Isolation and Characterization of a Novel Immunomodulating Fraction from Soybeans

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A novel immunomodulating fraction, SP-MAF1, was isolated from soybeans as a polysaccharide-protein complex by hot water extraction followed by acid treatment, ethanol treatment, and chromatography on a DEAE-Sepharose CL-6B column, and was characterized in some biological activities.

SP-MAF1 increased glycosylation and IL-1 production by macrophages, but did not affect TNF production. SP-MAF1 also increased splenocyte proliferation that was induced by LPS and ConA, and ConA-induced production of IL-2 by splenocytes. In an in vivo test, the mean survival times of SP-MAF1-treated mice bearing FM3A were significantly longer than those of non-treated group. Since SP-MAF1 affected macrophages and splenocytes but not tumor cell growth, this effect may be mediated by the host immune system. Furthermore SP-MAF1 had a capacity to suppress the TNBS-induced DTH reaction. These results suggested that SP-MAF1 could either enhance or suppress immune functions.

Since macrophages act as regulatory and effector cells in the immune system, the enhancement of macrophage function is expected to be applicable for therapy of microbial infections and cancer. There are many indicators of macrophage stimulating activity. Until now, it was reported that many substances activated macrophages to various stages.

It has been reported that some peptides obtained by enzymatic digestion from food proteins and some components obtained by hot water extraction from food materials activate macrophages. Jolles et al. reported that the peptides from milk casein stimulated phagocytosis in vitro and enhanced resistance to infection in vivo.1,2) Suetsuna et al. reported that peptides from soybeans stimulated phagocytosis, increased natural killer cell activity, and increased proliferative responses of splenocytes to mitogens.3) Furthermore Yoshikawa et al. showed that the peptides from soybeans stimulated phagocytosis and enhanced the production of Tumor Necrosis Factor in vivo.4) Miwa et al. also reported that water extracts from various foods including soybeans enhanced nitrite formation by macrophage.5) These reports suggest that several foods with macrophage-stimulating activities, especially soybeans, might be important in the defense system. However the previous studies were all almost concerned with proteinous substances; there has never been a report about polysaccharide substances from soybeans with macrophage-stimulating activity.

In the course of screening for macrophage-stimulating substances among food extracts, we detected that an extract of soybeans increased macrophage glycosylation, which is an indicator of activated macrophages, and isolated a polysaccharide-protein complex with macrophage-stimulating activities. In this paper, we report a novel immunomodulating fraction from soybeans and also discuss its biological activities both in vitro and in vivo on the immune system.

Materials and Methods

Mice. Special pathogen-free female C3H/HeN and CDF, mice were obtained from Charles River Japan Co., Ltd., Tokyo.

Chemicals. Except as noted otherwise, all chemicals were from Wako Pure Chemical Industries, Osaka.

Isolation of the active fraction from soybeans. One kg of defatted soy flour (Nissin Oil Mills, Ltd., Tokyo) was extracted with 10 liters of distilled water at 100°C for 30 min. After the extraction was done by stirring at room temperature, the extract was acidified to pH 4.5 with 2 N HCl. The precipitate was collected, dissolved in distilled water, and mixed with four volumes of ethanol. The precipitate formed was removed by centrifugation. After the supernatant solution was concentrated by a rotary evaporator to about 1 liter, it was acidified again to pH 2.0 with 2 N HCl. The precipitate was collected by centrifugation and dissolved in 10mL N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.0). Then the solution was put onto a DEAE-Sepharose CL-6B column (Pharmacia LKB, Sweden) previously equilibrated with the HEPES buffer. After the column was washed with 0.2m KCl buffer, the elution was done by 0.6m KCl buffer. The active fractions assayed by glycosylation of macrophage were pooled, dialyzed against distilled water, and lyophilized. This fraction isolated from soybeans was designated SP-MAF1.

Macrophages and splenocytes. To induce inflammatory macrophages in the peritoneal cavity, 2ml of 3.85% thiglycollate medium (BBL, Cockeysville, MD, U.S.A.) was injected intraperitoneally (i.p.) into C3H/HeN mice 4 days before collection. Peritoneal exudate cells (PEC) were collected with Eagle's minimum essential medium (MEM, Nissui Seiyaku, Tokyo). The cells were washed three times and suspended in RPMI-1640 medium (Gibco Laboratories, New York, NY, U.S.A.) supplemented with 10% fetal calf serum (Gibco Laboratories), 20μM 2-mercaptoethanol, 50μg/ml kanamycin, and 8μg/ml tylon tartrate (Gibco Laboratories). PEC were used as macrophages throughout this work.

To obtain splenocytes, female C3H/HeN mice were killed and the spleen was teased with a plastic syringe in a Petri dish containing 5ml of MEM. The product was gently strained through a 100-mesh screen to remove clumps and prepared as a single-cell suspension. These cells were washed three times with MEM and resuspended in RPMI-1640 medium.

Cytokines. Standard cytokines, recombinant Interleukin-1 β (rIL-1β), recombinant Interleukin-2 (rIL-2), and recombinant Tumor Necrosis

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Abbreviations: HEPES, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid; i.p., intraperitoneal; PEC, peritoneal exudate cells; MEM, minimum essential medium; IL-1, Interleukin-1; IL-2, Interleukin-2; TNF, Tumor Necrosis Factor; ConA, concanavaline A; LPS, lipopolysaccharide; TNBS, 2,4,6-trinitrobenzene sulfonate; DTH, delayed-type hypersensitivity.
Factor α (α-TNFα) were obtained from R&D systems, Inc., Minneapolis, MN, U.S.A. Mitogens. Concanavalin A (ConA) and lipopolysaccharide (LPS, Salmonella typhosa, Difco Laboratories, Detroit, MI, U.S.A.) were used as mitogens through this work.

Assessment of glycolysis of macrophages. PEC (3 × 10^7 cells/ml) were incubated in RPMI-1640 medium in 96-well microculture plates (Sumitomo Bakelite Co., Ltd., Tokyo) at 37°C for 48 h. The concentration of glucose in the supernatant at the end of the culture was measured with an enzymatic method for blood analysis (Chugai Pharmaceutical Co., Tokyo). The glucose concentration was computed using the following formula:

\[
\text{Glucose consumption (\%)} = \frac{100 \times (1 - \text{concentration of glucose at the end of culture})}{(\text{initial concentration of glucose in medium})}
\]

Measurement of protein and total sugar content. The protein content of SP-MAFI was measured by the method of Lowry et al. using bovine serum albumin as the standard protein. Total sugar content was measured by the phenol–sulfuric acid method.

Production of IL-1 and TNF. PEC (2 × 10^6 cells/ml) were cultured with LPS or SP-MAFI in 2 ml of RPMI-1640 medium in 12-well microculture plates (Costar, Cambridge, MA, U.S.A.). After culture for 24 h at 37°C, the supernatants were collected and centrifuged to remove the cells. They were stored at −20°C until used.

The IL-1 activity in the supernatants was measured by proliferative response of a T helper cell line, D10.G4.1. The IL-1-containing supernatant was diluted serially and incubated with D10.G4.1 cells (5 × 10^4 cells/ml) in the presence of ConA (2 μg/ml) for 44 h. [3H]-Thymidine (3.7 × 10^8 Bq/well) was added during the final 4 h incubation and the incorporated radioactivity was counted by a liquid scintillation counter. One unit/ml corresponded to the concentration giving a half maximum incorporation in the D10.G4.1 assay.

The TNF activity in the supernatants was measured by the cytolytic response of a murine fibrosarcoma cell line, L929. The TNF cytotoxicity was assayed in the presence of actinomycin D. The TNF-containing supernatant was diluted serially and incubated with L929 cells (4 × 10^5 cells/ml) and actinomycin D at a final concentration of 1 μg/ml. After incubating for 18 h at 37°C, the remaining cells were fixed and stained with crystal violet, and the dye was measured as described.

Production of IL-2. Splenocytes (2 × 10^6 cells/ml) in 2 ml of RPMI-1640 medium in 12-well microculture plates were cultured in the presence of ConA, SP-MAFI, or both. After culture for 24 h at 37°C, the supernatants were collected and centrifuged to remove the cells. They were stored at −20°C until used.

The IL-2-containing supernatant was diluted serially and cultured for 24 h with CTL-L-2 cells (5 × 10^4 cells/ml), which is a killer T cell line and needs IL-2 for its growth. The [3H]-thymidine incorporative assay was done as described for the IL-1 assay procedure. Units of IL-2 activity were measured by reports. One unit/ml corresponded to the concentration giving a half maximum incorporation in the CTL-L-2 assay.

Antitumor activity against FMA3 cells in vivo. Mouse mammary tumor, FMA3 cells, were maintained in suspension culture in RPMI-1640 medium. FMA3 cells (1 × 10^6 cells) in 0.1 ml of PBS (phosphate buffered saline) were injected i.p. on day zero into six-week-old female C3H/HeN mice, and SP-MAFI or Lentinan (Yamanouchi Pharmaceutical Co., Ltd., Tokyo) in PBS was administered on days −4, −1, 1, 2, and 3 by i.p. injection. Each test group had 6 mice and values of T/C% to evaluate the antitumor effects were calculated from the mean survival time of treated mice compared to control mice.

Delayed-type hypersensitivity (DTH) assay in vivo. Two hundred μl of 10% 2,4,6-trinitrobenzene sulfonate (TNBS) in PBS was injected subcutaneously on day zero on the abdomen of eight-week-old female CDF1 mice. Seven days later 40 μl of the TNBS solution was subcutaneously injected into the left hind footpad. The increase in footpad thickness was measured 24 h after elicitation with a dial-caliper (Ozaki, Mfg Co., Ltd., Japan). The right footpad was also measured as a control. SP-MAFI in PBS was administered on days 1, 3, 5, and 7 by i.p. injection. Each group had 3 or 4 mice.

Results and Discussion

Isolation and some properties of SP-MAFI

Since an increase of the glycolysis reaction of macrophages is one of the indicators of activation of macrophages, we used this for the indicator in each fractionation step of SP-MAFI from soybeans. We obtained about 1 g of white powder as the active fraction from 1 kg of defatted soy flour. SP-MAFI consisted of 53.3% polysaccharide and 15.6% protein by weight. The constitutive sugars of the polysaccharide portion were mainly threose (approximately 80%, w/w).

In previous studies, some substances obtained from soybeans enhanced macrophage functions. The peptides obtained from soybeans by enzymatic digestion stimulated phosphocytosis of macrophages. Miwa et al. reported that the water extracts from soybeans enhanced nitrate formation and IL-1 production, and were identified as β-amylase and Kunitz-type trypsin inhibitor. All these reports pertained to the proteinous substances with macrophage stimulating activity. On the other hand, the major part of SP-MAFI was polysaccharide, and molecular mass was above 100 kDa by ultracentrifugation (data not shown). In addition, soybean trypsin inhibitor purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) had no effect on our assay system. Hence SP-MAFI may be different from substances previously reported, and this is the first report of the enhancement of some macrophage functions by polysaccharide–protein complex obtained from soybeans.

Biological activity of SP-MAFI in vitro

As shown in Table I, glycolysis of PEC was stimulated by addition of SP-MAFI. This effect depended on the concentration of SP-MAFI, and was observed at concentrations above 20 μg/ml. Since the production of monokines, such as IL-1 and TNF, is thought to be an important function of macrophages, we measured the monokine activities in the supernatant of PEC cultured with SP-MAFI. When PEC were cultured with LPS or SP-MAFI for 24 h, the IL-1 activity in the supernatant was increased significantly at concentrations above 20 μg/ml. These concentrations were almost comparable to those on glycolysis of PEC (Table I). The TNF activity was

<table>
<thead>
<tr>
<th>Dose (μg/ml)</th>
<th>Glucose consumption (%)</th>
<th>IL-1 (units/ml)</th>
<th>TNF (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20.1 ± 3.3*</td>
<td>20.4</td>
<td>N.D.*</td>
</tr>
<tr>
<td>LPS</td>
<td>44.4 ± 0.8* (220.9)#</td>
<td>121.5</td>
<td>6.5</td>
</tr>
<tr>
<td>SP-MAFI</td>
<td>4</td>
<td>28.3 ± 1.7 (140.8)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>36.1 ± 4.1 (179.6)</td>
<td>40.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>44.3 ± 2.0 (220.4)</td>
<td>72.9</td>
</tr>
</tbody>
</table>

* Mean ± SD of triplicate cultures.
# Not detected.
* Statistically significant (Student’s t-test, p < 0.05).
# Numbers in parentheses, percentage of control culture.
* Not determined.
Fig. 2. Stimulation of Proliferative Responses in SP-MAF1-pretreated and Non-treated Mouse Splenocytes by LPS and ConA.

The mouse splenocytes were treated with SP-MAF1 for 4h at 37°C in RPMI-1640 medium. After 3 washes, the proliferative responses by LPS (A) and ConA (B) were tested using the same method as described in Fig. 2. Results are the mean of triplicate assays. ○—○, non-treatment; □—□, SP-MAF1 50 μg/ml. Δ—Δ, SP-MAF1 100 μg/ml.

Table II. Effects of SP-MAF1 on IL-2 Production

<table>
<thead>
<tr>
<th>Dose (μg/ml)</th>
<th>IL-2 (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>N.D.*</td>
</tr>
<tr>
<td>ConA</td>
<td>6.8</td>
</tr>
<tr>
<td>ConA + SP-MAF1</td>
<td>9.3</td>
</tr>
<tr>
<td>SP-MAF1</td>
<td>12.0</td>
</tr>
<tr>
<td>100</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* Not detected.

LPS at concentrations up to 50 μg/ml (Fig. 2A). Morrison et al. showed that LPS activated B cells directly. Regarding the concentration of pretreatment by SP-MAF1, the LPS response was more sensitive than the ConA response. These results suggested that SP-MAF1 might affect B cells directly, but T cells indirectly. The activation of T cells might be caused by macrophage stimulation through IL-1 production by SP-MAF1, because IL-1 is a factor that activates T cells.

Biological activity of SP-MAF1 in vivo

SP-MAF1 increased several functions of macrophages and splenocytes in vivo. To estimate activities of SP-MAF1 in vivo, we examined its effects on the life span of C3H/HeN mice bearing an ascites-type tumor, FM3A. The mean survival times of SP-MAF1 treated mice were longer than those of the non-treated group. The mean T(3%) values (Table III) were 110.8, 136.4, and 121.4% at the i.p. doses of 10, 20, and 100 mg/kg, respectively. The increase of the mean survival times at the optimum dose (20 mg/kg) was significant, and was almost comparable to that of Lentinax, which is a polysaccharide with antitumor activity. SP-MAF1 showed no effect on cell growth of FM3A and other tumors at concentrations ranging from 6.25 to 100 μg/ml in an in vitro test (Fig. 3).

The mechanism of the antitumor effect of SP-MAF1 is not known. We showed that SP-MAF1 increased some functions of macrophages and splenocytes in vitro, but did...
macrophages (Table I). LPS is a polyclonal mitogen for B cells. In comparison with LPS, SP-MAFI has no direct effects on the proliferative response of B cells, but increased B cell proliferation induced by LPS (Fig. 2A). Since LPS can either enhance or suppress the immune response, it is possible that SP-MAFI suppressed the DTH reaction through a similar mechanism to that by LPS in vivo.

Soybean is a typical and useful food for man. In this report, we assessed a capacity of SP-MAFI obtained from soybeans to modulate the immune response in vitro and in vivo. It is noteworthy that soybean components has many activities in the immunological cell system. Recent studies, in which many food components including soybeans have immunomodulating activities, permit some speculations as to the physiological functions of food components in addition to nutrition. Further investigation will be needed for characterization of the action of SP-MAFI and application of it to therapy or analysis of immune systems.

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References

Table IV. Effects of SP-MAFI on DTH Reaction

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Increase in footpad thickness (cm × 10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>9.4 ± 0.7</td>
</tr>
<tr>
<td>SP-MAFI</td>
<td>2.6 ± 1.1*</td>
</tr>
<tr>
<td>500</td>
<td>5.0 ± 3.5</td>
</tr>
</tbody>
</table>

* Statistically significant (Student’s t-test p < 0.05).

Table III. Effects on the Life-span of Mice Bearing Ascites-type Tumor

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>MST (days)*</th>
<th>T/C (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>PBS</td>
<td>17.8 ± 3.8</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>19.7 ± 0.0</td>
<td>110.8</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>24.3 ± 3.1</td>
<td>136.4</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>21.6 ± 3.6</td>
<td>121.4</td>
</tr>
<tr>
<td>Lentilans</td>
<td>100</td>
<td>24.6 ± 3.3</td>
<td>138.3</td>
</tr>
</tbody>
</table>

* Mean survival time ± SD of six mice.

References...
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