〈Original Article〉

Enhancement of cytokine (IL-2, INF-γ) production in Th1 cells by crude protein extract of Equisetum arvense LINNE

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Summary We previously reported that the highest cell proliferation-inhibitory effect of a crude protein extract solution of Equisetum arvense LINNE (crude E. arvense protein) on cultured cells was noted in the group treated with the highest concentration of crude E. arvense protein. We also performed in vivo experiments on the life-prolonging effect on mice using L-1210 and B16F1 cells, and observed the highest effect at the lowest crude E. arvense protein concentration in experiments using either cell type. The Equisetaceae family is known to have various physiological effects. In this study, the influence of crude E. arvense protein on immune responses was investigated by measuring interleukin-2 (IL-2) and interferon-γ (IFN-γ) produced by Th1 cells playing the central role in cellular immunity. After 24-hour culture with 0.2 mg/ml of crude E. arvense protein in the presence of 5 μg/ml ConA, 1,434.5 pg/ml of IL-2 was produced, showing 1.7 times greater production than that in the control. In cells cultured for 48 hours, 2,130.9 pg/ml was produced by cells treated with 0.2 mg/ml of crude E. arvense protein in the presence of 10 μg/ml ConA, showing 1.9 times greater production than that in the control. Regarding the IFN-γ production-enhancing effect, 929.3 pg/ml was produced by cells cultured for 24 hours with 0.2 mg/ml of crude E. arvense protein in the presence of 5 μg/ml ConA, suggesting that Th1 cells were activated.

Key words: Th1, IL-2, INF-γ

1. Introduction

Equisetaceae broth has been used for compress and oral ingestion in folk remedies for a long time. Equisetum hyemale LINNE var has been used for hemostasis of intestinal hemorrhage and hemorrhoids[1] and polishing of craft products, wood, horn, and bone because it contains silicate in the stem surface tissue. Equisetum arvense LINNE (E. arvense) is used as a diuretic[1), 2)], and studies have been reported[3), 4), 5)]. We previously reported that crude protein extract of E. arvense (crude E. arvense protein) inhibited cancer cell proliferation in cell cultures of L-1210 (mouse-derived leukemia cells), 3T3 (mouse-derived SV-transformed fibroblasts), and HMV-I (human-derived melanin-producing melanoma cells)[6]. We also reported a strong life-prolonging effect on mice in an in vivo study using L-1210 and B16F1 (mouse melanoma

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cells) cells[7]. The Equisetaceae family has various physiological effects, as described above. In this study, we investigated the influence of crude E. arvense protein on the immune response system in vitro by measuring productions of interleukin-2 (IL-2) and interferon-γ (IFN-γ) by Th1 cells.

2. Materials and Methods

1. Preparation of crude E. arvense protein

Shade-dried E. arvense (200 g) was boiled in a beaker containing 2 L of distilled water for 4-5 hours. Cooled and filtered E. arvense broth was combined with the same volume of saturated ammonium sulfate and mixed. The mixture was kept standing for several hours and centrifuged in a cold centrifuge (1,500 × g, 15 min). The supernatant was removed, and the precipitate was dissolved in a small volume of distilled water. The dissolved solution was transferred into a dialysis membrane and dialyzed against distilled water in a refrigerator (distilled water was changed every 2-3 hours). The dialysate was centrifuged in a cold centrifuge (3,500 × g, 15 min), and the supernatant was collected. This supernatant was combined with the same volume of saturated ammonium sulfate and mixed. This procedure was repeated 3 times, and the last dialysate was cold-centrifuged (3,500 × g, 15 min) and lyophilized. This lyophilized substance was used as crude E. arvense protein.

2. Cytokine (IL-2 and IFN-g) production enhancement experiment

1) Materials

(1) Preparation of mouse splenocytes

Five-week-old female B6D2F1/Crlj mice (Charles River Laboratories Japan Inc.) weighing 20 g on average were anesthetized with chloroform, and the spleen was excised after blood sampling from the heart. The spleen was placed in a dish containing RPMI-1640 medium (GIBCO) with L-glutamine, 100 units/ml penicillin G (Banyu Pharmaceutical), 100 μg/ml streptomycin (Meiji), and 10% fetal bovine serum (Nippon Bio-Supply Center), loosened into small pieces using forceps, and pipetted with a Pasteur pipette to prepare a fine cell suspension. The loosened cell suspension was filtered through a stainless mesh to remove masses. The filtered cell suspension was transferred into a culture flask (75 cm^2, CORNING) and cultured in 5% CO_2 incubator (37℃) for one hour, and non-adhering cells (floating cells) were collected. The collected non-adhering cells were centrifuged (1,000 rpm, 5 min) to remove the supernatant. The cell pellet was suspended with 10% FCS-containing RPMI-1640. The cells were counted using a Bürker-Türk counting chamber, and the count was adjust to 5 × 10^6 cells/ml with 10% FCS-containing RPMI-1640.

All animals received humane care in compliance with the guidelines of the Management of Laboratory Animals in Fujita Health University, Japan.

(2) Crude E. arvense protein solution

Solutions at a concentration of 0.6, 0.06, and 0.006 mg/ml (final concentration: 0.2, 0.02, and 0.002 mg/ml, respectively) were prepared with 10% FCS-containing RPMI-1640.

(3) Preparation of crude E. arvense protein solution

Three mg/ml crude E. arvense protein solution was prepared with 10% FCS-containing RPMI-1640 and sterilized by filtration using a Millipore filter (0.22 μm). The sterile 3 mg/ml solution was 5 times diluted with 10% FCS-containing RPMI-1640 to prepare 0.6 mg/ml solution, 0.06 mg/ml solution was prepared by 10 times dilution of 0.6 mg/ml solution, and 0.006 mg/ml solution was prepared by 10 times dilution of 0.06 mg/ml solution.

(4) Preparation of Concanavalin A (ConA) solution

Thirty mg/ml of ConA Type Ⅳ (SIGMA) was prepared with 10% FCS-containing RPMI-1640 and sterilized by filtration using a Millipore filter (0.22 μm). This sterile 30 μg/ml ConA solution was 2 times diluted with 10% FCS-supplemented RPMI-1640 to prepare 15 μg/ml ConA solution. These 15 and 30 μg/ml solutions (final concentration: 5 and 10 μg/ml, respectively) were used in experiments.

2) Experiment

To 24-well flat-bottom multiwell culture plates (FALCON), 500 ml each of 5 × 10^7 cells/ml splenocyte suspension, ConA solution (15 and 30 μg/ml), and crude E. arvense protein solution (0.006, 0.06, and

0.6 mg/ml)were added to wells and incubated for 24 or 48 hours in a CO_2 incubator. To the negative control group, splenocyte suspension and 10% FCS-supplemented RPMI-1640 were added. To the positive control group, splenocyte suspension and ConA (15 and 30 μg/ml) were added. To the experimental groups, splenocyte suspension, ConA (15 and 30 μg/ml), and crude E. arvense protein solution (0.006, 0.06, and 0.6 mg/ml) were added. Each group was triplicated. After culture for 24 or 48 hours, the supernatants were collected by centrifugation as samples for IL-2 and IFN-γ measurements.

3) Cytokine (IL-2 and IFN-γ) measurement

Cytokines were measured using mouse IL-2 and IFN-γ measurement ELISA kits (BIOSOURCE). Calibration curves were prepared using standard solutions included in the kits (7.8, 15.6, 31.2, 62.5, 125, 250, and 500 pg/ml), and the IL-2 and IFN-γ levels in samples were determined using these curves.

① IL-2 measurement

Wash buffer (400 μl) was added to each well of a microplate for measurement, and the wells were washed for 15-30 seconds. Fifty μl of the sample was added to each well, followed by adding 50 μl of biotinylated anti-IL-2 (Biotin Conjugate) and mixing using a mixer. The plate was sealed and incubated at 37℃ for 2 hours. After discarding the solution in wells, 400 μl of wash buffer was added and discarded after 30 seconds. This procedure was repeated 4 times. Streptavidin-peroxidase (HRP) (100 μl) was added to each well and mixed using a mixer. The plate was sealed and incubated at room temperature for 30 minutes. After discarding the solution, wells were washed with 400 μl of wash buffer 4 times. Stabilized chromogen was added (100 μl per well), and the plate was incubated at room temperature for 30 minutes in a light-protected room. Precisely after 30 minutes, Stop Solution was added (100 μl per well), and the absorbance at 450 nm was measured (within 2 hours after stopping the reaction).

② IFN-γ measurement

Wash buffer (400 μl) was added to each well of a microplate for measurement, and the wells were washed for 15-30 seconds. One hundred μl of the sample was added to each well, and the plate was sealed and incubated at 37℃ for 2 hours. After discarding the solution in wells, 400 μl of wash buffer was added for washing, and this procedure was repeated 4 times. Biotinylated anti- IFN-γ (Biotin Conjugate) was added (100 μl/well) and mixed using a mixer. The plate was sealed and incubated at 37℃ for 30 minutes. After discarding the solution in wells, the wells were washed with 400 μl of wash buffer 4 times. Streptavidin-peroxidase (HRP) was then added

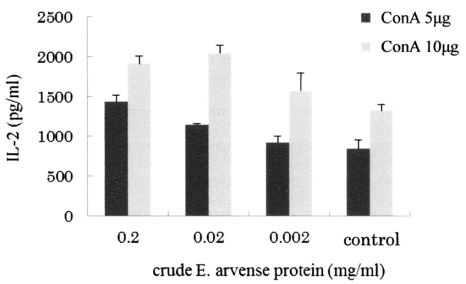

Fig. 1 IL-2 production enhancement by crude E. arvense protein (24 Hr Treatment)

Table 1 IL-2 production enhancement by crude E. arvense protein (24 Hr Treatment)

ConA	crude E. arvense protein (mg/ml)	IL-2 (pg/ml)	SE	p-value (t-test)
5 μg/ml	0.2	1434.8	83.7	0.013
	0.02	1136.2	16.9	0.057 (NS)
	0.002	924.1	77.6	0.580 (NS)
	Control	841.7	113.0	
10 μg/ml	0.2	1914.9	96.4	0.008
	0.02	2045.0	96.5	0.004
	0.002	1574.3	227.6	0.354 (NS)
	Control	1845.0	73.1	

Mean±SE (N=3)

(100 μl/well) and mixed using a mixer. After adding 100 μl of stabilized chromogen to each well, the plate was incubated at room temperature for 30 minutes in a light-protected room. Precisely after 30 minutes, Stop Solution was added (100 μl/well), and the absorbance at 450 nm was measured (within 2 hours after stopping the reaction).

4) Statistical analysis

All data are presented as mean ± SEM (N=3) and statistical analyses were done using Analyze-It software for Excel (version 12.0). All reported p values were two-sided and calculated using the Student's paired t test. In all cases p values of <0.05 were considered statistically significant.

3. Results

After 24-hour culture, 1,434.5 pg/ml of IL-2 was produced by cells treated with 5 μg/ml ConA and 0.2 mg/ml crude E. arvense protein, and this was 1.7 times greater than that (841.7 pg/ml) produced by the control cells. However, no significant difference was noted at the other concentrations. In the experiment in the presence of 10 mg/ml ConA, 1,914.9 and 2,045.0 pg/ml of IL-2 were produced by cells treated with 0.2 and 0.02 mg/ml crude E. arvense protein, respectively, showing significant differences from that (1,324.0 pg/ml) produced by the control (Table 1, Fig. 1).

The production was reduced in 48-hour compared to that in 24-hour cultures. The most marked enhancement of production was noted in cells treated with 0.2 mg/ml crude E. arvense protein in the presence of 5 μg/ml ConA, and the production was 602.1 pg/ml, showing a 6.4 times higher production than that (94.4 pg/ml) in the control. The productions at 0.02 and 0.002 mg/ml were 329.5 and 264.5 pg/ml, respectively, showing 3.5 and 2.8 times greater productions than those in the control, respectively. In cells treated in the presence of 10 μg/ml ConA, the productions were 2,130.9 and 1,629.5 pg/ml at 0.2 and 0.02 mg/ml crude E. arvense protein, respectively, showing 2.0 and 1.5 times greater productions than that (1,077.7 pg/ml) in the control (Table 2, Fig. 2).

Regarding the enhancement of IFN-γ production in cells treated in the presence of 5 μg/ml ConA, 929.3, 915.3, and 842.7 pg/ml of IFN-γ were produced by cells cultured for 24 hours with 0.2, 0.02, and 0.002 mg/ml crude E. arvense protein, respectively, showing no marked difference, but it was enhanced by 1.4, 1.4, and 1.3 times compared to the production (646.7 pg/ml) in the control. In cells treated in the presence of 10 μg/ml ConA, 0.2 mg/ml crude E. arvense protein induced no significant differ-

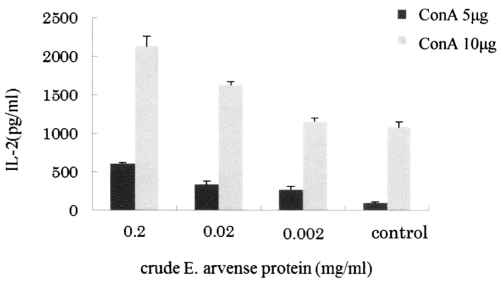

Fig. 2 IL-2 production enhancement by crude E. arvense protein (48 Hr Treatment)

Table 2 IL-2 production enhancement by crude E. arvense protein (48 Hr Treatment)

ConA	crude E. arvense protein (mg/ml)	IL-2 (pg/ml)	SE	p-value (t-test)
5 μg/ml	0.2	602.1	19.5	0.2×10-5
	0.02	329.5	44.0	0.007
	0.002	264.5	41.7	0.017
	Control	94.4	11.6	
10 μg/ml	0.2	2130.9	125.4	0.002
	0.02	1629.5	38.0	0.003
	0.002	1157.0	39.3	0.395 (NS)
	Control	1845.0	73.1	

Mean±SE (N=3)

ence, but the productions by cells treated at 0.02 and 0.002 mg/ml were 893.3 and 812.0 pg/ml, respectively, showing 1.4 and 1.3 times greater productions, respectively, compared to the production (616.0 pg/ml) in the control (Table 3, Fig. 3).

In cells cultured for 48 hours in the presence of 5 μg/ml ConA, no enhancement of production by crude E. arvense protein was noted, showing no significant difference at any concentration. In the presence of 10 μg/ml ConA, a significant difference was noted only in cells treated with 0.02 mg/ml crude E. arvense protein, and the production was 844.0 pg/ml, showing 1.4 times higher production than that (602 pg/m) in the control (Table 4, Fig. 4).

4. Discussion

We previously reported that when the cell proliferation-inhibitory effect of crude protein extract of Equisetum arvense LINNE (E. arvense) was investi-

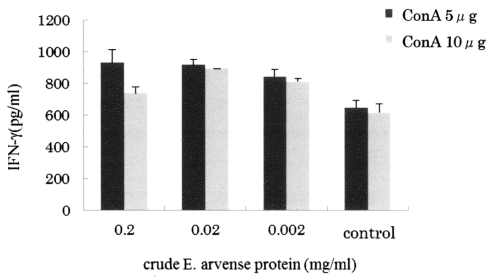

Fig. 3 IFN-γ production enhancement by crude E. arvense protein (24 Hr Treatment)

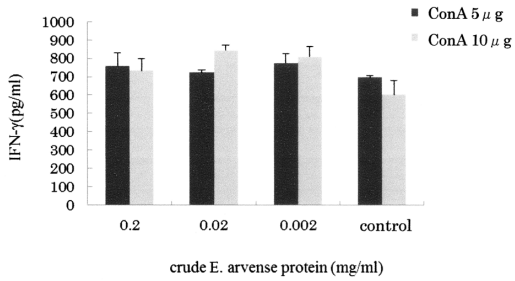

Fig. 4 IFN-γ production enhancement by crude E. arvense protein (48 Hr Treatment)

Table 3 IFN-γ production enhancement by crude E. arvense protein (24 Hr Treatment)

ConA	crude E. arvense protein (mg/ml)	IFN-γ (pg/ml)	SE	p-value (t-test)
5 μg/ml	0.2	929.3	81.0	0.039
	0.02	915.3	34.7	0.009
	0.002	842.7	46.8	0.040
	Control	646.7	45.2	
10 μg/ml	0.2	738.0	37.5	0.429 (NS)
	0.02	893.3	0.7	0.006
	0.002	812.0	17.9	0.024
	Control	616.0	52.6	

Mean±SE (N=3)

Table 4 IFN-γ production enhancement by crude E. arvense protein (48 Hr Treatment)

ConA	crude E. arvense protein (mg/ml)	IFN-γ (pg/ml)	SE	p-value (t-test)
5 μg/ml	0.2	758.7	71.3	0.429 (NS)
	0.02	721.3	16.6	0.258 (NS)
	0.002	774.0	52.5	0.216 (NS)
	Control	695.3	10.7	
10 μg/ml	0.2	734.7	65.3	0.259 (NS)
	0.02	844.0	25.7	0.041
	0.002	809.3	55.4	0.093 (NS)
	Control	602.0	76.9	

Mean±SE (N=3)

gated using cell cultures (L1210, HMV-I, and 3T3 cells), the highest inhibitory effect was noted in the group treated with crude E. arvense protein at the highest concentration. We also previously performed an in vivo experiment of the life-prolonging effect in mice using L-1210 and B16F1 cells, and observed that the highest life-prolonging effect was noted in the groups treated with crude E. arvense protein at the lowest concentration in experiments using either cell type.

These findings suggested that the inhibition observed in vitro was due to the cytotoxicity and cell proliferation-inhibitory effect of crude E. arvense protein. However, in the in vivo reaction, crude E. arvense protein may have activated biological (physiological) activities, such as immunity, and exhibited a strong life-prolonging effect, in addition to cytotoxicity and inhibition of cell proliferation. In this study, we measured cytokines produced by Th1 cells to investigate immune activation as an immune response. The most marked enhancement of IL-2 production was noted when cells were cultured for 24 hours with 5 μ g/ml ConA and 0.2 mg/ml crude E. arvense protein, and the production was 1,434.5 pg/ml, showing enhancement of production by 1.7 times compared to that in the control. In 48-hour cultures, 2,130.9 pg/ml of IL-2 was produced by cells cultured with 0.2 mg/ml crude E. arvense protein in the presence of 10 μ g/ml ConA, and the production was enhanced by 1.9 times compared to that in the control. Regarding the IFN-γ production-enhancing effect, 929.3 pg/ml of IFN-γ was produced by cells cultured for 24 hours with 5 μ g/ml ConA and 0.2 mg/ml crude E. arvense protein, showing enhancement by 1.4 times compared to that in the control. These findings suggested that crude E. arvense protein acts on Th1 cells and enhances immune responses. Although this study investigated the reactions involving Th1 cells, since Th1 and Th2 cells cross-regulate each other[8], it is possible that the increased IFN-g and IL-2 productions shifted the Th1/Th2 balance toward the Th1 cell side[9, 10]. It was also suggested that cellular immunity is enhanced. We are planning to investigate reactions of immunocytes, such as macrophages, cytotoxic T cells, and NK cells, and measure cytokines produced by Th2 cells to elucidate the action mechanism of the influence of crude E. arvense protein on the immune response system.

5. Conclusion

It was confirmed that crude protein extract of Equisetum arvense LINNE enhanced cytokine production by Th1 cells (IL-2 and IFN-γ).

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