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In vitro immunomodulating effect of protein-bound polysaccharide, PSK on peripheral blood, regional nodes, and spleen lymphocytes in patients with gastric cancer

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Summary. PSK, a protein-bound polysaccharide, has been widely used for cancer immunotherapy in Japan. However, the mechanism of its immunomodulatory effect has not been fully clarified. In the present study the in vitro effect of PSK on the lymphocytes of patients with gastric cancer was studied. Culturing lymphocytes with PSK at 5–100 μg/ml increased the level of DNA synthesis, and augmented the cytotoxicities against K562 and KATO-3. Flow-cytometric analysis also showed an increase in the proportion of interleukin-2 (IL-2)-receptor-positive cells after the lymphocytes were cultured with PSK. However the cytotoxicity of cells cultured with PSK was not augmented by the addition of recombinant interferon γ (rIFNγ) and rIL-2. Further experiments using fractionated PSK showed that its biological action is present mainly in fractions having molecular masses $>10^5$Da. However, these immunomodulations were not seen in all patients. These results suggest that the susceptibility of lymphocytes to PSK may be different in each patients, and that the immunomodulation by PSK may be mediated by mechanisms independent of IFN and IL-2.

Key words: PSK – NK cells – Immunotherapy – Gastric cancer

Introduction

PSK is a protein-bound polysaccharide, prepared from *Coriolus vesicolor* of the class Basidiomycetes, and has been widely used for cancer immunotherapy. Clinically, PSK does not inhibit tumor growth when administered alone. However, in combination with chemotherapy, PSK has life-prolonging effects on patients with gastrointestinal cancer after surgery [7, 13, 15], or with leukemia [18]. In murine experiments, PSK, alone or in combination with chemotherapy, effectively inhibited tumor growth and also inhibited recurrence and metastasis after the removal of the tumor by surgery or after chemotherapy [1, 5, 6, 11, 12, 14, 16]. Previous studies have suggested that the effect of PSK may be mediated by the host's immune system [22, 24]. However, the mechanism of the immunomodulating effect has not been fully elucidated, and only a few studies have employed human subjects. The present study was designed to evaluate whether or not PSK augments the antitumor immunity of human lymphocytes *in vitro*, and to determine which lymphocyte subpopulation is associated with the immune response evoked by PSK, and which part of PSK has a biological activity.

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Materials and methods

PSK and its fractions. PSK (lot-92 A) is a protein-bound polysaccharide prepared from basidiomycetes and obtained from Kureha Chemical Industry Inc., Tokyo, Japan. The preparation consists of heterogeneous polysaccharides, that contain 18%–38% proteins and have a mean molecular mass of about 10^5Da [14]. The fractions of PSK were prepared by ultrafiltration using an Amicon diaflow system. The molecular masses of fractions 1, 2, 3 and 4 (lot 92 A) are $<5\times10^4$, $(5-10)\times10^4$, $(10-20)\times10^4$, and $>20\times10^4$ Da, respectively.

Interleukin-2 and interferon-γ. Recombinant human interleukin-2 (rIL-2; TGP-3, lot Z3 206 141) was obtained from Takeda Pharmaceutical Co., Ltd., Tokyo, Japan. Recombinant human interferon γ (rIFNγ, lot GTA001) was obtained from Tore Co. Ltd., Tokyo, Japan.

Preparation of lymphocytes. The lymphocytes were separated from peripheral blood, regional lymph nodes (perigastric nodes), and the spleen of 22 patients with gastric cancer, who had undergone surgery at Kyoto University Hospital (Table 1). Peripheral blood lymphocytes (PBL) were separated by the centrifugation of heparinized blood on Ficoll-Paque (Pharmacia, Uppsala, Sweden) at 600 *g* for 30 min. Regional node lymphocytes were separated by mincing regional nodes, which were macroscopically not metastasized, with scissors, passing them through a no. 100 mesh stainless-steel screen, and centrifuging the cell suspension on Ficoll-Paque at 600 *g* for 30 min. After the cells were harvested from the interface, they were washed three times in Hanks' balanced salt

Table 1. Gastric cancer patients

No.	Age (years)	Sex	Stage[a]	Histology[b]	Operation[c]
1	40	M	I	Tub 2	PG
2	56	F	IV	Por	TG
3	58	M	III	Tub 1	TG
4	40	F	IV	Por	TG
5	64	M	III	Por	TG
6	40	F	I	Tub 2	PG
7	52	F	II	Mucinous	TG
8	42	M	II	Por	PG
9	65	F	I	Tub 1	TG
10	60	F	IV	Por	TG+SPX
11	50	M	I	Por	PG
12	71	F	III	Tub 2	TG
13	62	F	I	Tub 2	PG
14	35	M	III	Tub 2	TG+SPX
15	59	F	III	Por	PG
16	64	M	I	Por	PG
17	47	M	I	Tub 1	PG
18	61	F	I	Por	PG
19	51	M	III	Tub 2	TG+SPX
20	67	F	IV	Por	TG+SPX
21	43	F	II	Tub 1	PG
22	72	M	I	Por	PG

[a] Classified according to the general rule for gastric cancer study from the Japanese Research Society [20]

[b] Tub 1, well differentiated tubular adenocarcinoma; Tub 2, moderately differentiated tubular adenocarcinoma; Por, poorly differentiated adenocarcinoma

[c] PG, partial gastrectomy; TG, total gastrectomy; SPX, splenectomy

solution (HBSS; M. A. Bioproducts), and suspended in the appropriate media. A similar procedure was used to separate spleen cells from the spleen.

Subpopulation of lymphocytes. The phenotypes of the cells were assessed by flow cytometry using Orthospectrum III (Ortho-mune). OKT3, 4 and 8 monoclonal antibodies (Ortho-mune), Leu7, 11 and 19 monoclonal antibodies, and anti-IL-2 receptor monoclonal antibody (Becton Dickinson) were employed.

Cytotoxicity assay. The K562 human erythroleukemia cell line and KATO-3 human gastric cancer cell line were used as targets. Both cell lines had been passed in culture media: RPMI-1640 (M. A. Bioproducts) supplemented with 10% fetal calf serum (Gibco, Grand Island, NY, USA), 1% non-essential amino acids (Gibco), 1% pyruvate (Gibco), 1% L-glutamine (Gibco) and 50 μg/ml gentamicin (Shering). The cytotoxicity of lymphocytes against K562 and KATO-3 was assessed by a ^{51}Cr-release assay, and every assay was performed in triplicate. The target cells were labeled with $Na_2{}^{51}CrO_4$ (3.7×10^6 Bq/10^6 cells, NEN) in culture media for 3 h. After washing three times in HBSS, the concentration of the cells was adjusted to 5×10^4/ml in culture media. Samples of 100 μl target cell suspensions and 100 μl effector cell suspension were cultured in a 96-well round-bottom microtiter plate (Corning) for 12 h. After culturing, the supernatants were collected using the Titertek supernatant collection system (Flow Lab.). The radioactivities of the collected supernatants were measured in an γ-counter (Minaxy, Packard). The cytotoxicity was expressed as the percentage specific lysis, which was calculated as follows:

$$\text{Specific lysis (\%)} = \frac{\text{(test lysis} - \text{spontaneous lysis)}}{\text{(total lysis} - \text{spontaneous lysis)}} \times 100$$

For the total lysis and spontaneous lysis determinations 100 μl 2% Triton X and 100 μl culture medium, respectively, were added instead of effector cell suspension. Every experiment was performed in triplicate and the mean values were used for the calculations.

Proliferative response of lymphocytes. The proliferative response was measured by [^{3}H]thymidine ([^{3}H]dThd) (Amersham, Bucks, UK) incorporation assay, and every assay was performed in triplicate. The cells were cultured with PSK and its fractions on a 96-well, flat-bottom microtiter plate (Corning) at a cell concentration of 10^5 in 0.2 ml/well. 1.85×10^4 Bq [^{3}H]dThd/25 μl was added into each well 18 h before harvesting. After culturing, DNA in the cells was precipitated by trichloroacetic acid (Sigma, St. Louis) and trichloroacetic acid precipitates were harvested onto a glass-fiber filter (Flow Lab., Irvine Calif., USA) using a three-channel, semiautomatic cells harvester (titertek, Flow Lab.). After drying at room temperature, the glass-fiber filters were placed into minivials with 3 ml scintillation fluid (PCS, Amersham). The radioactivities of the filters were measured in a liquid scintillation counter (Aloka LS 1000). All of the data were expressed using the stimulation index (SI) which is calculated as follows:

SI = (test ^{3}H –background ^{3}H)/(control ^{3}H –background ^{3}H) ^{3}H was recorded as cpm, every experiment was performed in triplicate and the mean values were used for the calculations.

Results

The mitogenic effect of PSK on peripheral blood lymphocytes (PBL) and regional node lymphocytes was assessed by evaluating the level of DNA synthesis in the cells in the presence of PSK (Table 2). The level of DNA synthesis in the lymphocytes increased in the presence of PSK (at 5–500 μg/ml), and the maximum increase was obtained when the cells were cultured with PSK at 100 μg/ml for 5 days. However, the number of the cultured cells was unchanged after culturing with PSK for 7 days (data not shown). The effect of PSK on the natural killer (NK) activity of lymphocytes was assessed by a 12-h ^{51}Cr-release assay against K562 (Table 3). In the presence of PSK, the NK activity of PBL was significantly augmented in 3 out of 6 patients, but the cytotoxicity of regional lymph node cells was not augmented. The maximum augmentation was produced when

Table 2. Mitogenic activity of PSK[a]

Cells	Concentration of PSK (μg/ml)	Stimulation index to PSK (mean ± SD)		
		Day 3	Day 5	Day 7
PBL	1	0.95 ± 0.18	0.92 ± 0.17	0.99 ± 0.47
(n = 6)	5	1.07 ± 0.16	1.15 ± 0.21	0.91 ± 0.53
patients	10	1.08 ± 0.24	1.20 ± 0.23	0.97 ± 0.21
1–6	50	1.01 ± 0.16	1.52 ± 0.40	1.19 ± 0.23
	100	1.15 ± 0.19	1.68 ± 0.33*	1.32 ± 0.45
	500	0.94 ± 0.19	1.48 ± 0.44**	1.28 ± 0.53
	1000	1.02 ± 0.35	0.90 ± 0.45	0.80 ± 0.53
RLN	1	1.26 ± 0.30	0.93 ± 0.22	0.93 ± 0.25
(n = 5)	5	1.05 ± 0.12	1.07 ± 0.33	0.95 ± 0.23
patients	10	1.13 ± 0.26	1.06 ± 0.48	0.95 ± 0.10
1, 3–6	50	1.13 ± 0.11*	1.42 ± 0.30*	0.87 ± 0.18
	100	1.28 ± 0.06***	1.43 ± 0.34*	1.70 ± 0.27***
	500	0.84 ± 0.14*	0.71 ± 0.29	1.15 ± 0.25
	1000	0.48 ± 0.11***	0.36 ± 0.20***	0.36 ± 0.06***

[a] The cells were cultured with PSK or medium alone in 96-well flat-bottom microtiter plates. Mitogenic activity of PSK was assessed by DNA synthesis ([^{3}H]dThd incorporation) in cells according to the method described in Materials and methods, and was expressed using the stimulation index. PBL, peripheral blood lymphocytes; RLN, regional lymph node cells

* P <0.05, ** P <0.01, *** P <0.001

Table 3. Effect of PSK on natural killer cell activity[a]

Cells	Patient	Specific lysis (%) against K562 (mean ± SD; E/T = 10) in the presence of PSK at				
		0 μg/ml (medium alone)	10 μg/ml	50 μg/ml	100 μg/ml	500 μg/ml
PBL	4	13.2±2.0	26.8±3.5**	16.0±2.8	11.0±2.5	5.8±3.9*
	5	17.4±3.6	31.8±3.4**	28.3±2.6*	24.0±3.5	24.0±3.4
	6	20.1±3.4	16.7±3.0	13.2±4.4	11.5±2.5	9.5±2.3*
	7	6.1±1.9	4.7±1.5	5.4±1.5	6.8±2.2	3.0±1.3*
	8	10.5±2.4	18.2±2.0*	7.4±2.3	7.8±1.9	3.4±2.4*
	9	36.6±3.1	35.5±1.7	34.2±3.2	29.9±3.5	26.4±3.3*
RNL	4	2.7±2.2	0.5±3.4	0.6±1.7	0.6±2.1	2.9±1.7
	5	7.8±2.3	8.7±1.8	8.9±2.5	9.9±1.3	9.0±1.6
	6	4.9±1.6	3.2±2.5	3.2±1.3	2.6±1.5	2.9±1.2*
	9	0.9±1.5	4.4±2.4	0.9±1.2	0.7±2.0	0.4±1.3

[a] Cytotoxicity of cells against K562 was assessed in the presence of PSK at the indicated concentrations by 12-h ^{51}Cr-release assay, according to the method described in Materials and methods
* P <0.05, ** P <0.01, versus medium alone

Table 4. Dose-dependent effect of PSK on the generation of cytotoxic effector cells[a]

Target	Concentration of PSK (μg/ml)	Specific cytotoxicity (%) after culture with PSK (mean ± SD; E/T = 10)			
		Before culture	2 days	4 days	7 days
K562	Medium alone	13.7±0.9	27.4±2.1	42.0±2.9	40.9±2.2
	5	–	23.2±3.2	46.1±3.1	43.3±4.1
	10	–	32.7±2.1*	54.1±3.3**	46.5±2.8*
	50	–	34.9±1.6**	46.6±1.7	40.5±3.6
	100	–	31.1±2.5	46.0±2.3	39.6±2.9
	500	–	27.7±1.7	41.9±3.4	28.8±2.6**
KATO-3	Medium alone	26.1±3.2	41.1±3.8	61.7±3.3	33.0±1.9
	5	–	42.8±3.6*	60.2±3.1	35.9±1.9
	10	–	56.1±4.3**	57.2±2.9	38.3±2.3
	50	–	63.7±3.9***	56.9±3.7	36.8±2.4
	100	–	54.3±5.0*	66.2±3.4	36.9±1.8
	500	–	40.8±4.0	64.0±2.7	30.9±2.2

[a] The cytotoxic activity of spleen lymphocytes from patient 10 against K562 and KATO-3 was assessed after culture with PSK at the indicated concentrations. Cytotoxicity of spleen lymphocytes was assessed by 12-h ^{51}Cr-release assay, according to the method described in Materials and methods
* P <0.05, ** P <0.01, *** P <0.001, vs medium alone

Table 5. Effect of PSK on the Generation of cytotoxic effector cells[a]

Patient	Lympho-cytes	Concentration of PSK (μg/ml)	Specific lysis (%) (mean ± SD; E/T = 10)					
			Against K562			Against KATO-3		
			Before culture	Days 2–4	Days 6–8	Before culture	Days 2–4	Days 6–8
15	PBL	Medium alone	9.0±1.5	30.9±2.3	30.9±2.7 } **	10.0±1.1	18.9±2.0	20.2±1.7
		100	–	27.3±2.5	45.4±3.6 }	–	15.0±1.7	24.9±2.1
16	PBL	Medium alone	7.4±0.6	28.0±2.1 } ***	42.8±3.4 } **	6.3±0.7	10.9±0.9	26.5±2.7 } *
		10	–	14.0±1.7 }	29.3±2.6 }	–	9.3±0.9	18.5±1.7 }
17	PBL	Medium alone	4.3±0.5	15.6±1.7 } *	47.1±4.0	4.0±0.4	10.9±1.3	20.2±2.3 } **
		100	–	19.5±1.7 }	49.0±3.3	–	11.4±1.5	36.5±3.1 }
18	SPL	Medium alone	17.8±2.2	46.0±4.4	17.3±1.8 } ***	20.2±1.8	43.7±3.5	34.3±2.9 } *
		10	–	39.7±3.8	32.1±2.2 }	–	41.0±3.2	50.6±4.6 }
19	SPL	Medium alone	10.7±1.8	22.7±1.6	13.3±0.9	4.4±0.3	10.2±0.7	7.9±0.5
		10	–	22.5±1.5	11.3±1.7	–	13.3±0.9	6.1±0.7

[a] The cytotoxic activities of PBL and spleen lymphocytes (SPL) were assessed after culture with PSK at 10 μg/ml or 100 μg/ml. Cytotoxicity of the cells was assessed by 12-h ^{51}Cr-release assay according to the method described in Materials and methods
* P <0.05, ** P <0.01, *** P <0.001

Table 6. Lymphocyte subpopulations after culturing with PSK[a]

Culture conditions	Conc. of PSK (μg/ml)	Positive cells (%)						
		OKT3$^+$	OKT4$^+$	OKT8$^+$	IL-2 Rcp$^+$	Leu7$^+$	Leu11$^+$	Leu19$^+$
Before culture	–	33.3	19.6	13.7	0.8	10.7	15.0	11.4
Medium alone	–	23.0	15.0	10.9	9.5	10.5	5.7	3.7
PSK	5	22.4	13.6	9.1	10.2	10.5	6.3	5.1
	10	23.5	12.9	8.6	11.8	10.1	4.5	4.6
	50	22.6	12.0	8.5	11.3	11.2	4.5	4.2
	100	24.5	14.9	9.5	17.5	10.1	5.3	3.6
	500	21.2	13.3	8.6	21.6	11.6	6.5	4.7

[a] Spleen lymphocytes of patient 17 were cultured with PSK at the indicated concentrations, or in medium alone for 4 days. After culture cells were washed three times in Hanks' balanced salt solution (HBSS), and the subpopulations of the cells were analyzed by flow cytometry as described in Materials and methods

the PSK was added into the media at 10 μg/ml. At higher concentrations (500 μg/ml), PSK suppressed the cytotoxicity. The dose-dependent effect of PSK on the generation of cytotoxic effector cells was assessed after spleen lymphocytes were cultured with various concentrations of PSK, using K562 and KATO-3 as targets (Table 4). The cytotoxicity of the cells was significantly augmented after 2–7 days of culturing with PSK at 5–100 μg/ml; however, culturing at 500 μg/ml for 7 days decreased the cytotoxicity against K562. In other experiments, the cytotoxicities of PBL and spleen lymphocytes were significantly augmented in three out of five patients after culturing with PSK at 10 μg/ml or 100 μg/ml, and in one patient they significantly decreased (Table 5). The changes in the lymphocyte subpopulations after culturing with PSK are summarized in Table 6. Similar results was obtained in two other experiments. The populations of OKT3$^+$, OKT4$^+$, OKT8$^+$, Leu7$^+$, Leu11$^+$, and Leu19$^+$ cells did not change after PSK culturing; however, the number of IL-2 receptor$^+$ cells increased after culturing with PSK at 100 μg/ml and 500 μg/ml. The number of cells did not increase during 7 days of culturing with PSK at 5–100 μg/ml; however, it decreased after 7 days of culturing with PSK at 500 μg/ml. The effect of PSK culture duration on the cytotoxicity against K562 was assessed in three patients (Table 7). It was found that a short period (3 h) of lymphocyte stimulation by PSK, followed by washing and culturing with PSK-free media, resulted in the generation of cells with a higher cytotoxicity than that of cells produced by continuous long-term culturing with PSK. The effect of combining PSK with rIL-2 or rIFNγ was also assessed; however, the cytotoxicities of the cells against K562 and KATO-3 were

Table 7. Cytotoxicity comparison between cells cultured with PSK for a short period and those cultured for a long period

Culture conditions	Cytotoxicity (%) against K562 (mean ± SD; E/T = 10)		
	Patient 18 5 days	Patient 21 7 days	Patient 22 5 days
Before culture	12.0 ± 1.0	4.0 ± 0.7	4.0 ± 0.5
Medium alone	24.0 ± 2.2	13.0 ± 1.1	46.0 ± 4.6
PSK-treated (3 h)[a]	43.0 ± 4.1**	42.0 ± 3.7****	50.0 ± 4.7
PSK-treated (continuous)[b]	2.0 ± 2.0	23.0 ± 1.9	45.0 ± 3.9

[a] NPBL were cultured with PSK at 10 μg/ml for 3 h, washed in HBSS three times and cultured with PSK-free media for the indicated period
[b] PBL were cultured with PSK at 10 μg/ml for the indicated period. After culture the cytotoxicity of the cells was assessed by a 12-h ^{51}Cr-release assay as described in Materials and methods
* $P < 0.05$, ** $P < 0.05$, *** $P < 0.001$

Table 8. Mitogenic activities of PSK fractions[a]

Cells	Fraction	Stimulation indices to PSK and its fractions (mean ± SD) for concentrations of PSK and its fractions of			
		10 μg/ml	50 μg/ml	100 μg/ml	500 μg/ml
PBL	PSK	1.13 ± 0.29	1.44 ± 0.39*	1.76 ± 0.47**	1.72 ± 1.71
($n = 5$, patients 4–6,11,13)	Fraction 1	0.89 ± 0.43	0.78 ± 0.22*	0.83 ± 0.18*	0.68 ± 0.16**
	Fraction 2	0.81 ± 0.28	0.81 ± 0.17*	0.85 ± 0.20*	0.81 ± 0.14*
	Fraction 3	1.28 ± 0.71	1.07 ± 0.31	1.11 ± 0.33	1.13 ± 0.24
	Fraction 4	1.37 ± 0.37*	1.48 ± 0.35*	1.69 ± 0.40**	1.94 ± 1.14
RNL	PSK	1.08 ± 0.23	1.31 ± 0.21**	1.54 ± 0.35**	0.92 ± 0.32
($n = 5$, patients 4–6,11,12)	Fraction 1	1.14 ± 0.61	1.06 ± 0.34	1.00 ± 0.21	0.72 ± 0.12***
	Fraction 2	1.12 ± 0.19	1.16 ± 0.37	0.95 ± 0.28	0.73 ± 0.16**
	Fraction 3	1.00 ± 0.42	1.22 ± 0.39	0.92 ± 0.22	0.72 ± 0.08***
	Fraction 4	1.23 ± 0.66	1.42 ± 0.38*	1.27 ± 0.41	1.12 ± 0.45

[a] PBL and RNL were cultured with PSK fractions at the indicated concentrations in 96-well flat-bottom microtiter plate for 4 days. Mitogenic activity was assessed by DNA synthesis ([^{3}H]dThd incorporation) in the cells according to the method described in Materials and methods, and was expressed using the stimulation index
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 9. Effect of PSK fractions on the generation of cytotoxic effector cells[a]

Culture conditions	Conc. of PSK or fraction (μg/ml)	Specific lysis (%) (mean ± SD; E/T = 10)			
		Patient 19		Patient 20	
		K562	KATO-3	K562	KATO-3
Before culture	–	17.5 ± 1.5	8.4 ± 1.2	22.2 ± 1.9	4.1 ± 0.7
Medium alone	–	33.0 ± 2.9	9.8 ± 1.1	37.1 ± 3.3	1.0 ± 1.3
PSK	10	29.1 ± 2.7	10.0 ± 1.1	57.0 ± 4.9**	13.2 ± 3.3**
	100	35.3 ± 2.9	13.2 ± 1.1	58.1 ± 5.9**	15.9 ± 1.8***
Fraction 1	10	28.8 ± 3.1	0.1 ± 1.7**	39.8 ± 4.2	5.7 ± 2.1*
	100	38.2 ± 3.3	4.2 ± 0.9**	46.2 ± 4.2*	1.9 ± 1.9
Fraction 2	10	36.2 ± 3.2	0.6 ± 1.4**	45.9 ± 4.2*	0.9 ± 1.5
	100	24.9 ± 3.0*	8.9 ± 0.7	39.3 ± 3.5	–0.8 ± 1.3
Fraction 3	10	43.7 ± 3.6*	13.8 ± 1.2	39.2 ± 4.0	19.7 ± 2.3***
	100	36.3 ± 3.2	10.2 ± 0.9	41.9 ± 3.7	11.0 ± 2.5**
Fraction 4	10	43.1 ± 4.0*	10.3 ± 0.8	37.0 ± 3.3	11.9 ± 2.3**
	100	50.0 ± 3.8**	25.0 ± 1.9***	48.7 ± 4.1*	14.3 ± 1.7***

[a] SPL were cultured with PSK fractions at the indicated concentrations for 4 days, and the cytotoxicity against K562 and KATO-3 was assessed by a 12-h ^{51}Cr-release assay as described in Materials and methods
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs medium alone

Table 10. Lymphocyte subpopulations after culturing with PSK fractions[a]

Culture conditions	Positive cells (%)						
	$OKT3^+$	$OKT4^+$	$OKT8^+$	IL-2 Rcp^+	$Leu7^+$	$Leu11^+$	$Leu19^+$
Before culture	30.9	16.7	12.7	9.2	3.0	4.7	9.2
Medium alone	44.2	23.7	15.3	2.8	2.3	2.2	7.2
PSK	44.1	24.0	16.9	14.9	3.1	4.5	8.4
Fraction 1	45.0	25.8	16.5	16.9	2.7	3.9	10.1
Fraction 2	47.5	27.3	18.0	10.2	2.3	4.6	6.8
Fraction 3	41.8	24.0	15.4	24.5	4.1	4.0	9.5
Fraction 4	45.2	22.8	15.1	13.8	2.8	4.5	9.1

[a] Spleen lymphocytes of patient 20 were cultured with PSK fractions at 100 μg/ml or in medium alone for 4 days. After culture cells were washed in HBSS three times, and the subpopulations of the cells were analyzed by flow cytometry as described in Materials and methods

not augmented by either of these combinations (data not shown).

PSK consists of heterogeneous protein-bound polysaccharides, and in order to determine which part of PSK has biological activity, the immunomodulating effect of PSK fractions was studied. In regard to mitogenic activity, Fraction 4 was found to increase the level of DNA synthesis. The maximum increase was obtained for PBL at 100 μg/ml and for regional node lymphocytes at 50 μg/ml of fraction 4 (Table 8). However, none of the fractions augmented the NK cell activity when they were added into the 12-h release assay (data not shown). The effect of the fractions on the generation of cytotoxic cells was assessed after culturing the cells with the fractions for 4 days (Table 9). The results indicate that the cytotoxicity was augmented by culturing the cells with fraction 3 or fraction 4. However, the cytotoxicity augmented by the fractions did not necessarily surpass that augmented by PSK. The effects of the fractions on the subpopulations are summarized in Table 10. It can be seen that the proportion of IL-2 receptor$^+$ cells increased after culturing with fraction 3.

Discussion

It has been reported that a host-mediated action may be responsible for the effect of PSK, however the mechanism for this has not yet been elucidated [2–4, 23]. It has also been reported that intraperitoneally or orally administered PSK induces cytotoxic peritoneal exudate cells and augments the NK cell activity of splenocytes in mice [2, 4, 21, 23]. Other reports have indicated that the administration of PSK increases the production of IFN and IL-2 by lymphocytes, which may explain the augmenting effect of PSK on the cytotoxicity of lymphocytes [9, 10]. Other mechanisms must also be responsible. One such mechanism is the blockage by PSK of the suppressive activity of soluble suppressor factors in cancer patients [19]. Neutrophils may also play an important role in immunomodulation by PSK, because it has been reported that cytotoxic neutrophils are induced in the peritoneal cavity by the intraperitoneal injection of PSK [8].

The present study demonstrated that PSK stimulates the lymphocytes to increase DNA synthesis *in vitro*, and

that the cytotoxic activity of the cells is augmented by culturing with PSK. The analysis of the effect of PSK administration on the subpopulations of the cell phenotypes demonstrated that PSK administration increases the proportion of IL-2 receptor$^+$ cells, which is consistent with the results of a previous report [9]. There are two possible mechanisms for this increase in the proportion of IL-2 receptor$^+$ cells; one is that the production of IL-2 receptors is stimulated by the administration of PSK, and the other is that IL-2 receptor$^+$ cells proliferate after PSK stimulation. The former may be more reasonable, because when the lymphocytes were cultured with PSK, the increase of IL-2 receptor$^+$ cells occurred before the number of cells increased during culturing. The increased proportion of IL-2 receptor$^+$ cells may be one of the mechanisms responsible for the augmented cytotoxicity. However, in the present study, the cytotoxicity of cells cultured with PSK was not augmented by the addition of rIFNγ or rIL-2. Further, our recent study has also demonstrated that the addition of anti-IFN antibody or anti-IL-2 antibody did not affect the cytotoxicity generated by PSK culture (unpublished results). These suggest that IFN and IL-2 may not be involved in the augmentation of cytotoxicity by PSK, however, the possibility that other cytokines may be involved still remains.

The administration of PSK did not augment the *in vitro* cytotoxicity of lymphocytes in all the patients. The reason for this selective phenomenon is not clear. It has been reported that the therapeutic effect of PSK correlates with the types of HLA antigens in patients and that, among patients who were administered PSK after surgery for esophageal cancer, the survival rate of patients who were HLA-B40$^+$ was significantly better than that of patients who were HLA-B40$^-$ [17]. This fact suggests that the susceptibility of the lymphocytes to PSK may be different in each patient. In the present study, culturing lymphocytes with PSK increased their level of *in vitro* DNA synthesis in all patients; however, cytotoxicity was not augmented in 40%–50% of the patients. One possible reason for this discrepancy may be that the activation of cytotoxic effector cells by PSK is restricted to those persons having a certain type of HLA antigen.

The present study also showed that high-molecular-mass (>10^5 Da) PSK fractions have both a mitogenic action and an augmenting effect on the cytotoxicity of lymphocytes. This is somewhat inconsistent with our previous study on murine lymphocytes [21], which showed that high-molecular-mass fractions had a mitogenic action and low-molecular-mass fractions had an augmenting effect on the cytotoxicity. This difference may be due to the different species used in the experiments. However these facts suggest that various combinations of PSK fractions should be studied, and ultimately employed to augment the antitumor immunity of cancer patients.

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