Stimulation of IgM Production in Human-Human Hybridoma HB4C5 Cells and Human Lymphocytes by Soybean Hull Hemicellulose

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We screened immunoglobulin production stimulating factor (IPSF) in several types of dietary fibers using human-human hybridoma HB4C5 cells cultured in a serum-free medium. Among the fibers, soybean hull hemicellulose (SHH) showed the strongest IPSF activity; 1 mg/mL of SHH stimulated IgM production approximately 4-fold. SHH stimulated IgM production of human-human hybridomas derived from fusion partner NAT-30 cells and mouse-mouse hybridomas derived from P3U1 but not as much as those of human-human hybridomas derived from HO-323 cells. When the SHH preparation was fractionated on a Sepharose CL-4B gel filtration column, IPSF activity was observed in a carbohydrate peak at MW 32 000 containing small amounts of protein. The IPSF activity of SHH was reduced by heating at 100 °C and by digestion with trypsin or β-galactosidase. These results suggested that the IPSF activity of SHH was dependent on both polysaccharide and protein components. SHH also stimulated IgM production of human lymph node lymphocytes but not their IgG and IgE productions.

INTRODUCTION

Human-human hybridomas producing monoclonal antibodies (MAbs) specific to various antigens have been established for various purposes (James et al., 1987) and used for mass production of human MAbs (Murakami et al., 1987). For medical uses, purity of human MAbs is most important, and serum-free culture of hybridoma markedly cut down the purification cost of MAbs (Murakami et al., 1987). Some of hybridomas proliferate and produce MAbs in serum-free media, but their MAbs productivities in serum-free media are often lower than those in serum-supplemented medium (Aihara et al., 1988; Yamada et al., 1989b). Thus, we screened immunoglobulin production stimulating factor (IPSF), using human-human hybridoma cells cultured in a serum-free medium, and found various proteinous IPSFs in cell products (Shinmoto et al., 1988; Yamada et al., 1989b; Toyoda et al., 1990) and in foodstuffs (Yamada et al., 1989b, 1990a). It was also found that some of the IPSFs stimulated IgM and IgG production of human lymphocytes, especially in the presence of lipopolysaccharides (Yamada et al., 1990b). These findings indicated that human-human hybridomas cultured in serum-free medium were useful for screening of IPSFs that are effective in the human immune system. This paper describes the IPSF activity in a dietary fiber, whose various biological effects are reported (Robbins et al., 1977; Ayano et al., 1986).

MATERIALS AND METHODS

Materials and Chemicals. ERDF medium (a 2:1:1 mixture of RPMI1640, Dulbecco's modified Eagle and Ham's F12 media enriched with amino acids and vitamins) (Murakami, 1989) was purchased from Kyokuto Seiyaku Co., Ltd., and fetal bovine serum (FBS) from Hyclone Co., Ltd. Polyclonal anti-antibodies (human IgM, human IgG, mouse IgM, and those conjugated with peroxidase) were purchased from Tago Inc. Soybean and rice bran were supplied from the Breeding Material Laboratory and the Rice Breeding Laboratory of Chugoku National Agricultural Experiment Station (Hiroshima, Japan), respectively.

Preparation of Soybean Hull Hemicellulose B (SHH) (Ayano et al., 1986). Separated soybean (*Glycine max* var. Tamahomare) hull was washed with 99% ethanol and milled to 30 mesh. SHH was extracted from the milled hull by 1 N NaOH solution under N_2 gas with shaking for 1 day. The extract was neutralized by acetic acid, and then trichloroacetic acid was added to a final concentration of 7%. After standing for 1 h, it was centrifuged at 400*g* for 20 min. The supernatant was then dialyzed against distilled water for 3 days. Four times the volume of 99% ethanol was added to the dialyzed supernatant, and the resulting precipitate was freeze-dried. SHH was dissolved in PBS and filtered through a 0.22-μm nitrocellulose filter for sterilization.

Preparation of Rice Bran Hemicellulose (RBH). The rice (*Oryza sativa* var. Koshihikari) bran hemicellulose was prepared according to the method of Ayano et al. (1986), except that well-milled rice bran was treated with glucoamylase (Nagase Sangyo Co., Ltd.) in a 40 mM acetate buffer (pH 4.8) overnight before extraction with 1 N NaOH solution to remove starch (Aoe et al., 1989).

Preparation of Barley Gum. The preparation of barley gum was by the method of Wood et al. (1977). Well-milled barley was extracted by 10% $NaCO_3$ solution (pH 10) with shaking at 60 °C for 1 h. The extract was separated by centrifugation and adjusted to pH 4.5 with acetic acid to precipitate protein. After centrifugation, the supernatant was mixed with 4 volumes of ethanol to precipitate barley gum.

Cells and Cell Culture. Human-human hybridoma HB4C5 cells producing IgM specific to human lung adenocarcinoma cells (Murakami et al., 1985) were used to screen IPSF. HB4C5 cells were generated by fusing human lymphocytes of a lung cancer patient with human fusion partner NAT-30 cells (Murakami et al., 1985). The cells were inoculated at a cell density of 0.5×10^5 cells/mL and cultured at 37 °C for 2 days in ERDF medium supplemented with 10 μg/mL insulin, 35 μg/mL transferrin, 10 μM ethanolamine, and 2.5 nM selenium (ITES) (Murakami et al., 1982). The test samples were added to the medium. Determination of cell number using an electric cell counter (Toa Iyou Denshi Co., Ltd.) and the determination of the Ig content in the culture supernatant by the ELISA method were as described previously (Yamada et al., 1989a). Ig productivity was

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0021-8561/91/1439-0820$02.50/0

Table I. Effect of Dietary Fibers on IgM Production of Human–Human Hybridoma HB4C5 Cells[a] (Means ± SD)

samples	addition concn, μg/mL	rel cell no.[b]	rel IgM concn[b]	rel IgM productivity[b]
barley gum	10	0.94 ± 0.04	1.07 ± 0.05	1.14 ± 0.05
	100	1.09 ± 0.06	1.18 ± 0.10	1.08 ± 0.09
	1000	0.95 ± 0.02	1.23 ± 0.01	1.28 ± 0.03
soybean hull hemicellulose	5	1.01 ± 0.05	1.31 ± 0.08	1.30 ± 0.04
	50	1.19 ± 0.04	1.96 ± 0.13	1.64 ± 0.11
	500	1.41 ± 0.05	3.23 ± 0.18	2.27 ± 0.10
rice bran hemicellulose	5	0.97 ± 0.01	0.91 ± 0.06	0.93 ± 0.05
	50	1.04 ± 0.09	0.95 ± 0.08	0.90 ± 0.02
	500	1.05 ± 0.08	1.06 ± 0.08	1.00 ± 0.02

[a] Cells (0.5×10^5 cells/mL) were cultured in ITES–ERDF medium supplemented with various samples for 2 days. [b] Nonaddition was as control.

calculated by dividing the Ig concentration in the medium by the cell number on each sampling day.

To determine the specificity of IPSFs, five types of human–human hybridomas (HB4C5, G4G7, AF11D2, CD2C16, and EMK-F7) and two types of mouse–mouse hybridomas (D6 and F6) were used. IgM-producing G4G7 cells were derived from NAT-30 cells; AF11D2 and CD2C16 cells producing IgM were derived from human fusion partner HO-323 cells, which was human B lymphoblastoid line derived from WIL2-NS (Ohashi et al., 1986). Both IgG and IgM producing EMK-F7 cells were derived from HO-323; D6 cells and F6 cells were IgM producers derived from mouse fusion partner P3-X63-Ag8(P3U1) cells.

Column Chromatography. SHH was applied to a Sepharose CL-4B column (bed volume 800 mL; column size 32 i.d. × 1000 mm) equilibrated with 50 mM sodium phosphate buffer (pH 7.7). The elution speed was regulated at 1 mL/min, and the elution was collected with 10 mL per tube. The protein content in the fractions was monitored from the absorbance at 280 nm and the carbohydrate content by the phenol–sulfuric acid method (Dubois et al., 1956). The IPSF activity of each fraction was determined after dialysis. Pullulan (Showa Denko Co., Ltd.) was used as the molecular weight marker for the chromatography.

Analysis of Heat Stability of SHH. SHHs heated at various temperatures for 30 min were added to culture media of HB4C5 cells (final concentration, 500 μg/mL), and IgM content of the culture supernatants were determined after 2 days.

Enzymatic Digestions of SHH. β-Galactosidase was purchased from ICN Biomedicals Inc. The enzyme (5 IU) was added to the SHH solution (500 μg/mL). The same amount of enzyme was also added to the control PBS solution. After incubation at 37 °C for 24 h, the reaction mixture was added to culture medium of HB4C5 cells (final concentration 450 μg/mL), and the cells were cultured for 2 days to determine IPSF activity of SHH. Treatment with trypsin was performed, as described previously (Yamada et al., 1989a).

Effect of SHH on Ig Production of Human Lymphocytes. Human lymphocytes were isolated from the lymph nodes of mammary cancer patients. The lymph nodes were cut into small pieces and pressed between two slide glasses in ERDF to squeeze out lymphocytes, and the cells were washed two times with the ERDF medium. After washing, cells were suspended in ITES–ERDF and 5% FBS–ERDF supplemented with SHH and then innoculated at a cell density of 1×10^6 cells/mL into 24-well plates. After 5 days cultivation, IgM, IgG, and IgE contents in the culture supernatant were determined by the ELISA method.

RESULTS AND DISCUSSION

Effect of SHH on Proliferation and Ig Production of Hybridomas. Three types of dietary fibers were added to the culture medium of HB4C5 cells at various concentrations. As shown in Table I, barley gum (1000 μg/mL) and SHH stimulated IgM production of the human–human hybridomas but RBH did not. All IPSFs so far reported stimulate proliferation of hybridomas as well as IgM production (Yamada et al., 1989a,b). Barley gum was unique for its stimulation of IgM production without stimulating cell proliferation. SHH also stimulated IgM production without stimulating proliferation of the cells at 5 μg/mL to the extent that barley gum did at 1000 μg/mL. At 500 μg/mL, SHH stimulated IgM production more strongly.

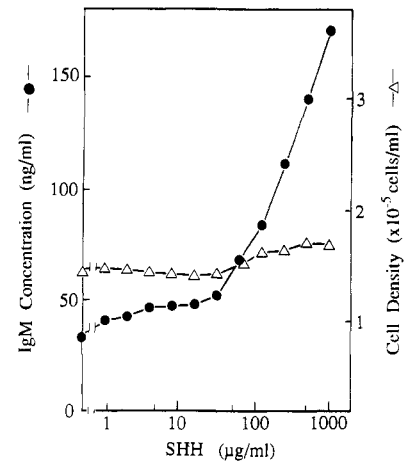

Figure 1. Dose-dependent stimulation of proliferation and IgM production of HB4C5 cells by SHH. HB4C5 cells (0.5×10^5 cells/mL) were cultured in ITES–ERDF medium containing various concentrations of soybean hull hemicellulose (SHH) for 2 days. Cell number and IgM concentration of the culture supernatants were then examined.

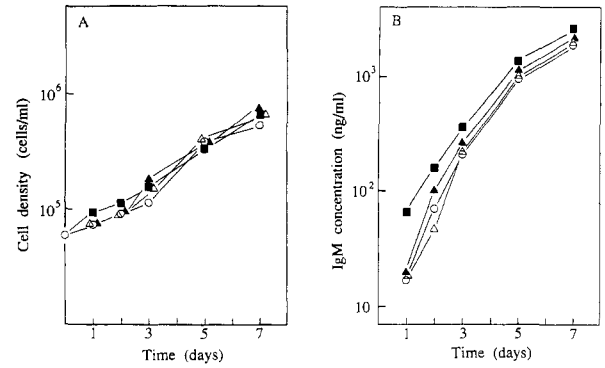

Figure 2. Time courses of the proliferation and IgM accumulation of HB4C5 cells in the presence of SHH. HB4C5 cells were cultured in ITES–ERDF medium supplemented with 0 (○), 5 (△), 50 (▲), and 500 μg/mL (■) of SHH for various periods to examine the effect of SHH on proliferation (A) and IgM production (B) of the cells.

Figure 1 shows the dose–response curves of SHH on proliferation and IgM production of HB4C5 cells. Weak stimulation of IgM production could be induced without stimulation of proliferation at concentrations below 30 μg/mL. At higher SHH concentrations, IgM concentration increased linearly with the increase in SHH concentration, accompanying weak stimulation of proliferation. The stimulation of cell proliferation by SHH (below 1.3-fold of control on the second day) was weaker than those of proteinous IPSFs (1.5–2.4-fold) (Yamada et al., 1989b). At 1 mg/mL, SHH gave a maximum IPSF activity (4.2-fold increase in IgM production), but the culture medium was too viscous to handle the cells. Thus, the maximum SHH concentration was set at 500 μg/mL thereafter.

Figure 2 shows the time course of proliferation and IgM production of HB4C5 cells in the presence of various amounts of SHH. The proliferation stimulating effect of SHH was negligible, but it stimulated IgM production of the cells, especially at low cell densities. Proteinous IPSFs also stimulate well proliferation and IgM production of the cells at low cell densities (Yamada et al., 1989b,

Table II. Effect of Soybean Hull Hemicellulose on Ig Production of Various Hybridomas[a]

hybridomas	parent cells	Ig class	rel cell no.	rel Ig concn	rel IgM productivity
HB4C5	NAT-30	M	1.26	2.82	2.23
G4G7	NAT-30	M	1.16	2.21	1.89
AF11D2	HO-323	M	1.05	1.16	1.11
CD2C16	HO-323	M	1.29	1.44	1.11
EMK-F7	HO-323	M	1.50	1.50	1.00
EMK-F7	HO-323	G	1.50	1.61	1.07
D6	P3U1	M	0.94	1.67	1.77
F6	P3U1	M	0.91	1.52	1.66

[a] Various human–human and mouse–mouse hybridomas (5×10^4 cells/mL) were cultured in ITES-ERDF containing SHH (final concentration 500 μg/mL) for 2 days.

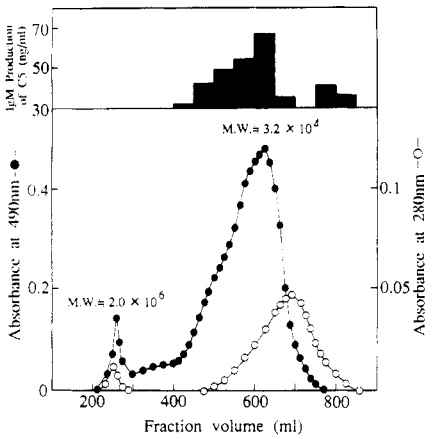

Figure 3. Gel filtration profile of SHH on a Sepharose CL-4B. Each fraction was dialyzed and then added to the culture medium of HB4C5 cells. Cells (1×10^5 cells/mL) were cultured for 6 h in 96-well plates, and IgM concentration was then determined by the ELISA method.

1990a). Since the HB4C5 cells are producing cellular IPSFs by themselves (Yamada et al., 1989a), the effect of extracellular IPSFs is difficult to detect at high cell densities.

When SHH was added to various human–human and mouse–mouse hybridomas, the effects on their proliferation and Ig production varied with cell lines (Table II). Human–human hybridomas derived from human fusion partner NAT-30 cells (HB4C5 and G4G7) and mouse–mouse hybridomas derived from P3U1 cells (D6 and F6) showed higher IgM productivities in the presence of SHH, but the effect on the IgM productivity of human–human hybridomas derived from human fusion partner HO-323 cells (AF11D2, CD2C16, and EMK-F7) was negligible. Similar results are also obtained in cellular IPSFs (Yamada et al., 1989a; Toyoda et al., 1990) and proteinous IPSFs (Yamada et al., 1989b). In the case of EMK-F7 cells producing both IgG and IgM, SHH stimulated proliferation of the cells, but it stimulated neither IgG nor IgM production.

Purification and Characterization of IPSF in SHH. To purify IPSF, SHH was gel filtrated by using a Sepharose CL-4B column (Figure 3). The first peak containing protein and carbohydrate had no IPSF activity. The second carbohydrate peak eluted slightly earlier than the second peak of protein. The highest IPSF activity was observed in the fraction corresponding to the second carbohydrate peak, whose molecular size was about 32 kDa. Figure 4 shows the heat stability of the SHH fraction. The IPSF activity of SHH was stable below 60 °C but decreased with heating at 80 and 100 °C. However, about 30% of the activity was retained even after 30 min of heating at 100 °C. When SHH was treated with various

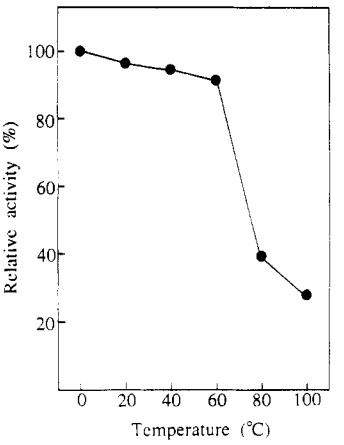

Figure 4. Heat stability of IPSF activity of SHH. SHH was heated for 30 min at various temperatures and added to culture medium of HB4C5 cells to examine its IPSF activity.

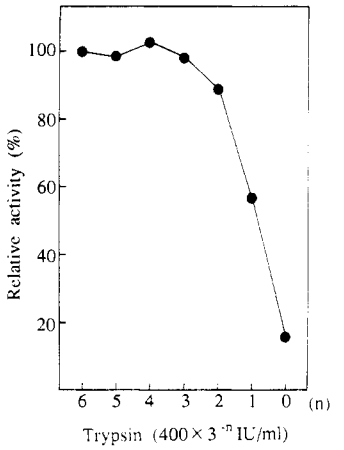

Figure 5. Susceptibility to trypsin of SHH. SHH (500 μg/mL) was mixied with the $^1/_{10}$ volume of various concentrations of trypsin solutions. After 1 h of incubation, trypsin inhibitor solution (43 μg/mL) was added to inactivate trypsin and then added to the HB4C5 cultures. For the positive control (100% activity), the mixture of SHH, trypsin (400 IU/mL), and trypsin inhibitor was added to the HB4C5 culture.

Table III. β-Galactosidase Digestion of SHH

	rel activity
PBS + enzyme (50 IU)	1.00
SHH	2.18
SHH + enzyme	1.20

concentrations of trypsin for 1 h, the activity decreased linearly at trypsin concentrations over 400×3^{-2} IU/mL, and less than 20% of the activity was retained after the treatment with 400 IU/mL of trypsin (Figure 5). It has been reported that soybean hemicellulose is composed of arabinogalactan, xyloglucan, or galactomannan (Kawamura, 1967). Thus, the effect of β-galactosidase digestion on the IPSF activity of SHH was examined. As shown in Table III, the activity of SHH was decreased by the treatment. These results suggest that both polygalactopyranoside and protein moieties are involved in the IPSF activity of SHH.

Effect of SHH on Ig Production of Human Lymph Node Lymphocytes. Table IV shows the effect of SHH on Ig production of human lymphocytes. SHH stimulated IgM production of lymphocytes in ERDF medium supplemented with ITES or 5% FBS, but it did not stimulate IgG and IgE production. Similar results were obtained in three experiments using human lymph node lymphocytes and two experiments using human peripheral blood

Table IV. Effect of SHH on Ig Production of Lymph Node Lymphocytes[a]

additives	cell no. × 10^5 cells/mL	Ig concn, ng/mL IgM	IgG	IgE
ITES	6.79	10	169	8
ITES-SHH[b]	7.12	27	152	8
5% FBS	9.34	82	173	14
FBS-SHH[b]	9.76	139	187	14

[a] Cells (9×10^5 cells/mL) were cultured in ERDF medium supplemented with various additives for 5 days. [b] SHH was added 500 μg/mL to the ERDF medium.

lymphocytes. This indicates that SHH predominantly stimulates IgM production of human lymphocytes, as well as that of human–human hybridomas.

Various types of proteinous IPSF have been previously studied (Yamada et al., 1989a,b, 1990a; Toyoda et al., 1990). We showed here that a hemicellulose preparation of soybean composed of protein and polysaccharides had IPSF activity and that the polysaccharide moiety was important for the activity as well as the protein moiety. These IPSFs stimulate the Ig production of human lymphocytes (Yamada et al., 1990b; present observation), as well as those of human–human and mouse–mouse hybridomas. These results suggest that various types of macromolecules, including protein and polysaccharides, are affecting Ig production of human B lymphocytes. Human–human hybridomas cultured in serum-free medium could be very useful for the screening of in vivo effectors and clarification of a mechanism of their stimulation activity of Ig production.

ABBREVIATIONS USED

SHH, soybean hull hemicellulose; PBS, phosphate-buffered saline; Ig, immunoglobulin; FBS, fetal bovine serum; IPSF, immunoglobulin production stimulating factor.

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Received for review May 2, 1990. Revised manuscript received September 4, 1990. Accepted October 7, 1990.

Registry No. Hemicellulose, 9034-32-6.