Cytotechnology **25:** 205–211, 1997.

Immunomodulating activities of polysaccharide fractions from dried safflower petals

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Received 13 January 1997; accepted 9 July 1997

Key words: B cell, *Carthamus tinctorius* L., cytokine, lipopolysaccharides, macrophages, polysaccharide, safflower

Abstract

In the course of screening for immunomodulators, we found a significant blastogenic activity specific for splenic B cells in the extracts of safflower (*Carthamus tinctorius* L.). Active fractions termed SF1 and SF2 were purified from dried petals of safflower by boiling water extraction, ethanol precipitation and Sepharose CL-2B column chromatography. The elution profiles of the gel filtration indicated that the molecular weight of SF1 and SF2 was estimated to be more than 100 kD. Major components of SF1 and SF2 seem to be polysaccharides, and structural analysis of alditol acetate derivatives by GC-MS revealed some differences between SF1 and SF2 in the sugar component. Biological activities of SF1 and SF2 on B cells and macrophages were examined in comparison with lipopolysaccharides (LPS). SF1 and SF2 induced both the proliferation and the IgM production of B cells to the equivalent level as those induced by LPS. In macrophages, SF1 and SF2 effectively stimulated the production of NO. However, SF1 stimulated the production of IL-1, IL-6, and TNF as much as LPS, while SF2 induced them only weakly or not at all. Thus, these results suggest that SF1 and SF2 activate B cells and macrophages in different mechanisms.

Abbreviations: BSA – bovine serum albumin; IL-1 – interleukin-1; IL-6 – interleukin-6; IgM – immunoglobulin M; LBP – lipopolysaccharide binding protein; LPS – lipopolysaccharide; PEC – peritoneal exudate cells; PHA – phytohemagglutinin; PSK – protein-bound polysaccharide extracted from *Coriolus versicolor*; TdR – thymidine deoxyribase; TG – thioglycollate; TNF-α – tumor necrosis factor-α

Introduction

Immune system protects us from the invasion of infectious microorganisms and viruses, and cancer. Sometimes, these infectious agents and tumor cells can escape from the immune surveillance and cause serious diseases. Thus, substances that specifically activate such immune responses will be expected to be of important use for clinical intervention. To date, a number of papers have reported that polysaccharide preparations from higher plants (Kurahata *et al*., 1989, Stimpel *et al*., 1994), fungi (Brander *et al*., 1959, Brander *et al*., 1958, Itoh *et al*., 1990, Chihara *et al*., 1969), and bacteria, (Kawaguchi *et al*., 1983), stimulate the immune responses, and some of them have been used for the clinical treatment. To obtain such immunomodulators, we have established an assay system using mouse spleen cells, and screened the natural products such as the microbial metabolites and plant extracts. Recently, we have found a strong blastogenic activity in the safflower extracts. In this paper, we have described the purification and biological activities of the high molecular weight substances from the safflower extracts. Safflower is currently used for food materials such as cooking oil and a source for natural red dyes. Moreover, safflower petals are also used as

tradiotional drugs for women's diseases, poor circulation and menopausal disorders.

Materials and methods

Mouse

C57BL/6 and C3H/HeN mice (female, 6 wk-old) were purchased from Charles River Co. Ltd. (Yokohama, Japan). C3H/HeJ mice (female, 6 wk-old) were purchased from Sankyo Labo Service (Tokyo, Japan). Six to eight wk-old mice were used for experiments.

Culture medium

RPMI 1640 medium (Life Technologies Inc., Grand Island, NY) supplemented with 10% (v/v) fetal calf serum (FCS; Bioserum, Victoria, Australia), 50 μM 2-mercaptoethanol, 50 μg/ml kanamycin, and 8 μg/ml tylosin tartrate was used for experiments.

Purification of SF1 and SF2 from dried safflower petals

Purification steps are shown in Fig. 1. Dried petals of safflower (*Carthamus tinctorius* L.) (Kampo Science fundation, Tokyo, Japan) were extracted with boiling water for 15 min, and compressed with the gauze. The resultant extracts were centrifuged at 10,000 × g for 20 min, and the supernatants were precipitated with ethanol at a final concentration of 70%. The precipitates were collected by centrifugation (10,000 × g, 10 min), and washed three times with 70% ethanol. After being freeze-dried, the resultants were dissolved in water, and the aqueous solution was adjusted to pH 3 by HCl. The insoluble materials were removed by centrifugation (10,000 × g, 10 min), and the recovered supernatants were again adjusted to pH 7 by NaOH. Active components were collected by 70% ethanol precipitation and freeze-dried. The dried ethanol precipitates were dissolved in 10 mM Tris-HCl (pH 7.4) and directly applied to Sepharose CL-2B column (2.5 × 90 cm) equilibrated with 10 mM Tris-HCl (pH 7.4). The samples were eluted with the same buffer at a flow rate of 1.63 ml/min, and 5 ml-fractions were collected. SF1 and SF2 were collected by 70% ethanol precipitation and freeze-dried.

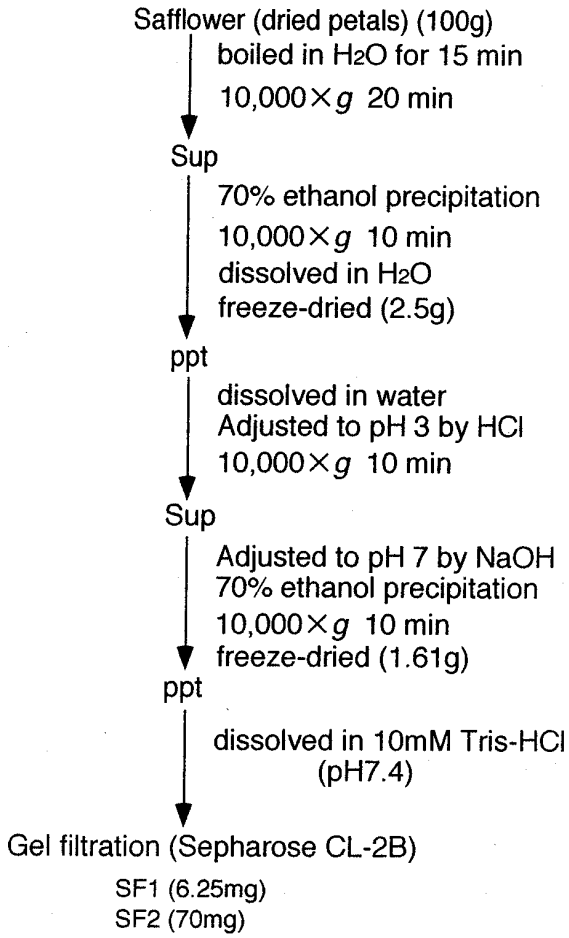

Figure 1. Purification steps of SF1 and SF2 from safflower dried petals. The recoveries of dried materials were represented in parentheses.

Components of polysaccharides

SF1 and SF2 were first hydrolyzed with 2 N trifluoroacetate at 120 °C for 2 h in the closed pipe. After the organic materials were removed by ether extraction, the aqueous phase was reduced with $NaBH_4$ and acetylated at 40 °C for 2 h. The resultant alditol acetate derivatives were analyzed by GC-MS. The organic phase was reacted with 5% methanol-HCl at 60 °C for 2 h, and analyzed by GC-MS. Protein content in SF1 and SF2 was determined by using Bio-Rad Protein Assay system.

Assay for blastogenic activity

Spleen cells from C57BL/6 mice were treated with 0.83% ammonium chloride, 10 mM Tris-HCl (pH 7.5) for 2 min to remove red blood cells. Spleen cells (1×10^6 cells/ml, 100 μl) were cultured with test samples for 48 h in flat-bottom microtiter plates. For the last 4 h of incubation, the cells were pulse-labeled with 9.25 kBq/well of [^{3}H] thymidine (TdR, ICN Biomedicals Inc., Costa Mesa, CA). The cells were harvested on glass-fiber filters (Whatman GF/A) and the radioactivity was counted in a scintillation counter.

Measurement of IgM

Spleen cells from C57BL/6 mice were prepared as described above. Spleen cells (1×10^6 cells/ml, 100 μl) were cultured with test samples for 72 h in flat-bottom microtiter plates, and the supernatants were collected. The amount of IgM in the supernatants was determined by the ELISA method (Lim *et al*., 1995). Round-bottom microtiter plates were coated with anti-mouse IgM (H&L) A107US (American Qualex, California, USA). Culture supernatants or standard mouse IgM were diluted with 0.1 M $NaHCO_3$ containing 0.02% NaN_3 and incubated in the anti-IgM-coated plates. Bound IgM was detected with horseradish peroxidase-conjugated anti-mouse IgM (H&L) A107PS (American Qualex) in 0.1 M $NaNO_3$ containing 1% BSA.

Isolation of macrophages

C3H/HeN mice were injected i.p. with 2.5 ml of 3.8% thioglycollate (TG) medium. After 4 days, peritoneal exudate cells (PEC) were collected. PEC (1×10^6 cells/ml, 1 ml) were cultured in the presence of test samples in 24-well plates for 24 h. The culture supernatants were harvested, and measured for the following biological activities.

Measurement of IL-1

Thymocytes from C3H/HeJ mice (5×10^6 cells/ml, 100 μl) were cultured with serially diluted culture supernatants or standard mouse rIL-1 in the presence of 2 μg/ml of PHA for 48 h in flat-bottom microtiter plates (Kalechman *et al*., 1990), and pulse-labeled with 9.25 kBq/well of [^{3}H] TdR for the last 4 h.

Measurement of IL-6

IL-6-dependent mouse hybridoma clone, MH60.BSF2, was cultured with optimally diluted culture supernatants or standard mouse rIL-6 for 48 h in a final volume of 200 μl of 2.5×10^4 cells/ml in flat-bottom microtiter plates (Matsuda *et al*., 1988), and the cell viability was measured by MTT method.

Measurement of TNF-α

Mouse fibrosarcoma L929 (7.5×10^5 cells/ml, 100 μl) was cultured with serially diluted culture supernatants in the presence of 2 μg/ml of actinomycin D in flat-bottom microtiter plates for 18 h. Adherent cells were stained with 0.2% crystal violet (Takasuka *et al*., 1991), and absorbance at 595 nm was measured. One unit of TNF was defined as the concentration that induces 50% cell lysis.

Measurement of NO

The amount of NO was determined by measuring nitrite, as described previously (Kalechman *et al*., 1996). One hundred μl of culture supernatants or dilution thereof were mixed with an equal volume of 10% Griess-Romijn Nitrite Reagent (Wako Pure Chemical Industries Ltd., Osaka, Japan), and absorbance at 540 nm was measured. Total nitrite were determined by using the standard curve of $NaNO_2$ (0.1 to 100 μM).

Results

Purification of SF1 and SF2 from safflower petals

The purification procedure of SF1 and SF2 was described in the section of Materials and methods. The ethanol precipitates were applied to Sepharose CL-2B column chromatography. The blastogenic activity was separated into two fractions termed SF1 and SF2. These fractions seem to be entirely free of proteins, since they did not have UV absorbance. The elution profiles of the gel filtration revealed that the molecular weight of SF1 and SF2 were estimated to be more than 100 kD. 6.25 mg of SF1 and 70 mg of SF2 were obtained from 100 g of safflower dried petals.

Structural analysis of SF1 and SF2

The ethanol precipitates of safflower extracts were positive for PAS staining, and SF1 and SF2 contained 2.98% and 1.05% protein by weight, respectively. No lipid was detected by GC analysis in 500 μg of SF1 or SF2 (data not shown). These observations suggest that major components of SF1 and SF2 are polysaccharides. SF1 and SF2 were first hydrolyzed, and the resultant sugar monomers were converted to their alditol acetate derivatives, and analyzed by GC-MS. The relative ratio of the sugar components was calculated in comparison with the least sugar component, mannose (Table 1). Following three conclusions were obtained: (1) SF1 contains hexoses more abundantly than SF2, (2) SF1 contains glucose more than galactose, and (3) there is no difference between SF1 and SF2 in the content of deoxysugars and pentoses.

Effects of SF1 and SF2 on B cells

FACS analysis revealed that safflower extracts induced only B220$^+$, but not Thy1$^+$, cell population of spleen cells (data not shown). Thus, we tested the effects of SF1 and SF2 on the proliferation and differentiation of B cells in comparison with the typical B cell mitogen, lipopolysaccharides (LPS). The maximum activities of SF1 and SF2 were comparable to that of LPS in the proliferation (Fig. 3) and the IgM production (Fig. 4). As far as effective doses are concerned, SF1 was about 10-fold more effective than SF2.

Effects of SF1 and SF2 on macrophages

We further examined the effects of SF1 and SF2 on the function of macrophages. Four soluble mediators produced by TG-elicited PEC in response to SF1, SF2 or LPS were measured: IL-1 (Fig. 5), IL-6 (Fig. 6), TNF-α (Fig. 7), and NO (Fig. 8). SF1 induced the production of IL-1 as strong as LPS, while SF2 was inactive (Fig. 5). In the case of IL-6, the equivalent amount of IL-6 was detected in the culture supernatants of SF1- and LPS-treated PEC (Fig. 6). However, the induction of IL-6 by SF2 was relatively weak in the maximum level and the effective dose (Fig. 6). The same tendency was observed in the TNF-α production (Fig. 7). In the case of the NO production, SF1 and SF2 exhibited the comparable activity with LPS (Fig. 8). However, higher doses of SF1 and SF2 were required to draw the maximum production. In this point, SF1 was more effective than SF2.

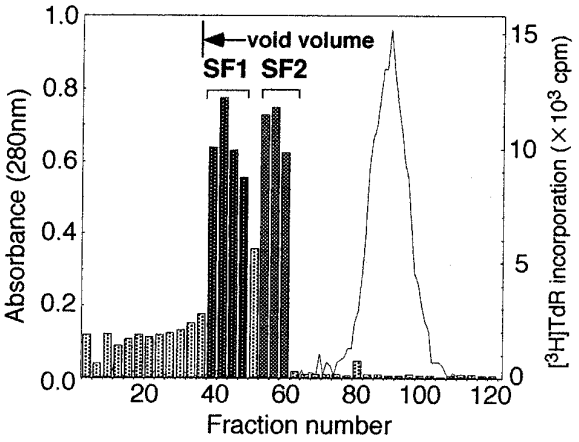

Figure 2. Profiles of gel filtration of SF1 and SF2. The ethanol precipitates were applied to Sepharose CL-2B column chromatography. Mouse spleen cells were cultured with the 25% (v/v) of eluents which were the mixture of every three fractions for 48 h and pulsed with [^{3}H]-TdR for the last 4 h. [^{3}H]-TdR incorporation (bars) and absorbance at 280 nm (solid line) were shown.

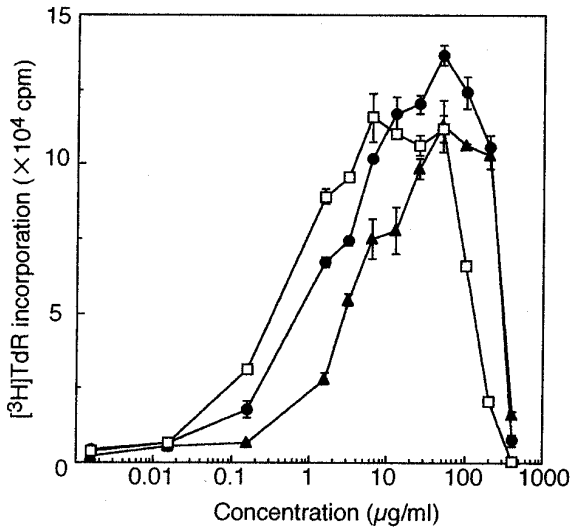

Figure 3. Effects of SF1 and SF2 on the proliferation of spleen cells. Mouse spleen cells were cultured with SF1 (filled circles), SF2 (filled triangles), or LPS (open squares) for 48 h, and pulse-labeled with [^{3}H] TdR for the last 4 h.

Discussion

In this paper, we reported the purification procedure of the high molecular weight substances from safflower extracts and their biological activities on B cells and macrophages. Dried safflower petals were subjected to boiling water extraction, ethanol precipitation, and Sepharose CL-2B column chromatography. High

Table 1. Relative ratio of sugar components of SF1 and SF2

	Relative ratio of sugar components					
	Rhamnose	Arabinose	Xylose	Mannose	Glucose	Galactose
SF1	2.9	7.5	3.8	1	11.6	8.9
SF2	2.9	10.3	4.2	1	5.1	8.5

The content of mannose was set up to 1, and the relative ratio of each sugar was calculated.

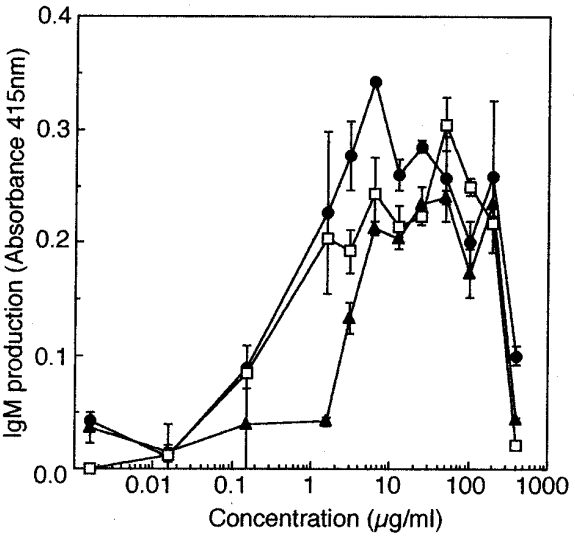

Figure 4. Effects of SF1 and SF2 on the IgM production of spleen cells. Mouse spleen cells were cultured with SF1 (filled circles), SF2 (filled triangles), or LPS (open squares) for 72 h. The amount of IgM in culture supernatants was measured by ELISA method.

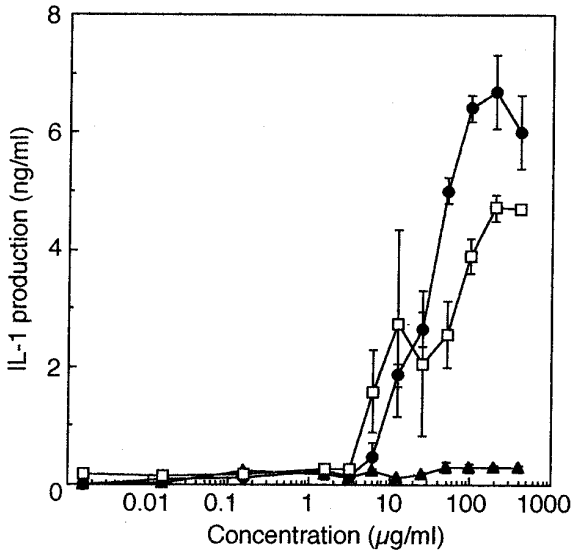

Figure 5. Effects of SF1 and SF2 on the IL-1 production of PEC. TG-elicited PEC were cultured with SF1 (filled circles), SF2 (filled triangles), or LPS (open squares) for 24 h. The amount of IL-1 was measured as described in Materials and methods.

molecular weight fractions termed SF1 and SF2 were identified to exhibit a specific blastogenic activity on B cells. SF1 and SF2 induced the proliferation and differentiation of B cells as strong as LPS. Moreover, SF1 effectively stimulated macrophages to produce various cytokines as comparable as LPS, while the effects of SF2 on them were relatively weak.

LPS contains polysaccharide chains and lipid A both of which can exert the blastogenic activity (Hofstad *et al*., 1993). In contrast, a number of immunomodulators, such as lentinan (Chihara *et al*., 1970), and schizophillan (Itoh *et al*., 1990), are composed of only polysaccharides, and have a common structure of β-1,3-D-glucan (Nemoto *et al*., 1994). SF1 and SF2 contain neither proteins and lipids, nor a simple repetition of the sugar chains (data not shown), but mainly consisted of neutral sugars, i.e. rhamnose, arabinose, xylose, mannose, glucose and galactose. Thus, the structures of SF1 and SF2 seem to be different from the immunomodulatory polysaccharides so far reported (Wang *et al*., 1993, Mizuno *et al*., 1995). In our assay systems, PSK (Sakagami *et al*., 1993) did not show any effects on spleen cells and PEC, although polysaccharide fractions from *Glycyrrhizae Radix* showed the blastogenic activities on mouse spleen cells (data not shown).

Although LPS induces the activation of the host immune responses, concomitant inflammatory responses result in the pathogenesis of sepsis and the adult respiratory distress syndrome (Nick *et al*., 1996). These fatal effects preclude LPS from the clinical use for infectious diseases and cancer. In contrast, some polysaccharides, such as PSK (Kato *et al*., 1995), are currently used for the therapeutic treatments of cancer. Moreover, as compared with LPS, the production of inflammatory factors such as IL-1 and TNF-α was weakly induced especially by SF2. In these points, SF1

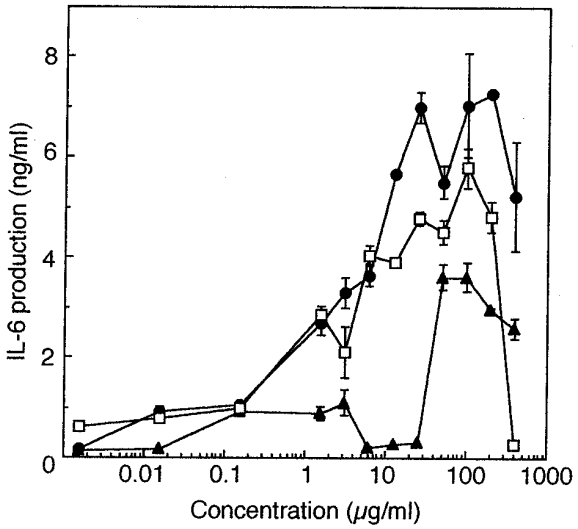

Figure 6. Effects of SF1 and SF2 on the IL-6 production of PEC. TG-elicited PEC were cultured with SF1 (filled circles), SF2 (filled triangles), or LPS (open squares) for 24 h. The amount of IL-6 was measured as described in Materials and methods.

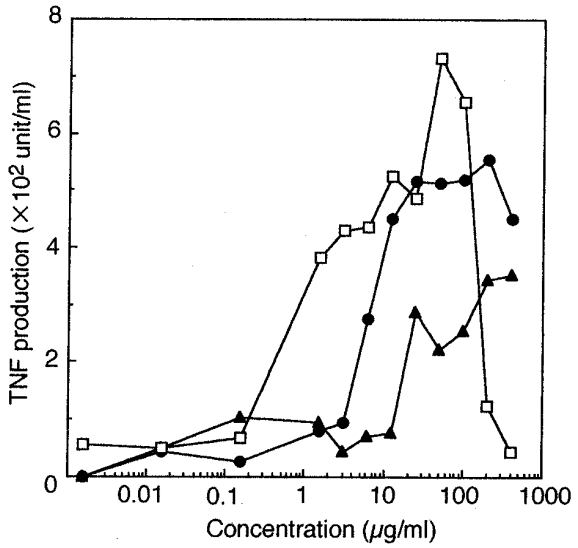

Figure 7. Effects of SF1 and SF2 on the TNF-α production of PEC. TG-elicited PEC were cultured with SF1 (filled circles), SF2 (filled triangles), or LPS (open squares) for 24 h. The amount of TNF-α was measured as described in Materials and methods.

and SF2 will be expected to be useful clinical agents. Trials to determine anti-tumor effects of SF1 and SF2 *in vivo* are currently in progress.

In the case of B cells, SF1 and SF2 showed the similar effects on the proliferation and the IgM production, although the effective dose of SF2 was 10-fold higher than that of SF1. Since the molecular weight of SF1

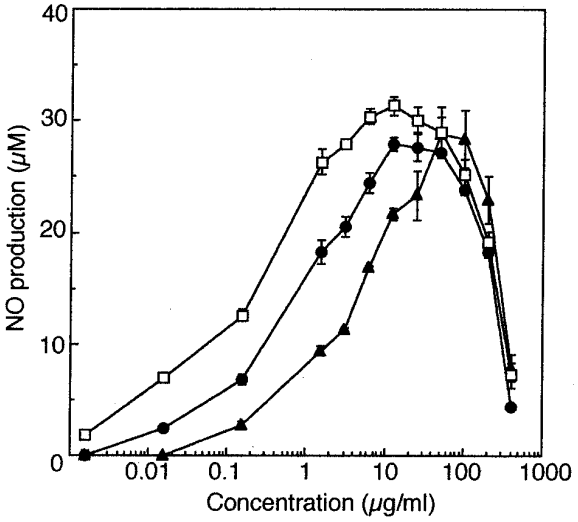

Figure 8. Effects of SF1 and SF2 on the NO production of PEC. TG-elicited PEC were cultured with SF1 (filled circles), SF2 (filled triangles), or LPS (open squares) for 24 h. The amount of NO was measured as described in Materials and methods.

was higher than SF2, SF1 is likely to cross-link surface receptors more efficiently than SF2.

LPS can activate macrophages via at least two distinct pathways (Cohen *et al*., 1995). One pathway is transmitted by CD14. CD14 is expressed strongly on monocytes and weakly on neutrophils. CD14 binds LPS complexed to LBP (LPS binding protein) (Ziegler-Heitbrock *et al*., 1993). The other pathway is independent of CD14 and LBP, and putative receptors have not been identified. In the presence of high concentrations of LPS (>100 ng/ml), CD14-independent pathway is shown to be operative (Haziot *et al*., 1996). In TG-elicited macrophages, SF1 and SF2 exhibited significant differences in their ability to induce the production of soluble mediators. SF1 induced the production of IL-1, IL-6, TNF-α, and NO to the equivalent level as detected in LPS-treated cells. Particularly, SF2 did not induce the production of IL-1, more weakly that of IL-6 and TNF-α, but strongly that of NO. Thus, SF2 and possibly SF1 seem to stimulate macrophages via distinct pathways from that of LPS. Further studies on the mode of action of SF1 and SF2 will reveal novel pathways of activation of immune cells.

Acknowledgements

This work was partly supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Sci-

ence, and Culture of Japan, and a research fund from the Kampou Science Foundation, Tokyo, Japan.

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