Effect of the Water-Soluble and Non-dialyzable Fraction Isolated from Senso (Chan Su) on Lymphocyte Proliferation and Natural Killer Activity in C3H Mice

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We found lymphocyte proliferating substances in the water-soluble and non-dialyzable fraction prepared from the crude drug Senso (Chan Su). The effect of this fraction was increased by affinity chromatography using the concanavalin A–agarose. By analyzing the fraction using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and lectin blotting, we estimated that one of the active substances of this fraction is a glycoprotein that has about 13 kDa of molecular weight and α-mannose within the molecule. The purified fraction increased the IL-2 and the IL-12 level in the supernatant of spleen cell culture, and increased the natural killer activity of spleen lymphocytes in C3H/HeN mice. These results show that Senso contains immunopotentiating substances that may serve as an immunomodulator in an organism.

**Key words** Senso; Toad venom; mitogenic activity; natural killer activity; interleukin-12

Senso (Chan Su, Toad venom) is the dried venom of toad (*Bufo bufo gargarizans* CANTOR or *Bufo melanostictus* SCHNEIDER) and has been used as an Oriental drug.¹ The pharmacological effects of this drug have been reported to be cardiotonic, an excitatory for respiration, a diuretic, anti-inflammatory and anti-tumor development.¹,² Many of these effects are attributed to bufadienolides, one of the active compounds in Senso.¹,² Recently, new biological activities of bufadienolides, such as a differentiating effect on tumor cells of bufalin,³ an inhibitory effect of several bufadienolides on tumor cell growth⁴ and an inhibitory effect on the IL-6 activity were reported.⁵,²¹

On the other hand, although Senso contains a considerable amount of water-soluble substances, few reports about their biological activities except for biological amines (epinephrine and tryptamine derivatives) have been confirmed.¹,⁶,⁷ In a family of toad skin and its secretions, there are several peptides and proteins that have antimicrobial activities.⁵,⁹ So we tried isolating any substances that have biological activity in the high molecular and water-soluble fraction of Senso, and found some using the lymphocyte proliferation study that have been used as a screening test for investigating immunological effect of testing substances²⁰

**MATERIALS AND METHODS**

**Materials** Senso (JP, made in China) was obtained from Matsura Yakugyo (Aichi, Japan). Concanavalin A–agarose (Wako Pure Chemistry, Osaka, Japan), concanavalin A–alkaline phosphatase conjugate (Molecular Probes, OR, U.S.A.), protease (PRONASE, Calbiochem, CA, U.S.A.), α-mannosidase (ICN Biochemicals, OH, U.S.A.), cell proliferation assay kit (Cell titer 96 Aqueous, Promega, WI, U.S.A.), cell cytotoxicity assay kit (Cytotox 96, Promega) and ELISA kit for cytokines (for IL-2, IL-4 and IL-12, Biosource International, CA, U.S.A.) were used. All chemicals used were analytical grade.

**Animals** Male C3H/HeN and C3H/HeJ mice (4–6 weeks old) were obtained from SLC Japan (Shizuoka, Japan), and kept in a special room at 20–22°C, RH 45–58%, 12 h light–dark cycle at least 6 d before use.

**Cells** YAC-1 cells were purchased from Dainihon Pharmaceutical (Osaka) and cultured in RPMI 1640 medium (Nihonseiyaku, Tokyo) containing 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin G and 100 μg/ml streptomycin. These cells were used as the target cells for natural killer assay.

**Fractionation of Senso** Senso (4.44 g) was washed 3 times with 180 ml (60 ml each) of methanol and the residual methanol evaporated under an N₂ stream. To the residue, 60 ml of diluted PBS (10 mM phosphate buffered saline, pH 7.4, containing 4 mM 2-mercaptoethanol and 0.1 mM phenylmethanesulfonyl fluoride) was added and extracted using an ultrasonic generator (B-20, Branson, CT, U.S.A.) for 1 h. This liquid was centrifuged (20000×g, 25 min, 4°C), and the supernatant was dialyzed against water for 3 d using a cellophane tube (Sanko Pure Chemistry, Tokyo). Contents in the tube were lyophilized and the fraction 1 was obtained (0.546 g). Fraction 2A and fraction 2B were obtained from fraction 1 using the concanavalin A–agarose as follows: 50 mg of fraction 1 dissolved in diluted PBS (1 ml) containing 0.1 mM MnCl₂ and 0.1 mM CaCl₂ was added to the concanavalin A–agarose (8 ml) and reacted at 4°C for 18 h. Then the resin was washed using the same buffer, and substances which have affinity with the concanavalin A were eluted using 10 ml of 0.2 M α-methyl-β-mannoside containing diluted PBS. The washed (effluent) and the eluted liquid were dialyzed against water separately, and the fractions 2A (45.1 mg) and 2B (4.9 mg) were obtained by the lyophilization. To test the contamination of lipopolysaccharides in fraction 2B, a reagent kit (Limrus-ESII test Wako, Wako Pure Chemicals) was used. Content of protein and saccharides in fractions was determined by the Bradford method¹⁰ and the phenol–sulfuric acid method¹¹ using bovine serum albumin and glucose as standard, respectively. For the estimation of active constituent in the fraction, fraction 2B was treated with protease (PRONASE) in PBS (pH 7.4) containing 1 mM CaCl₂ or α-mannosidase in 0.5 M citrate buffer (pH 4.5) at 37°C for 48 h. The enzyme-treated fractions were used for the lymphocyte proliferation assay and for analysis using the sodium dodecyl

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Lymphocyte Proliferation Spleen cells of C3H/HeN mice (C3H/HeJ mice were also used to estimate the probability of the contamination of lipopolysaccharides in the fractions) were obtained by squeezing the organ in the culture medium (RPMI1640 containing 5% FBS, 100 IU of penicillin and 100 µg/ml of streptomycin) using frosted slide glasses aseptically. After centrifugation (500×g, 5 min, 4 °C), erythrocytes in the pellet were removed by washing the pellet with a solution containing 0.829% NH₄Cl, 0.1% KHCO₃ and 0.367% EDTA–2Na. After the centrifugation, the pellet was washed with the culture medium 2 times. The washed spleen cells were suspended in the culture medium and the cell count measured using a counter (MEK-4300, Nihon Kohden, Tokyo). The spleen cell count was adjusted to 2×10⁶/20 µl using the culture medium and used as the spleen cell suspension. Into each well of the 96 well microplate (Becton Dickinson, NJ, U.S.A.), 10 µl of test drug (dissolved in aseptic water or sterilized PBS), 5 µl of 1 mM 2-mercaptoethanol, 45 µl of culture medium and 40 µl of spleen cell suspension were poured and cultured at 5% CO₂ and 37 °C for 48 h. The liquid of the cell proliferation assay kit (20 µl) was added to each well of the plate at 4 h before the end of culture, and the absorbance of each well at 492 nm was measured using the microplate reader (MTP-300, Corona, Ibaraki). To estimate the probability of the contamination of lipopolysaccharides in the fractions, the test drugs were also treated by a solution of polymyxin B (50 µg/ml, ICN Biochemicals). Augmentation of the lymphocyte proliferation (stimulation index) was calculated by the following formula:

\[
\text{stimulation index (％)} = \frac{OD_{492\text{nm}} \text{of the lymphocyte culture with test drugs} - OD_{492\text{nm}} \text{of the culture medium with test drugs} - OD_{492\text{nm}} \text{of the culture medium with vehicle}}{OD_{492\text{nm}} \text{of the culture medium with vehicle}}
\]

SDS–PAGE Analytical polyacrylamide slab gel electrophoresis in the presence of SDS (2%) was carried out on 15 W/4% acrylamide gel under a reduced condition using mini-gel electrophoresis apparatus (Sanplatec, Osaka) as reported. Lectin Blotting Samples separated by SDS–PAGE were transferred to polyvinylidene difluoride membrane (0.45 µm, Osmonics, MA, U.S.A.) using a wet transferring apparatus (Sanplatec, Osaka, 200 mA, 4 h, 14 °C) in transferring buffer (14.4 g glycine, 3.03 g Tris and 1 g SDS were dissolved in 1000 ml of 20% methanol). After washing with 20 mM Tris buffered saline (TBS), the membrane was soaked in the 1% bovine serum albumin containing TBS for 16 h at 4 °C for blocking. Then the membrane was washed with 0.05% Tween 20 containing TBS (TTBS), and reacted with 5 µg/ml of concanavalin A–alkaline phosphatase conjugate (Molecular Probes) for 1 h at 4 °C. After washing with TTBS and TBS, the membrane was soaked in the substrate solution (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, Bio-Rad, CA, U.S.A.).

Cytokine Content 4×10⁶/well of spleen cells of C3H/HeN mice were cultured with fraction 2B (10 µg/ml or 100 µg/ml) or concanavalin A (0.375 µg/ml) under the same condition as the lymphocyte proliferation study. The culture medium was taken at 48 h of culture and centrifuged. Levels of the IL-2, IL-4 and IL-12 in the supernatant were assessed using ELISA.

NK Assay Fraction 2B was dissolved in the aseptic saline and injected intraperitoneally to the C3H/HeN mice 1 mg/kg/d for 3 d. On the 4th day, mice were sacrificed, the spleen removed aseptically and spleen cells obtained by the same procedure as for the lymphocyte proliferation assay. Spleen cells were treated with a nylon column (0.5 g, Wako Pure Chemical) for 1 h at 37 °C and 5% CO₂. The cells passing through the column were used as the effector cells; YAC-1 cells were used as the target cells. The effector and target cells were poured into test tubes and centrifuged at 500×g for 5 min. Then the tubes were incubated in a CO₂ incubator for 4 h at 37 °C and 5% CO₂. The enzyme (lactose dehydrogenase) activity released by specific lysis of the target cells in the supernatant was measured using a kit (Cytotox 96, Promega). RPMI1640 medium containing 10% FBS, 100 IU of penicillin, 100 µg/ml of streptomycin and 0.05 m 2-mercaptoethanol was used as the culture medium for the NK assay. The NK activity of effector cells was calculated by the following formula:

\[
cytotoxicity(\%) = 100 \times \frac{(A-B)-(C-D)}{(C-D)/(E-F)}
\]

\(A\): experimental release

\(B\): spontaneous release of effector cells

\(C\): spontaneous release of target cells

\(D\): blank for spontaneous release of target cells

\(E\): maximum release of target cells (by Triton X-100 treatment)

\(F\): blank for maximum release

Statistical Analysis Statistical differences in the cytokine concentration in the supernatant of lymphocyte culture were evaluated using the one-way analysis of variance (ANOVA) followed by the Tukey or the Bonferroni test. Students’ t-test was used to determine the NK activity.

RESULTS AND DISCUSSION

Augmentation of lymphocyte proliferation was confirmed dose-dependently by the addition of the water-soluble and non-dialyzable fractions of Senso (Fig. 1). The effect of the fraction through the concanavalin A–agarose (fraction 2B) was more potent than fraction 1. As concanavalin A has affinity with α-mannose or α-glucose, the active substance contained in this fraction may contain a polysaccharide or glycoprotein, which has α-mannose or α-glucose within the molecule.

These effects on the lymphocyte were similar to the effect of lipopolysaccharides. We then tested the contamination of lipopolysaccharides in the fraction and found their content in fraction 2B was under 0.0004% by the Limtest. The augmentation of lymphocyte proliferating effect of this fraction was confirmed under the polymyxin B treatment (which has binding activity to protein A of lipopolysaccharides) and on the spleen cells of C3H/HeJ mice. These results show that the effects on the lymphocyte from fraction 2B isolated from Senso were not originated from the contaminant of lipopolysaccharides (data not shown).

For the estimation of active substances in the fraction of Senso, fraction 2B was separated using SDS–PAGE and divided into 8 fractions to test their effects on the spleen cells.
Fractions with molecular weight over 118—214 kDa and 6.8—20.8 kDa showed the augmentation of proliferating activity on the spleen cells (Fig. 2). Comparing each fraction with its protein content, the fraction of 6.8—20.8 kDa was about 54.6% of fraction 2B. As the lymphocyte proliferating effect of the fraction was increased by purification using concanavalin A–agarose affinity chromatography, the lectin blotting using concanavalin A is believed useful to estimate the active substances in the fraction. Concanavalin A binding substances were recognized at the molecular weight of about 13 kDa and about 18 kDa by SDS–PAGE and the lectin blotting of fraction 2B (Fig. 3A). When fraction 2B was digested with the α-mannosidase or the protease, the augmentative activity of this fraction on the spleen lymphocytes was weakened (Fig. 3B) and the band of about 13 kDa and about 18 kDa shown by the lectin blotting became thinner (Fig. 3C). The augmentative activity on the lymphocyte proliferation was also shown the fraction that contains 13 kDa (Fig. 3D). Combining the above results, we could estimate that at least one active substance isolated from Senso is a glycoprotein that has about 13 kDa of molecular weight and has d-mannose within its molecule.

Intraperitoneal injection of fraction 2B significantly increased the NK activity of spleen cells in C3H/HeN mice (Fig. 4). Fraction 2B also significantly increased IL-2 and IL-12 content in the supernatant of spleen cell culture in vitro (Table 1), and these cytokines are known to increase NK activity.15) Although further investigation (effect of this fraction by another route of administration) is needed, these results show the probability that fraction 2B has an immunopotentiating effect both in vitro and in vivo.

Fraction 2B was also increased the IL-2 and IL-12 level and decreased the IL-4 level in the supernatant of spleen cell culture at 100 µg/ml (Table 1). Compared with the effect of concanavalin A, the increasing effect of this fraction on the IL-12 level was remarkable. IL-12 is known as an important cytokine for innate resistance and adaptive immunity for vertebrates, and inclines the helper T-cell balance (Th1/Th2 balance) toward Th1 by inducing the interferon-γ production from NK cells and T-cells.16) Leaning of the Th1/Th2 balance toward Th2 is recognized in subjects who have some allergic disorders17) or are bearing some tumors.18) The effect of fraction 2B on the cytokine content may suggest that this fraction serves to prevent or treat these disorders by reinforcing the Th1 response.19)

In conclusion, we found immunopotentiating substances in the water-soluble and non-dialyzable fraction of the crude drug Senso, and at least one of the active substances is glycoprotein that has about 13 kDa of molecular weight and has d-mannose within the molecule. Investigation into other biological effects and structures of these substances is in progress.
Fig. 3. Lectin Blotting and Effect on the Proliferation of Spleen Lymphocyte in C3H/HeN Mice of Fraction 2B and Its Digested Substances

(A) Lectin blotting of fraction 2B. Fraction 2B (25 μg) was separated using SDS-PAGE and blotted to the PVDF membrane using the electro-blotting apparatus. The blotted membrane was treated with concanavalin A-alkali phosphatase conjugate, and molecules that have affinity with the lectin were shown by a color change on the membrane when it soaked into the substrate liquid.

(B) Effects of fraction 2B and its digested substances on the proliferation of spleen lymphocyte in C3H/HeN mice. The data are expressed as the mean±S.E. (n=3).

(C) Lectin blotting of fraction 2B and its digested substances. Fraction 2B (25 μg) and its digested substances (equivalent to the non-digested fraction 2B) using α-mannosidase or protease (PRONASE) were separated using SDS–PAGE and blotted on the PVDF membrane. The blotted membrane was treated as Fig. 3A.

(D) Effects of fractions of fraction 2B on the spleen lymphocyte in C3H/HeN mice. Fraction 2B (50 μg) was separated using SDS–PAGE, divided into 2 fractions by molecular weight and the materials in each gel were extracted with saline and salts and detergent removed by dialysis. The volume of each fraction was adjusted to 1 ml using sterilized PBS. The data are expressed as the mean of 2 samples.

Table 1. Effects of Fraction 2B and Concanavalin A on the Level of Cytokines in the Supernatant of Spleen Cell Culture Prepared from C3H/HeN Mice

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (μg/ml)</th>
<th>Contents (pg/ml)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IL-2</td>
<td>IL-4</td>
<td>IL-12</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>4.7±0.4</td>
<td>8.2±0.1</td>
<td>2.8±0.8</td>
</tr>
<tr>
<td>Fraction 2B</td>
<td>10</td>
<td>6.7±0.9</td>
<td>8.0±0.5</td>
<td>4.0±0.7</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>31.1±1.1*</td>
<td>2.6±1.0*</td>
<td>64.8±6.9*</td>
</tr>
<tr>
<td>Concanavalin A</td>
<td>0.375</td>
<td>32.0±2.8*</td>
<td>7.8±0.2*</td>
<td>7.7±2.1*</td>
</tr>
</tbody>
</table>

The data are expressed as the mean±S.E. (n=3—6). *p<0.05 as compared with the multiple comparison test. Spleen cells were seeded 4×10⁵/well with 0.1 ml of RPMI 1640 medium containing 5% fetal bovine serum, 50 μM 2-mercaptoethanol and drug, and cultured in 5% CO₂ at 37 °C for 48 h. Cytokines in the medium supernatant were measured using ELISA.

Fig. 4. Effect of Fraction 2B on the Natural Killer Activity of Spleen Lymphocyte in C3H/HeN Mice

Mice were treated by intraperitoneal injection of fraction 2B or vehicle (as control) once a day for 3 d, and sacrificed to remove the spleen on the fourth day. Effector cells were purified by the nylon column treatment, and reacted with the target cells (YAC-1) by their ratio of 50 : 1 (effector : target) for 4 h. *p<0.05 as compared with the control group using Students’ t-test.
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