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Activation of human natural killer cells by the protein-bound polysaccharide PSK independently of interferon and interleukin 2

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1. Summary

The protein-bound polysaccharide PSK was tested for the ability to activate human natural killer (NK) cells. When blood lymphocytes and purified $CD3^-CD16^+$ large granular lymphocytes (LGL) were treated in vitro overnight with PSK, they demonstrated enhanced NK cell activity against K562. The PSK-activated killer cells also lysed NK-resistant targets and freshly isolated autologous and allogeneic tumor cells. The PSK effect was observed with concentrations that could be obtained in the blood of cancer patients receiving oral administration of PSK. PSK-induced enhancement of NK activity was not abrogated by monoclonal antibodies (mAb) that neutralized interferon (IFN)α, IFNγ, or interleukin-2 (IL-2). In addition, mAb reactive with p55 (α chain) or p75 (β chain) glycoproteins of IL-2 receptors had no effects on PSK-enhanced NK activity even when used simultaneously. These results indicate that the PSK could activate human NK cells independently of IFN and IL-2/IL-2R systems.

2. Introduction

Natural killer (NK) cells damage tumor cells without prior sensitization and major histocompatibility complex [1, 2]. Our recent evidence on the population and single cell levels indicates that $CD3^-CD16^+$ large granular lymphocytes (LGL) of cancer patients can lyse autologous tumor cells [3, 4], and release a cytotoxic factor, termed LGL-derived cytotoxic factor (LGL-CF), with lytic effects on autologous and allogeneic tumor cells [5].

Considerable attention has been paid to the augmentation of NK activity and the generation of cytotoxic lymphocytes capable of lysing autologous tumor cells and the use of such activated killer cells for adoptive cellular immunotherapy in patients. NK cells are rapidly activated by IL-2, resulting in enhancement of NK cell activity and generation of lymphokine-activated killer (LAK) cells that could kill autologous and allogeneic tumor cells [6, 7]. The response appears to be mediated through interaction of IL-2 with β chain (p75 molecule) of IL-2 receptors (IL-2R/p75) [8]. On the other hand, the protein-bound polysaccharide PSK derived from basidiomycetes has been undergoing clinical trial as an anti-cancer agent and reported to prolong the survival time of patients [9]. While a number of studies suggested that the primary effect of PSK is the restoration of host immunity impaired by tumor and/or antitumor chemotherapeutic agents [10, 11], the mechanisms responsible for its antitumor activity are not thoroughly understood. The present study was designed to investigate the effect of PSK on the cytolytic activity of $CD3^-CD16^+$ LGL.

Key words: Natural killer cell; PSK; Interleukin-2; IL-2R

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3. Materials and Methods

3.1. *Effector cells*

Effector cells were prepared from blood as described [3 – 5]. Mononuclear cells were isolated from blood by Ficoll-Hypaque centrifugation, and were depleted of adherent cells using autologous serum-coated dishes, Sephadex G10 columns and nylon wool columns. The nonadherent cells were then fractionated by centrifugation on discontinuous seven-step Percoll gradients. The cells collected from low-density fractions 2 and 3 usually consisted of more than 75% LGL, as judged by morphology. The Percoll-purified LGL were further depleted of T cells by treatment with anti-CD3 monoclonal antibody (mAb) (OKT3; Ortho Pharmaceutical, Tokyo, Japan) and magnetic beads. The fraction contained more than 85% LGL, as judged by reactivity to anti-CD16 mAb (OKNK; Ortho Pharmaceutical). The cells collected from high-density fractions 6 and 7 contained more than 95% T lymphocytes with less than 2% LGL.

3.2. *Target cells*

Target cells used in the present study were the NK-sensitive K562 human erythroid leukemia cell line, the relatively NK-resistant Daudi Burkitt's lymphoma line, and freshly isolated human tumor cells. Fresh human tumor cells were prepared as described [3 – 5]. Briefly, specimens of tumor tissues were obtained from 16 patients with adenocarcinoma of the lung and 11 with squamous cell carcinoma of the lung by surgical removal, and were mechanically minced and treated with DNase, collagenase and hyaluronidase. Tumor cells were then isolated by three-step discontinuous Percoll gradient centrifugation, followed by adherence to plastic dishes. Tumor cell populations having less than 10% contamination with nonmalignant cells and more than 93% viability were accepted for use.

3.3. *PSK, IFN, and IL-2*

PSK was supplied by Kureha Chemical, Tokyo, Japan [8]. Purified human recombinant IFNα (A/D) with specific activity of 1×10^8 U/mg protein was obtained from Japan Roche, Tokyo, Japan, and recombinant human IFNγ with specific activity of 1×10^8 U/ml from Daiichi Pharmaceutical, Tokyo, Japan. Recombinant human IL-2 was from Shionogi Pharmaceutical, Osaka, Japan. Effector cells at a concentration of 1×10^6/ml were incubated in serum-free T medium (Daigo, Osaka, Japan) alone, or with 100 μg PSK/ml, 3000 U IFNα/ml, 500 U IFNγ/ml, or 1000 U IL-2/ml for 18 h at 37 °C in serum-free T medium (Daigo, Osaka, Japan), unless otherwise stated, as described previously [12]. There were no differences in the recovery of viable cells cultured alone and with PSK.

3.4. *Monoclonal antibodies*

mAb directed against IFNα at 2×10^5 neutralizing activity units/mg protein, mAb against IFNγ at 2×10^4 neutralizing activity units/mg protein, and anti-IL-2 mAb containing 1×10^6 neutralizing activity units/mg protein and control serum were obtained from Cosmo-Bio, Tokyo, Japan. These mAb were added to PSK-stimulated cultures at final concentrations that neutralized 1000 U of IFNα, 200 U IFNγ, or 100 U IL-2, as described previously [12]. Tac mAb reactive with IL-2R/p55 and 2RB mAb reactive with IL-2R/p75 were supplied by Dr. T. Uchiyama, Kyoto University, Kyoto, Japan. Tac and 2RB mAb were added to lymphocyte cultures with saturating amounts (20 – 50 nmol) [13].

3.5. *Cytotoxicity assay*

A 4-h ^{51}Cr release assay was performed as described in detail elsewhere [3 – 5]. Briefly, 100 μl ^{51}Cr-labeled target cells and 100 μl effector cells were assigned at different ratios (E:T) to each well of microtiter plates. The plate was incubated for 4 h at 37 °C. Samples were then harvested, and the activity was counted in an autogamma scintillation counter. The percent cytotoxicity was calculated by the formula: % cytotoxicity = [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

3.6. *Statistical analysis*

All determinations were made in triplicate, and results were calculated as the means plus or minus

the standard deviation. Statistical significance was established by Student's t-test.

4. Results

To evaluate whether PSK augments NK cell activity, preliminary attempts were made to determine optimal conditions for activation of NK cells. Blood lymphocytes were incubated with varying concentrations of PSK for 18 h before they were tested for cytotoxicity against K562. Results of a representative experiment are shown in Fig. 1A. The maximum enhancement of NK activity was observed when lymphocytes or purified $CD3^-CD16^+$ LGL were treated with a dose of 10 – 100 μg PSK/ml. The concentrations of PSK have been found in the blood of cancer patients who had received the drug orally (unpublished observation). Lower doses of PSK had little or no effect on NK cells, and higher doses were inhibitory.

Kinetic studies in which lymphocytes were preincubated with 100 μg PSK/ml for time intervals varying from 0 to 48 h demonstrated that the augmentation of cytotoxicity became noticeable by 18 h (Fig. 1B). No further enhancement was observed after 24 – 48 h incubation.

Treatment with PSK of $CD3^-$ LGL induced cytotoxicity also against the relatively NK-resistant Daudi cells (Table 1). In addition, the PSK-activated $CD3^-$ LGL lysed autologous and allogeneic freshly isolated human tumor cells.

A possible role of IFN in the enhancement of NK activity induced by PSK was then considered. Addition of mAb that neutralized IFNα or IFNγ to PSK-stimulated lymphocyte cultures did not abrogate the PSK-induced enhancement of NK activity (Table 2). A mixture of the two mAbs was also without effect. In addition, cytotoxicity mediated by PSK-activated lymphocytes was higher than that mediated by IFN-treated ones. Furthermore, the supernatant of PSK-stimulated lymphocyte cultures contained no detectable amounts of IFN (data not shown).

The possibility that PSK activates NK cells through IL-2/IL-2R system was examined by the use of mAb directed against IL-2, IL-2R/p55, and

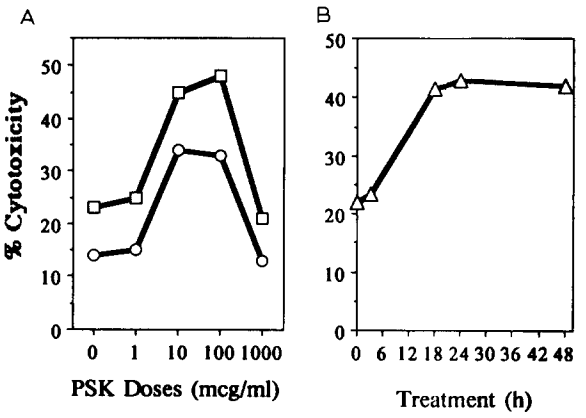

Fig. 1. PSK dose- and time-dependent augmentation NK cell activity. Lymphocytes (○) and purified $CD3^-CD16^+$ LGL (□) were treated alone or with varying concentrations of PSK for 18 h (A), or for time intervals varying from 0 to 48 h (B). They were then washed and tested for lysis of K562 cells in a 4-h Cr release assay at an E:T of 10:1. Similar results were obtained in three different experiments.

TABLE 1

Effects of PSK on cytotoxicity against NK-sensitive and NK-resistant tumor cell lines and fresh human tumor cells.

Expt.	PSK treatment[a]	% Cytotoxicity to			
		K562	Daudi	Autologous tumor	Allogeneic tumor
1	None	28 ± 3.5	5 ± 2.6	3 ± 1.2	4 ± 1.8
	PSK-treated	44 ± 4.8[b]	28 ± 3.3[b]	18 ± 4.1[b]	17 ± 2.3[b]
2	None	17 ± 2.1	4 ± 2.3	6 ± 1.9	3 ± 2.2
	PSK-treated	29 ± 3.1[b]	15 ± 1.6[b]	17 ± 1.8[b]	21 ± 3.3[b]
3	None	22 ± 1.5	8 ± 2.0	11 ± 3.1	0 ± 0.8
	PSK-treated	30 ± 1.8[b]	20 ± 2.4[b]	25 ± 2.6[b]	14 ± 1.1[b]

[a]Blood lymphocytes from a patient with squamous cell carcinoma of the lung were treated with 100 μg PSK/ml for 18 h. They were then tested for cytotoxicity against target cells shown at an E:T of 20:1. [b]Value is significantly higher than that of untreated lymphocytes at $P < 0.05$.

TABLE 2

Possible role of IFN in augmentation of NK activity by PSK.

Treatment[a]	% Cytotoxicity		
	Expt. 1	Expt. 2	Expt. 3
None	23 ± 1.9	11 ± 1.2	31 ± 2.5
PSK	39 ± 2.3[b]	20 ± 1.1[b]	43 ± 1.9[b]
PSK + control serum	36 ± 2.5[b]	19 ± 0.5[b]	44 ± 1.5[b]
PSK + anti-IFNα	38 ± 2.0[b]	22 ± 1.3[b]	42 ± 2.4[b]
PSK + anti-IFNγ	34 ± 3.8[b]	22 ± 2.0[b]	40 ± 2.3[b]
PSK + anti-IFNα + anti-IFNγ	35 ± 2.1[b]	20 ± 2.1[b]	41 ± 1.3[b]
IFNα	33 ± 2.2[b]	28 ± 1.6[b]	49 ± 3.0[b]
IFNγ	32 ± 1.7[b]	24 ± 1.7[b]	46 ± 2.7[b]

[a]Blood lymphocytes from a normal donor were treated with medium, PSK (100 μg/ml) in the presence or absence of anti-IFNα mAb and/or anti-IFNγ mAb, or with IFNα and/or IFNγ for 18 h. They were then washed and tested for cytotoxicity against K562 at an E:T of 20:1. [b]Value is significantly different from that of none at $P<0.05$.

IL-2R/p75. When lymphocytes were activated in vitro by PSK in the presence of anti-IL-2 mAb, they exhibited enhanced lysis of K562, the level of which was comparable to that seen in the absence of the mAb (Table 3). No IL-2 was detected in the supernatants produced by the PSK-stimulated lymphocyte cultures when determined in the biological CTLL assay and enzyme-linked immuno-assay (data not shown). Next, Tac mAb reactive with IL-2R/p55 and 2RB mAb directed against IL-2R/p75 were added to PSK-stimulated lymphocyte cultures. Neither anti-IL-2R/p55 mAb nor anti-IL-2R/p75 mAb abrogated the elevation of NK potential by PSK. The mixture of both mAb produced no inhibitory effect. In addition, lymphocyte samples of some individuals (#2, 6) responded only to the NK enhancing effect of PSK but not of IL-2, while others (#3, 7) responded only to IL-2 but not to PSK (Fig. 2). Thus, PSK may activate NK cells independently of IL-2/IL-2R interaction.

TABLE 3

Unlikely involvement of IL-2/IL-2R systems in PSK-induced cytotoxicity.

Treatment[a]	% Cytotoxicity	
	Expt. 1	Expt. 2
None	31 ± 2.6	16 ± 1.2
PSK	45 ± 2.7[b]	27 ± 1.5[b]
PSK + control serum	46 ± 2.3[b]	30 ± 1.6[b]
PSK + anti-IL-2	44 ± 2.5[b]	27 ± 0.8[b]
PSK + anti-IL-2R/p55	46 ± 1.9[b]	28 ± 2.0[b]
PSK + anti-IL-2R/p75	43 ± 2.2[b]	31 ± 2.1[b]
PSK + anti-IL-2R/p55 + anti-IL-2R/p75	44 ± 2.6[b]	29 ± 1.8[b]
IL-2	54 ± 3.9[b]	35 ± 3.0[b]

[a]$CD3^-$ LGL were treated with medium, PSK (100 μg/ml) in the presence or absence of anti-IL-2 mAb, anti-IL-2R/p55 mAb, anti-IL-2R/p75 mAb, or with IL-2 for 18 h. They were then washed and tested for cytotoxicity against K562 at an E:T of 10:1. [b]Value is significantly different from that of none at $P<0.05$. Similar results were obtained in two different experiments.

TABLE 4

Comparison of NK augmenting effect of PSk with that of IL-2.

Individuals	% Cytotoxicity		
	Control	PSK-treated[a]	IL-2-treated
#1	29 ± 3.5	44 ± 4.8[b]	43 ± 4.8[b]
#2	59 ± 2.6	79 ± 3.3[b]	39 ± 3.3[b]
#3	51 ± 1.2	38 ± 4.1[b]	61 ± 3.3[b]
#4	29 ± 1.8	32 ± 2.3	73 ± 3.3[b]
#5	15 ± 1.1	16 ± 3.0[b]	30 ± 3.3[b]

[a]Blood lymphocytes from a patient with squamous cell carcinoma of the lung were treated with 100 μg PSK/ml for 18 h. They were then tested for cytotoxicity against target cells shown at an E:T of 20:1. [b]Value is significantly higher than that of untreated lymphocytes at $P<0.05$. Similar results were obtained in three different experiments.

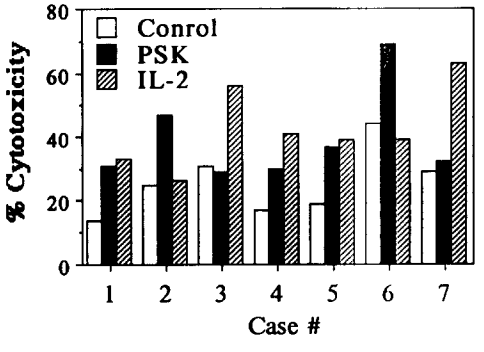

Fig. 2. Comparison of NK augmenting effects of PSK and IL-2. Lymphocyte samples of different individuals were treated either with PSK or IL-2. They were then washed and tested for lysis of K562 cells in a 4-h Cr release assay at an E:T of 20:1.

5. Discussion

The present study has demonstrated that the protein-bound polysaccharide PSK activates $CD3^-CD16^+$ LGL, resulting in a rapid and strong augmentation of the cytolytic activity. The PSK-activated killer cells were also cytotoxic to the relatively NK-resistant Daudi cells. In addition, lytic potential to freshly isolated autologous and allogeneic human tumor cells was induced by stimulation with PSK. The finding may be of clinical importance since the activation of NK cells was observed at the concentrations of PSK that are achieved in the blood of cancer patients who received standard oral administration of the drug (3 g daily). We have found that administration of PSK resulted in elevated NK activity of blood and tumor-infiltrating lymphocytes in some cancer patients.

The mechanism responsible for induction of cytotoxicity by PSK is not yet understood. IFN is a well known cytokine that enhances human and mouse NK cell activity [13]. IFN does not seem to be involved in the activation of NK cells by PSK because neutralizing mAb to IFNα or IFNγ did not abrogate the PSK-induced cytotoxicity. In addition, IFN was not detected in supernatants produced by PSK-stimulated lymphocyte cultures when determined in enzyme-linked immunosorbent assays.

Several attempts have been made to activate NK cells and generate cytotoxic lymphocytes capable of lysing fresh autologous tumor cells, and IL-2 has been implicated as an important cytokine in the induction of killer cells [7]. The activation signal is provided through specific interaction with IL-2R [8], which is composed of at least 2 subunits, p55 kDa and p75 glycoproteins [15]. The IL-2R/p75 alone is suggested to be sufficient for the internalization of IL-2 and to mediate the initial phase of a variety of IL-2-mediated responses of NK cells and T cells [16]. PSK, however, appears to induce cytotoxicity independently of the IL-2/IL-2R system for the following reasons: (1) mAb that neutralized IL-2 did not abrogate enhancement of NK activity by PSK; (2) anti-IL-2R/p55 mAb and anti-IL-2R/p75 mAb, which inhibited binding of IL-2 to the receptor, were ineffective in inhibiting the activation of NK cells by PSK; (3) cell-free supernatants from PSK-stimulated lymphocyte cultures contained no detectable amounts of IL-2 and failed to induce cytotoxicity; (4) the responsiveness of individual donors to PSK differs from that of IL-2. The findings are further supported by recent evidence that PSK does not induce IL-2 in human lymphocytes [17]. Thus, the NK effect could be up-regulated by PSK independently of the IL-2/IL-2R system.

In conclusion, PSK can augment NK cell activity in vitro independently of IFN and IL-2 induction. The mechanism by which PSK activates NK cells is not clarified in the present study. According to our preliminary experiments PSK does not augment the expression of adhesion molecules CD11a (LFA-1) and CD18 (β chain of CD11a, b, c).

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