/kg). Furthermore, swainsonine exhibited a strong inhibitory effect on the experimental B16 pulmonary metastasis in mice at high doses (100~1,000 mg/kg). In all experiments described above, these data indicate that the mode of action of swainsonine is different from most other immunopotentiating agents. It also differs from biological adjuvants, in that swainsonine exhibited no effect on the immune responses of normal mice. Therefore these results suggest that swainsonine may be proved a potential form of therapy in those situation where immunoresponsiveness is compromised by tumor, infections or other debilitating diseases.

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