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Immunoregulatory effects of Sizofiran (SPG) on lymphocytes and polymorphonuclear leukocytes

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SUMMARY

The effect of SPG on leukocytes has been studied in 20 patients with oral carcinoma and the actions have been analysed *in vitro*. SPG 1 mg/kg was administered intramuscularly twice weekly. Peripheral venous blood was collected before, and 1 week and 2 weeks after the initiation of SPG treatment. Both $CD16^+CD57^-$ and $CD16^-CD57^+$ cell populations were significantly increased after treatment, but no T cell subset varied. While enhancement of lymphokine-activated killer activity could not be found, an increase in natural killer (NK) activity was observed in 15 of the subjects, and the mean NK level was significantly increased from an initial $34{\cdot}7 \pm 18{\cdot}7\%$ to $46{\cdot}4 \pm 16{\cdot}5\%$ after two weeks of injections. O_2-production by polymorphonuclear leukocytes (PMNL) was stimulated 6 h after SPG injection. When PMNL were treated *in vitro* with SPG 32 μg/ml, enhanced O_2-generation was induced and protein kinase C (PKC) activity in a membrane fraction increased. SPG did not directly affect non-specific PMNL killing of K562 cells or antibody-dependent cell mediated cytotoxicity against Raji cells, but non-specific PMNL killing was enhanced by culture-conditioned medium from peripheral blood mononuclear cells (PBMC) containing 10 μg/ml SPG. Interleukin-1β, -3, -4, -6, tumour necrosis factor-α, granulocyte-macrophage colony stimulating factor and IFN-γ levels in the conditioned medium were not increased compared with medium from PBMC not treated with SPG. No clear increase of these cytokines was found in serum from the SPG-treated patients. From the above results, enhancement of PMNL O_2-generation by SPG seems to be a direct action of SPG, but the mechanism of elevation of the non-specific killing activity of PMNL and NK cells is not known. Perhaps other cytokines than those assayed have participated in increasing non-specific cytotoxicity.

Keywords Sizofiran oral cancer O_2^- generation NK cell cytotoxicity cytokines

INTRODUCTION

In recent years immunotherapy has been increasingly indicated for cancers. Inducing cancer cell-specific cytotoxic T lymphocytes (CTL) seems logically the most advantageous immunotherapy, but CTL therapy has not yet become widespread because of its technical difficulties (Darrow, Slingluff & Seigler, 1988). Consequently, non-specific immunotherapies are widely indicated, using various biological response modifiers (BRM) (Okamura *et al.*, 1989; Tamura *et al.*, 1989).

SPG, a polysaccharide isolated from culture medium of basidiomycetes, *Schizophyllum commune Fries*, has been confirmed to possess host-mediated anti-tumour and immunopotentiating activity (Sakagami *et al.*, 1988; Shimizu *et al.*, 1989; Tsuchiya *et al.*, 1989). SPG had clinically been used as a BRM, but no *in vitro* proof of direct stimulatory activity on lymphocytes or macrophages could be found and the *in vivo* anti-tmour mechanism of SPG is still obscure. We examined *in vivo* SPG effects on peripheral blood lymphocytes and neutrophils in oral cancer patients, and we also examined *in vitro* SPG influences on these cells, hoping to clarify SPG actions as a BRM.

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PATIENTS AND METHODS

Patients and treatment schedule

Twenty oral carcinoma patients were subjected to this study. The subjects were composed of 14 primary and six secondary cases who had not received any cancer therapy for at least 4 weeks before SPG administration. Patients had T2, T3 or T4 carcinoma with or without metastasis, but no terminal stage case was included. SPG 1 mg/kg was administered intramuscularly on day 1, 4, 7, 10 and 13, and heparinized blood samples were obtained at day 0, 8 and 14. There was no other treatment during SPG administration.

Cell preparation

Peripheral blood mononuclear cells (PBMC) and polymorphonuclear leukocytes (PMNL) were isolated from heparinized

blood samples by Ficoll–Paque (Pharmacia Fine Chemicals, Piscataway, NJ) separation. Layered cells lying above erythrocyte pellets were collected and contaminated erythrocytes were lysed by hypotonic shock with sterile distilled water for 30 s. The collected cells were washed twice in phosphate-buffered saline (PBS, pH 7·2) before readjustment to desired concentrations. Cytocentrifuged preparations of PMNL were stained with Giemsa, and it was microscopically ascertained that the preparations were composed of more than 96% PMNL. PBMC collected from the interface layer between upper plasma and lower Ficoll–Paque were washed with PBS, and non-adherent lymphocytes were separated from adherent cells by culturing in plastic dishes for 1 h. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum.

Two-colour analysis of lymphocyte surface phenotype

The lymphocyte surface phenotype was simultaneously examined with the cytotoxicity assay. Both monoclonal FITC-conjugated (anti-CD3; Leu4, anti-CD4, Leu3a, anti-CD8; Leu2a and anti-CD57; Leu7) and PE-conjugated (anti-CD16; Leu11c, anti-Leu8 and anti-HLA-DR, Becton Dickinson Immunocytometry Systems, Mountain View, CA) antibodies were used for double colour analysis. Combinations of anti-CD8 with anti-Leu8, anti-CD4 with anti-Leu8, anti-CD57 with anti-CD16, and anti-CD3 with anti-HLA-DR were used. Isolated lymphocytes of 5×10^5 cells were resuspended in 100 μl PBS containing 0·1% NaN_3 and packed into culture tubes. After saturating with both three-fold-diluted FITC- and PE-conjugated antibody solutions, the tubes were incubated for 30 min on ice in a dark place. Cells were then washed twice, resuspended in a 0·1% NaN_3 solution, and analysed with an EPICS V flow cytometer (Coulter, Hialeah, FL).

Cytotoxicity assay

Both natural killer (NK) and lymphokine-activated killer (LAK) activities were measured by a ^{51}Cr release assay using K562 cells and Daudi cells as targets for NK and LAK cells, respectively. LAK cells were induced from patients' PBMC by culturing in the medium containing 100 U/ml human recombinant IL-2 (Shionogi Pharmaceutical Co., Osaka, Japan) for 3 days. The targets were labelled with $Na_2\ ^{51}CrO_2$ (200 μCi/ml, New England Nuclear, Boston, MA), washed, and added to lymphocyte suspensions in microtitre plates at an effector/target (E/T) ratio of 25/1. After incubation at 37°C for 4 h, the plates were centrifuged and the radioactivity of the supernatants was measured by an auto-gamma counter. The percentage of specific tumour cell lysis was calculated by the formula:

$$\% \text{ Specific lysis} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100$$

where spontaneous isotope release was determined in the supernatant from the target cell culture only.

PMNL non-specific cytotoxicity was investigated using a procedure similar to that for NK activity assay. PMNL were cocultured with labelled K562 cells at an E/T ratio of 25/1 in the medium containing 10 μg/ml phorbolmyristate acetate (PMA, Sigma Chemical Co., St. Louis, MO) for 4 h. The radioactivities in the supernatants were measured. and the percentage of specific lysis was calculated by the above formula. PMNL antibody-dependent cell mediated cytotoxicity (ADCC) was also measured by ^{51}Cr release assay using Raji cells as the targets. Antiserum against Raji cells was prepared by immunizing rabbits with Raji cells. Labelled target cells were mixed with effector cells at an E/T ratio of 50/1, and heat (56°C)-inactivated anti-Raji antiserum was added to the assay mixture making the final concentration 2%. After 4 h incubation the ^{51}Cr release was determined in the supernatants. The percentage of cytotoxicity was calculated by the formula shown above. All cytotoxicity assays were triplicated, and each value shown in the tables and figures is the mean of the triplicates.

Quantification of cytokines

Cytokines in patients' serum and the culture supernatants were measured using ELISA kits for IL-1β (Cistron Biotechnology, Pine Brook, NJ), IL-3 (R & D Systems, Minneapolis, MN), IL-4 (R & D Systems), IL-6 (R & D Systems), tumour necrosis factor-α (TNF-α, T Cell Science, Cambridge, MA) and granulocyte-macrophage colony stimulating factor (GM-CSF, Genzyme, Boston, MA), and radioimmunoassay kit for IFN-γ (Centocor, Malvern, PA).

O_2^- assay

O_2^- generation of PMNL was assayed by a cytochrome C reduction method. PMNL (2×10^6 cells) were resuspended in 2 ml Hank's balanced salt solution (HBSS) containing 100 μM cytochrome C and 1 mM NaN_3. After adding each stimulation agent (50 ng/ml PMA, 5 μM A23187, 2·5 mg/ml opsonized zymosan, or 1 μM N-formyl-methionyl-leucyl-phenylalanine (FMLP) Sigma), cytochrome C reduction was estimated by the absorbance change at 550–540 nm using a Shimazu UV300 dual beam spectrophotometer. Generated O_2^- was calculated from the O_2^- dismutase-inhibitable cytochrome C reduction curve, using the linear phase.

PKC activity assay

PKC activity was assayed in both cytosol and membrane fractions. PMNL were disrupted by sonication for 15 s × 4 at 4°C in an extraction buffer solution as described elsewhere (Balazovich, Smolen & Boxer, 1986). Sonicates were centrifuged at 500 *g* for 10 min to remove nuclei and unbroken cells, and then centrifuged at 100 000 *g* for 15 min. The supernatant was defined as the cytosol fraction. The pellets were resuspended in the extraction buffer solution, sonicated again for 15 s × 4, and the obtained supernatant was defined as the membrane fraction. PKC activity was estimated by using a PKC assay kit (Amersham, Amersham, UK) according to the manufacturer's instructions. Each fractionated cytosol or membrane aliquot was added at 25 μl to 50 μl of the PKC assay mixture (final concentrations of 50 mM Tris-HCl; pH 7·5, 1 mM calcium acetate, 0·67 mole % Lα-phosphatidyl-L-serine, 2 μg/ml PMA, 75 μM peptide, 2·5 mM dithiothreitol, 50 μM ATP, 15 mM magnesium acetate, 0·05% w/v sodium azide, including 0·2 μCi/25 μl of assay solution of [γ-^{32}PI]ATP). The reaction was allowed to proceed for 15 min at 25°C and stopped by the addition of 100 μl of an ice-cold acidic reaction-quenching reagent. The terminated reaction mixture was allowed to soak into binding papers and washed twice in 5% acetic acid for 10 min. After washing, binding papers were placed in scintillation vials, and incorporated ^{32}P was quantified with a liquid scintillation counter. PKC activity was expressed as picomoles ^{32}P incorporated into peptide in 1 min per 10^7 cells.

Table 1. Influence of SPG on lymphocyte subset proportions

Subsets	Before	1 week	2 weeks
$CD8^+CD11b^-$	12·9±8·6*	13·3±7·3	14·3±7·2
$CD8^+CD11b^+$	1·7±0·6	1·7±0·5	1·8±0·6
$CD4^+Leu8^-$	13·4±6·2	13·3±6·0	13·6±4·9
$CD4^+Leu8^+$	12·1±7·9	12·6±7·3	14·6±8·7
$CD3^+$	48·8±17·2	51·6±13·1	51·0±12·9
$CD3^+HLA\text{-}DR^+$	3·0±2·8	2·6±2·0	2·4±1·9
$CD16^-CD57^+$	18·3±9·1	23·6±12·1†	24·6±13·4†
$CD16^+CD57^+$	3·1±3·5	3·2±2·6	4·4±3·1
$CD16^+CD57^-$	6·5±3·5	7·3±2·9	10·9±5·6†

*Indicated values represent mean (%)±1 s.d. of 20 patients.

†Statistically significant differences between before and after treatment were found in both $CD16^-CD57^+$ and $CD16^+CD57^-$ subsets (paired t-test, $P<0·01$ in both).

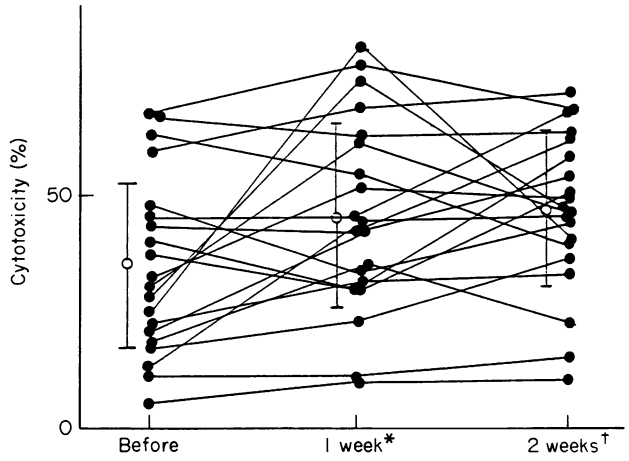

Fig. 1. NK activity against K562 cells in SPG-injected patients. Cytotoxicities were determined by 4 h ^{51}Cr release assay at an E/T ratio of 25/1. A statistically significant difference was found between before and after both 1 week and 2 week treatment (paired t-test, *$P<0·05$, †$P<0·01$). ⊻, mean±1 s.d.

Statistical analysis

Values were analysed by the paired t-test between, before and after SPG treatment, and each value after treatment was considered to be significantly different from the original value when $P<0·05$.

RESULTS

Lymphocyte subset populations

Flow-cytometric analysis of peripheral blood lymphocytes revealed that the T cell subset populations were not influenced by SPG, there being no clear difference between the original T cell subset populations and those after treatment (Table 1). As to NK subsets, $CD16^-CD57^+$ and $CD16^+CD57^-$ cell populations significantly increased after treatment ($P<0·01$), though the $CD16^+CD57^+$ cell proportion did not change.

Table 2. Effect of SPG-treated PBMC culture supernatant on PMNL cytotoxicity

Source of PMNL	PMA (−) (supernatant) (−)	PMA (−) (supernatant) I	PMA (−) (supernatant) II	PMA (+) (supernatant) (−)	PMA (+) (supernatant) I	PMA (+) (supernatant) II
A	4·7*	14·0	ND	9·0	15·7	ND
B	3·7	9·4	ND	8·0	11·5	ND
C	6·5	11·1	7·8	11·1	14·9	13·5

PBMC from healthy donor I and II were cultured with 50 μg/ml SPG for 24 h, and PMNL from three other healthy donors (A, B and C) were treated with or without each SPG-treated PBMC culture supernatant for 3 h. After washing, PMNL were co-incubated with ^{51}Cr-labelled K562 cells at the ratio of 25/1 for 4 h in the presence or absence of PMA (10 ng/ml).

*Values represent the mean (%) cytotoxicity of triplicated assays. Standard deviations were consistently <10% of the mean.

Cytotoxicities

Killing activity against Daudi cells (LAK activity) was about 60% even after treatment (data not shown), and enhancement of LAK activity by SPG could not be observed. NK cytotoxicity against K562 cells comparatively increased after SPG treatment (Fig. 1). Out of 20 subjects, NK activity in 15 patients increased within 2 weeks, and mean NK activity increased from its original 34·7±18·7% to 45·4±20·3% at 1 week of treatment and to 46·4±16·5% at 2 weeks of treatment. NK activity showed a wide distribution, and statistical significance of the upregulatory SPG effect was examined by paired t-test. Paired t-test revealed the increase at both times was significant ($P<0·05$ and $P<0·01$, respectively). However, *in vitro* SPG treatment of lymphocytes did not result in enhancement of NK activity (data not shown).

Non-specific PMNL cytotoxicity against K562 cells was 4–6% when the effectors were cultured with the supernatant of SPG-free PBMC culture, and the activity increased up to about 10% when PMNL were cultured with SPG-treated PBMC medium (Table 2). The addition of PMA (10 ng/ml) to effector and target-mixed cultures upregulated PMNL cytotoxicity to 8–11%, and the cytotoxicities were further enhanced by several percentage points by adding SPG-treated PBMC medium to the mixed culture. Killing activity of PMNL against Raji cells increased by rabbit anti-serum against Raji cells (Table 3). Thus, ADCC was noted in the PMNL–Raji cell system, but ADCC was not affected by pretreatment of PMNL with 50 μg/ml SPG.

Cytokine production

Serum cytokine levels were measured before and 3 h after the second administration of SPG (Table 4). In serum from non-treated cancer patients, IL-3 (~640 pg/ml), IL-4 (~290 pg/ml), IL-6 (~580 pg/ml) and TNF-α (~355 pg/ml) were detected in more than half of the patients. In the cytokines examined, IL-6 most clearly existed in serum, and its minimal level was 46 pg/ml. Contrary to those cytokines, IL-1β, GM-CSF and IFN-γ were scarcely detected in serum. Each serum cytokine level after treatment did not exceed each respective initial level except for a few patients. TNF-α, which was found in small amounts in serum before treatment, decreased in many cases after SPG

Table 3. ADCC activity of PMNL against Raji cells

		Healthy donor				
	Antiserum	A	B	C	D	Mean ± 1 s.d.
SPG-pretreatment (−)	−*	5·3	8·3	9·8	9·0	8·1 ± 2·0
	+†	21·1	22·6	37·6	48·9	32·6 ± 13·2
SPG-pretreatment (+)	−	4·9	9·0	9·8	9·8	8·4 ± 2·3
	+	29·8	30·1	36·8	41·4	34·5 ± 5·6

PMNL pretreated with or without 50 μg/ml SPG for 2 h were co-incubated with ^{51}Cr-labelled Raji cells (E/T = 100/1) in the medium containing 1% anti-Raji cell rabbit antiserum or normal rabbit serum. After 12 h, supernatant radioactivity was counted by a gamma counter, and per cent cytotoxicity was calculated. Values represent the mean of triplicates. Standard deviations were consistently < 10% of the mean.

*Normal rabbit serum.

†Anti-Raji cell antiserum.

Table 4. Cytokine levels in serum from SPG-injected cancer patients

	IL-1β		IL-3		IL-4		IL-6		TNF-α		GM-CSF		IFN-γ	
Patients	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
A	0	0	140	96	290	260	160	0	95	0	<6	7	<0·5	<0·5
B	0	0	0	0	39	<30	185	44	355	0	7	<6	<0·5	<0·5
C	0	0	0	0	0	0	66	0	225	0	7	8	<0·5	<0·5
D	0	0	640	700	180	240	580	500	25	0	<6	<6	<0·5	<0·5
E	0	0	54	35	55	<30	46	0	0	0	<6	<6	<0·5	<0·5
F	0	0	ND	ND	ND	ND	300	380	0	0	8	<6	ND	ND
G	0	0	ND	ND	ND	ND	400	440	0	0	10	11	ND	ND
H	40	0	ND	ND	ND	ND	94	75	50	20	10	8	ND	ND

SPG (40 mg) was intramuscularly injected on day 1 and 4, and blood samples were obtained 3 h after the second injection. Each value shows pg/ml except for IFN-γ (U/ml), and represents the mean of duplicates.

administration. Those cytokine kinetics were not related with T-stages.

PMNL spontaneously produced only low IL-6 (~236 pg/ml) during 24 h culture without SPG, whereas PBMC produced not only high level IL-6 (~2650 pg/ml) but also remarkable levels of IL-1β (~402 pg/ml) and TNF-α (~235 pg/ml) (Table 5). No enhanced cytokine production could be observed even though PMNL or PBMC were treated with 10 μg/ml SPG for 24 h.

PMNL O_2^- generation

PMNL O_2^- generation increased to 135·8 ± 52·1 at 6 h after the second SPG administration from its initial level of 96·7 ± 31·3 pmol/min, and the generation tended to decrease at 12 h (Fig. 2). In order to ascertain SPG effects on PMNL O_2^- generation, O_2^- productivity was tested *in vitro* using PMNL from both healthy controls and cancer patients (Table 6). O_2^- generation of healthy controls' PMNL was enhanced by SPG pretreatment. When A23187 was used as the inducer, O_2^- production of SPG (32 μg/ml)-pretreated PMNL was upregulated about two-fold, i.e. from 68·1 ± 7·5 in non-treatment to 115·2 ± 12·8 pmol/min in pretreatment with SPG. O_2^- generation of PMNL from cancer patients was inferior to that of controls, corresponding to about 80% of the latter in each O_2^- inducer. About 20% upregulation of O_2^- generation resulted from control PMNL treatment with 32 μg/ml SPG. SPG treatment in patients' PMNL also enhanced O_2^- generation by about 20%, and the respiratory burst was reached at the SPG-non-treated control level.

PKC activity

One minute after PMNL treatment with 50 ng/ml PMA, PKC activity in cytosol was 693 ± 95 pmol, being less than the control (without PMA stimulation) level of 801 ± 96 pmol (Table 7). On the contrary, PKC activity in membrane increased from the control level of 197 ± 205 pmol to 453 ± 87 pmol. When PMNL were treated with 50 μg/ml SPG, PKC activity changed little for 1 min after treatment, but at 5 min PKC activity in membrane increased to 443 ± 67 pmol, corresponding with the decrease of the activity in cytosol. At 30 min after PBMC treatment with 50 μg/ml SPG, PKC activity in cytosol also decreased to 539 ± 25 pmol from the control level of 654 ± 96 pmol (Table 8). Contrarily, PKC activity in membrane fraction increased to 327 ± 8 pmol, exceeding the control level of 235 ± 90 pmol.

Table 5. *In vitro* SPG influence on cytokine production by PMNL and PBMC

Donor cell		SPG	IL-1β (pg/ml)	IL-3 (pg/ml)	IL-4 (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)	GM-CSF (pg/ml)	IFN-γ (U/ml)
A	PMNL	—	0	<30	0	150	0	<6	<0·5
		10 μg	0	<30	0	185	0	<6	<0·5
	PBMC	—	75	<30	0	318	63	10	<0·5
		10 μg	85	<30	0	195	45	<6	<0·5
B	PMNL	—	0	<30	0	0	0	<6	<0·5
		10 μg	0	<30	0	0	0	0	<0·5
	PBMC	—	402	<30	0	2650	88	<6	<0·5
		10 μg	358	<30	0	2600	130	<6	<0·5
C	PMNL	—	0	<30	0	148	0	0	<0·5
		10 μg	0	<30	0	24	0	0	<0·5
	PBMC	—	208	<30	0	1900	45	<6	<0·5
		10 μg	208	<30	0	2175	45	<6	<0·5
D	PMNL	—	0	ND	ND	236	0	0	ND
		10 μg	0	ND	ND	145	0	<6	ND
	PBMC	—	390	ND	ND	950	235	<6	ND
		10 μg	285	ND	ND	810	178	0	ND

PMNL or PBMC from four healthy donors were cultured with or without 10 μg/ml SPG for 24 h, and cytokine levels in the culture supernatants were assayed.

Each value represents the mean of duplicated assays.

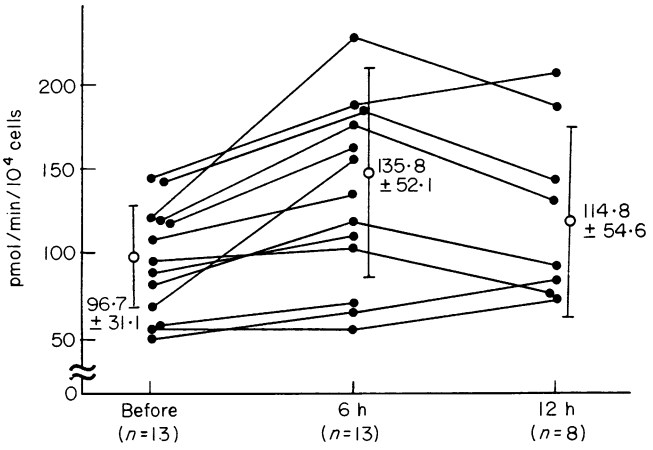

Fig. 2. Kinetics of O_2^- generation by PMNL after the second SPG injection. A significant increase was found at 6 h (paired *t*-test, $P < 0{\cdot}01$). ⊼, mean ± 1 s.d.

DISCUSSION

Upregulation of immunosurveillance against malignant tumours by BRMs has been clarified. Regarding SPG, actions such as enhancement of NK and macrophage cytotoxic activity (Sugawara, Lee & Wong, 1984), activation of CTL (Shimizu *et al.*, 1989), co-operation in induction of IFN production by lymphocytes (Sakagami *et al.*, 1988) and immunopotentiation of the body (Tsuchiya *et al.*, 1989) have been reported. However, many of these investigations were obtained *in vivo* using animals. Unlike the bacterial substrate OK-432 (Bonavida & Jewett, 1989) and BCG-CWS (Toko & Fujimoto, 1989), and also cytokines (Klasa, Silver & Kong, 1990), *in vitro* direct actions of SPG on macrophages and lymphocytes have not yet been examined. One of the reasons why the mechanisms of SPG actions have not been sufficiently analysed seems to be that SPG has mainly been studied *in vivo* and the obtained results were not ascertained *in vitro*. Thus, details of SPG actions in cancer patients remain unknown. We administered SPG to oral cancer patients and examined some immunological parameters, and also investigated SPG *in vitro* with the aim of analysing and identifying the clinical results.

In lymphocyte subsets, T cell subset populations did not change after SPG administration. Comparatively, NK subset populations of $CD16^-CD57^+$ and $CD16^+CD57^-$ cells significantly increased by SPG, and at the same time an increase of NK activity could be observed at 1 week after SPG administration. Meanwhile, *in vitro* examination revealed that unlike OK-432 (Uchida & Micksche, 1981), SPG does not directly enhance NK activity (data not shown). Tsuchiya *et al.* (1989) reported the existence of bone marrow-stimulating soluble factor in serum from SPG-injected mice, and they considered that the factor promotes NK cell differentiation and maturation resulting in enhanced NK activity. Together with their consideration and our results, it seems likely that *in vivo* SPG acts on white blood cells such as macrophages or neutrophils and causes them to produce NK cell-proliferating and maturing cytokine(s), with these cytokines then enhancing NK cell cytotoxicity. Thus, cytokine levels in serum from the patients and supernatants of SPG-stimulated PMNL and PBMC were examined. However, neither IL-1β, -3, -4, -6, TNF-α, GM-CSF nor IFN-γ was increased in serum or the supernatants, and the increase of NK activity by SPG could not be analysed.

PMNL O_2^- generation increased at 6 h after the second SPG injection. *In vitro* examinations revealed that the inferior O_2^- productivity of cancer patients' PMNL was upregulated by SPG

Table 6. Influence of SPG on PMNL O_2^- generation

	Stimulants					
	PMA (50 ng/ml)		OZ (2·5 mg/ml)		A23187 (5 μM)	
Pretreatment* SPG (μg/ml)	Control ($n=8$)	Patient ($n=4$)	Control ($n=5$)	Patient ($n=4$)	Control ($n=5$)	Patient ($n=4$)
0	109·9±7·8†	89·0±7·5	57·6±4·5	47·6±5·4	68·1±7·5	57·6±8·2
2	109·7±9·5	ND	57·1±6·8	ND	ND	ND
8	113·9±11·8	ND	68·1±8·5	ND	ND	ND
32	117·0±12·5	107·4±11·3	69·9±9·2	51·3±8·4	115·2±12·8	83·5±9·2

*PMNL were pretreated with each indicated volume of SPG for 5 min and each O_2^- stimulant was added.
†Generated O_2^- was assayed by a cytochrome C reduction method. Results represent a mean (pmol/min per 10^4 cells) ± 1 s.d.

Table 7. Influence of SPG and PMA on PMNL PKC activity

			SPG		PMA	
Fraction	Treatment time (min)	(Medium)	10 μg/ml	50 μg/ml	10 ng/ml	50 ng/ml
Cytosol	1	801±96	705±78	842±5	675±24*	693±95*
	5	907±127	888±29	867±46	805±73	675±42†
Membrane	1	197±205	199±89	205±48	247±67	453±87*
	5	258±188	292±148	443±67*	195±10	298±258

PMNL from healthy persons were treated with SPG or PMA for the indicated time, and the cells were fractionated into cytosol and membrane. PKC activity in each fraction was then estimated by the method shown in Patients and Methods. Each value indicated represents mean (pmol/min per 10^7 cells) ± 1 s.d. of five healthy donors. Significant differences against medium control were found in both SPG and PMN treatments (*, $P<0·05$; †, $P<0·01$).

Table 8. Influence of SPG and PMA on PKC activity of PBMC

			SPG		PMA	
Fraction	Treatment time (min)	(Medium)	10 μg/ml	50 μg/ml	10 ng/ml	50 ng/ml
Cytosol	1	759±12	ND	746±42	ND	506±34†
	5	624±125	655±107	597±42	ND	334±2†
	30	654±96	702±88	539±25*	196±46†	347±45†
Membrane	1	142±20	ND	143±295	ND	278±76†
	5	270±71	221±37	276±30	ND	337±2
	30	235±90	209±67	327±8*	589±25	359±11*

PKC activity in each fraction was estimated by the same method as described in Table 7. The values represent mean (pmol/min per 10^7 cells) ± 1 s.d. Significant differences against the medium control were found in both SPG and PMA treatments (*, $P<0·05$; †, $P<0·01$).

to near the healthy control level. The enhancing action of SPG was most clearly observed when A23187 (Ca^{++} ionophore) was used as an O_2^- inducer, and a slight upregulatory effect of SPG was noted when using PMA as the inducer. Therefore, it can be considered that SPG acts on multiple stimulating pathways for induction of O_2^- generation. PKC has been stated to be a key enzyme for neutrophil activation (Gerard *et al.*, 1986). Investigators (Nishizuka, 1986; Szamel *et al.*, 1990; Atluru *et al.*, 1990) mentioned that PKC is associated with activation of not only neutrophils but also many other kinds of cells, and that PKC translocates from cytosol to cell membrane when an activating stimulation is added . We observed the same

phenomenon on 50 μg/ml SPG-treated PMNL and PBMC, i.e. decrease of PKC activity in cytosol and contrary increase of the activity in membrane fraction (Tables 7 and 8). From these results, it may be a mechanism of upregulatory SPG action that causes SPG to act directly on PKC and increase membrane PKC activity, and results in enhanced cell reactivity to O_2^- generation stimulators. SPG did not affect NK activity and ADCC of PMNL even though SPG upregulated the O_2^- generation system. Morikawa *et al.* (1985) reported that neutrophil killing activity on tumour cells was mainly mediated by hydrogen peroxide (H_2O_2), and that bacterial substrates such as OK-432 upregulated neutrophil cytotoxicity by enhancing H_2O_2 production. As mentioned above, O_2^- generation was increased by SPG although H_2O_2 production was not examined. Superoxide is oxidized when reacting with H^+, and converted into H_2O_2 in an extremely short time (Nabi *et al.*, 1979; Makino *et al.*, 1986). Therefore, elevated O_2^- generation may be synonymous with elevation of PMNL killing activity. However, the increase of NK and ADCC activity caused by SPG could not be obtained. This may have been because SPG-induced O_2^- was at too low a level and thus insufficient to enhance PMNL cytotoxicity.

Direct enhancing ability of SPG was not observed in PMNL killing, but killing in K562 targets was enhanced by PMNL treatment with the culture supernatant of SPG-treated PBMC. PKC translocation from cytosol to cell membrane was, however, not so remarkable as in PMNL, also observed when PBMC were treated with SPG. Therefore, it can be thought that PBMC was directly stimulated to produce soluble factors which enhance PMNL killing. Certain levels of IL-3, -4, -6 and TNF-α existed in patients' serum, but such cytokines as IL-1β, GM-CSF and IFN-γ which can stimulate neutrophils to kill more non-specific targets, could hardly be observed even after SPG administration. Neither were these cytokines found in culture supernatants of SPG-treated PBMC. From these results, soluble factor(s) without the above cytokines which may enhance neutrophil killing seem to be generated from PBMC by SPG.

SPG increased NK cell counts and its activity and also enhanced PMNL O_2^- generation, regardless of the tumour stages. Among these SPG actions, only upregulation of O_2^- generation could be understood as a result of direct SPG action on PMNL. Mechanisms of other SPG actions on white blood cells remain to be analysed. More investigations on the influence of SPG are necessary, not only for analysis of the reagent actions but also regarding its usefulness in cancer therapy.

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