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Abstract

In order to improve the postoperative prognosis of gastric cancer patients we have performed preoperative endoscopic intratumoral administration of various biological response modifiers. In the present study we have investigated the kinetics and the immune response augmenting effect of intratumorally injected PSK, a protein-bound polysaccharide preparation, by immunohistochemical methods using anti-PSK antibody and various other antibodies. PSK-containing cells were located in the tumor tissues and follicular marginal zones of regional lymph nodes. Intratumorally administered PSK appeared to be phagocytized by the histiocytes and to cause them to become antigen-presenting cells. These cells may play a major role in augmenting immune responses in gastric cancer patients.

KEYWORDS: PSK, immunohistochemistry, gastric cancer

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The Effect and Distribution of a Protein-Bound Polysaccharide Preparation, PSK (Krestin[®]), Intratumorally Injected Prior to Surgery into Gastric Cancer Patients

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In order to improve the postoperative prognosis of gastric cancer patients we have performed preoperative endoscopic intratumoral administration of various biological response modifiers. In the present study we have investigated the kinetics and the immune response augmenting effect of intratumorally injected PSK, a protein-bound polysaccharide preparation, by immunohistochemical methods using anti-PSK antibody and various other antibodies. PSK-containing cells were located in the tumor tissues and follicular marginal zones of regional lymph nodes. Intratumorally administered PSK appeared to be phagocytized by the histiocytes and to cause them to become antigen-presenting cells. These cells may play a major role in augmenting immune responses in gastric cancer patients.

Key words : PSK, immunohistochemistry, gastric cancer

A variety of biological response modifiers (BRMs) have been utilized to enhance antitumor activity in cancer patients and to improve their prognosis. Since 1978, we have endoscopically infused a variety of BRMs prior to surgery in order to evaluate their antitumor effect. We reported that intratumoral injection of BRMs leads to the enhancement of both lymphocytic infiltration within the tumor site and antitumor activity (1-4). However, the mechanisms whereby BRMs augment antitumor activity have not yet been investigated in detail. Therefore, we investigated the kinetics and immune response-augmenting mechanism of locally

administered PSK, a protein-bound polysaccharide preparation (5) by immunohistochemical staining using anti-PSK and other antibodies.

Materials and Methods

Subjects and antibodies used. PSK (Krestin[®], Kureha Chemical Ind., Co., Ltd., Tokyo, Japan) is a protein-bound polysaccharide extracted and purified from cultivated mycelia of *Coriolus versicolor* CM-101 belonging to Basidiomycetes. This preparation contains 18-38% protein which consists of 18 kinds of amino acids. Mean molecular weight of PSK is about 10^5 by ultracentrifugation technique and its estimated structure of polysac-

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charide portion of PSK comprises α - or β -glucan with 1 \rightarrow 4, 1 \rightarrow 3 or 1 \rightarrow 6 branches. Two hundred fifty mg/5 ml of PSK was injected endoscopically into tumor sites of 18 gastric cancer patients 7 days before surgery. Resected stomach and dissected lymph node specimens were used for immunostaining (Table 1).

Table 1 Antibodies used

Antibody	Specificity
Anti-PSK	PSK
S-100 protein	Dendritic cells
OKT 6	Thymic T-lymphocytes
OKIa 1	B-cells, monocytes, activated T-cells
OKM 1	Monocytes, null cells, granulocytes

Polyclonal antibody against PSK and staining method. In order to observe localization of PSK, paraffin sections were subjected to indirect immunofluorescent staining with anti-PSK antibody (Kureha Chemical Ind., Co., Ltd.) (5). After the removal of paraffin, anti-PSK antibody was dropped onto the sections, which were then incubated for 1 h at room temperature in a moist chamber. The sections were rinsed three times with phosphate-buffered saline (pH 7.4) (PBS), stained with fluorescein-labelled anti-rabbit IgG antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA), incubated for 30 min at room temperature in a moist chamber and then rinsed in PBS. The stained tissue was examined under a Vanox Universal Microscope equipped with a Model BHS-RFK-AI attachment for fluorescent microscopy (Olympus Optical Co., Ltd., Tokyo, Japan).

Double immunohistochemical staining. Double staining with anti-PSK antibody and anti-S-100 protein antibody (α -chain-specific, Ohtsuka Pharmaceutical Co., Ltd., Tokyo, Japan) was performed. Deparaffinized sections were stained with anti-PSK antibody, followed by fluorescein-labelled anti-rabbit IgG antibody to produce a green reaction product. The sections were then stained with anti-S-100 protein antibody, followed by phycoerythrin-labelled anti-mouse IgG antibody (Vector Lab., Inc., Burlingame, CA, USA) to yield a red reaction product. Deparaffinized sections were also stained for α -naphthyl-acetate esterase (ANAE) (6).

Other monoclonal antibodies. Cryostat sec-

tions of fresh lymph nodes were treated with monoclonal antibodies. Acetone-fixed sections were allowed to react with the monoclonal antibodies OKT6, OKIa1 and OKM1 (Ortho Diagnostic Systems, Inc., Raritan, NJ, USA) overnight at 4°C. After being washed three times with PBS, they were incubated with biotin-conjugated horse anti-mouse immunoglobulin antibodies and avidin-biotin peroxidase complex (ABC Kit PK-4002, Vector Labs., Inc.). Finally the sections were soaked in 0.02% 3, 3-diaminobenzidine (DAB) solution with 0.03% H₂O₂ for 3 to 5 min and counterstained with Meyer's hematoxylin solution.

Results

Anti-PSK antibody positive (*i. e.* reactive with anti-PSK antibody) cells were observed at the PSK injected site (Figs. 1a and b). Fluorescent granular substances were observed in the cytoplasm of histiocytes in the tumor and its periphery as contrasted with hematoxylin-eosin staining of the same area. The injected PSK is seemed to be phagocytized by histiocytes.

In the regional lymph nodes (7), anti-PSK antibody positive cells were found at follicular marginal zones and marginal sinuses (Fig. 2).

The results of double staining with anti-PSK antibody and anti-S-100 protein antibody in the regional lymph nodes are shown in Fig. 3. Some cells were stained green by fluorescein indicating that these cells were anti-PSK antibody positive (Fig. 3a). Many of these cells were stained red by

Fig. 1 Paraffin section from the PSK-injection site. (a) Indirect immunofluorescent staining was performed with anti-PSK antibody and fluorescein-labelled anti-rabbit IgG antibody. The immunoreactivity is present in cytoplasm of histiocytes with granular immunostaining (arrowheads). (b) Hematoxylineosin staining of the same area as seen in (a).

Fig. 2 Paraffin section from regional lymph node immunostained with anti-PSK antibody and fluorescein-labelled anti-rabbit IgG antibody. Positive cells are mainly located in the marginal zone of the follicles (arrowheads).

Fig 1 ▶

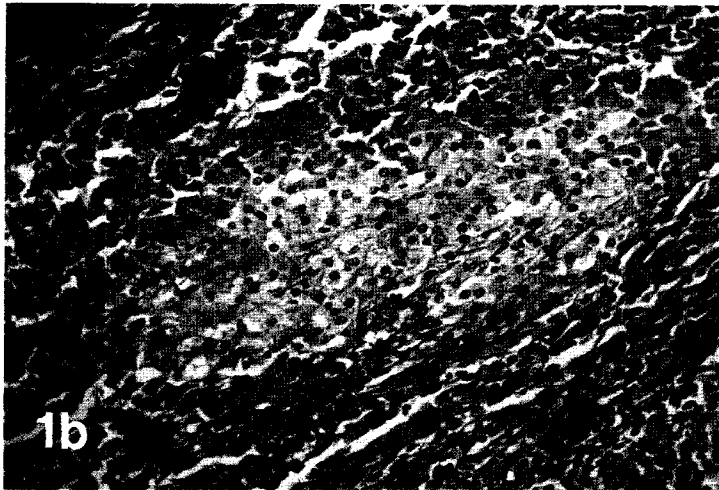
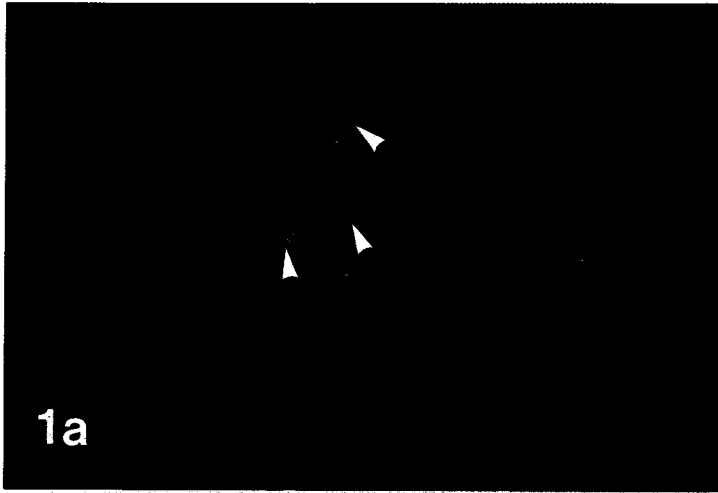
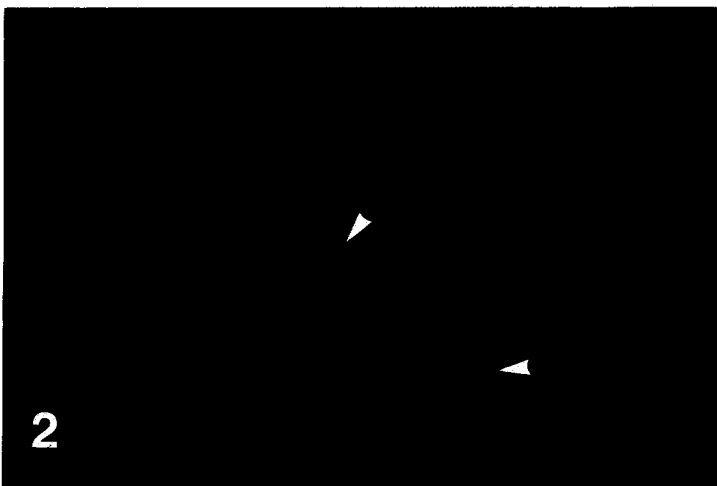


Fig 2 ▶



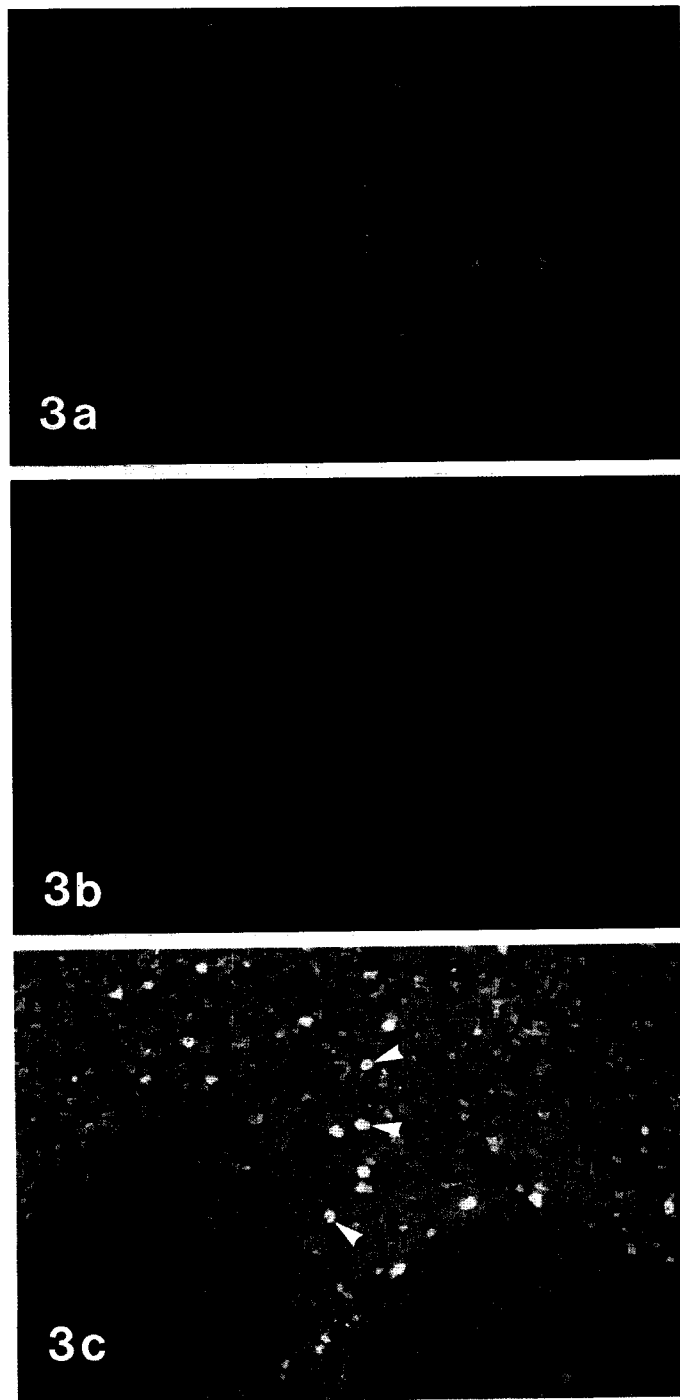


Fig. 3 Double immunochemical staining of the regional lymph node with anti-PSK antibody and anti-S-100 protein antibody. Anti-PSK antibody positive cells are stained green by fluorescein (a) and S-100 protein positive cells were stained red by phycoerythrin (b). Many of anti-PSK positive cells are S-100 protein positive which were stained yellow by the double immunochemical staining (c) (arrowheads).

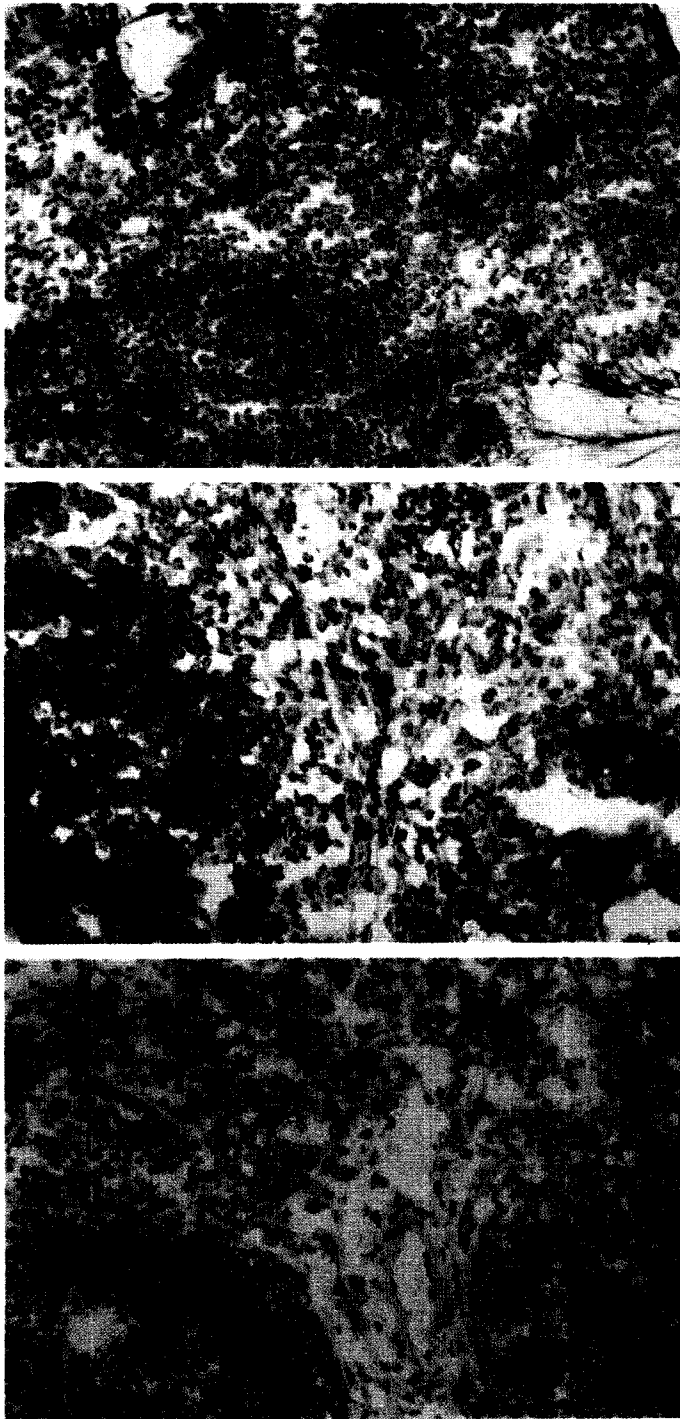


Fig. 4 Frozen section from anti-PSK antibody positive regional lymph node immunostained by indirect immunoperoxidase staining with OKT6 (a), OKIa1 (b) and OKM1 (c). Positive cells are visible in the marginal zone of the follicles (arrowheads).

phycoerythrin indicating they were S-100 protein positive (Fig. 3b). These anti-PSK antibody and S-100 protein positive cells were stained yellow by the double immunohistochemical staining (Fig. 3c).

In the anti-PSK antibody positive lymph nodes, OKT6 positive cells were located in the marginal zone of the follicles (Fig. 4a) and OKIa1 and OKM1 positive cells were visible in the follicles and follicular mar-

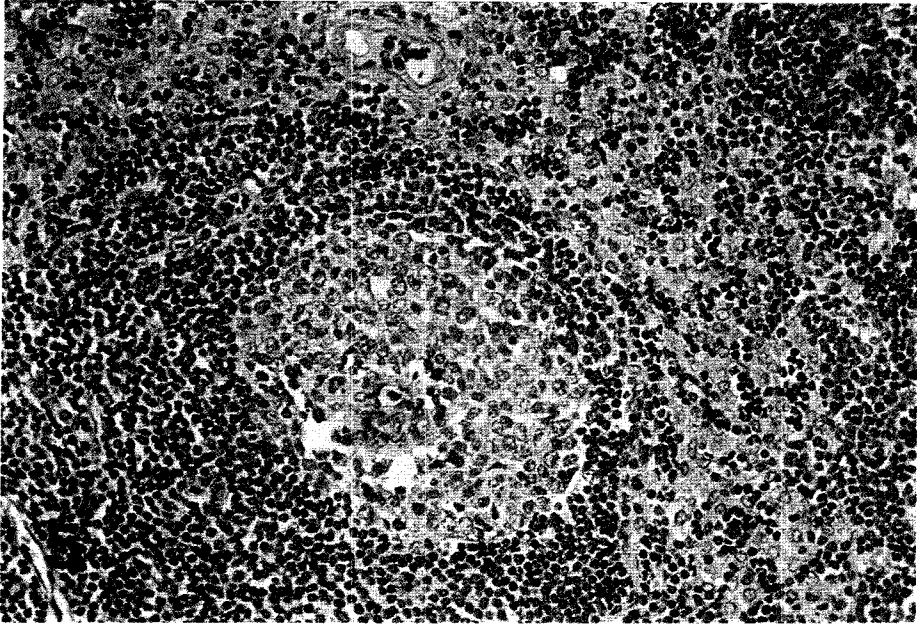


Fig. 5 Paraffin section from anti-PSK antibody positive regional lymph node stained for α -naphthyl-acetate esterase. A large number of positive cells are located in the marginal zone (arrowheads).

Table 2 Ratio of T-cell subsets of regional lymphnodes in untreated controls, and patients intratumorally administered PSK

Group ^a	T-cell subsets	Ratio ^b	
		Control (n=5)	PSK (n=9)
1	OKT4 ⁺ /OKT3 ⁺	0.72 ± 0.11	0.93 ± 0.16
	OKT8 ⁺ /OKT3 ⁺	0.34 ± 0.15	0.20 ± 0.09
	OKT4 ⁺ /OKT8 ⁺	2.56 ± 1.58	5.52 ± 2.53
	Leu7 ⁺ /OKT3 ⁺	0.04 ± 0.00	0.06 ± 0.03
2	OKT4 ⁺ /OKT3 ⁺	0.76 ± 0.09	0.86 ± 0.32
	OKT8 ⁺ /OKT3 ⁺	0.31 ± 0.09	0.27 ± 0.15
	OKT4 ⁺ /OKT8 ⁺	2.70 ± 0.99	5.13 ± 4.48
	Leu7 ⁺ /OKT3 ⁺	0.12 ± 0.14	0.11 ± 0.11

a : Regional lymph nodes defined and grouped according to the general rules for the gastric cancer study (see Ref. 7).

b : Mean ± SD.

ginal zones (Figs. 4b and c).

An anti-PSK antibody positive regional lymph node stained for α -naphthyl-acetate esterase (ANAE) is shown in Fig. 5. Many ANAE positive cells were found in the marginal zone.

T-cell subsets of the regional lymph node lymphocytes were examined by flow-cytometry (Table 2). The ratio of OKT4⁺ to OKT3⁺ cells tended to be higher in both of the PSK-treated groups than in the control group. The ratio of OKT8⁺ to OKT3⁺ cells was low, and no difference was observed in the ratio of Leu7⁺ to OKT3⁺ cells between the groups.

The distribution of anti-PSK antibody positive cells in the regional lymph nodes is shown in Table 3. Positive cells were found in group 1 and 2 lymph nodes on the

periphery of tumors in many cases. However, in one of the seven n_0 cases, anti-PSK antibody positive cells were also found in group 4 paraaortic lymph nodes, which were the most distant regional lymph nodes.

Discussion

Since 1978, we have endoscopically infused a variety of BRMs (BCG-CWS, N-CWS, OK-432, PSK, IL-2, *etc.*) prior to surgery in order to enhance the antitumor activity of regional lymph nodes of patients with gastric and colonic cancers. Little is known about how these BRMs generate antitumor activity. From our studies carried out so far, the involvement of an immune response in gastric cancer patients given

Table 3 Distribution of anti-PSK antibody positive cells in regional lymphnodes

Case	Age Sex	Site ^a and type ^b of tumor	R number ^c	n^d	Number of anti-PSK antibody ⁺ regional lymph node ^e				
					Proximal Group 1*	Group 2*	Group 3*	Distant Group 4*	
1	53 M	C Min	II c	R ₂	n_0	1, 3	7, 8		
2	60 M	C Post M	Min II c	R ₃ +16	n_0	1, 4d, 6	9	(-)	(-)
3	64 M	C Min	Borr IV	R ₂	n_2	1, 3	(-)	(-)	
4	71 M	C Min	Borr III	R ₃ +16	n_4	3	(-)	(-)	(-)
5	72 M	C Post	Borr I	R ₃ +16	n_0	3	8, 11	(-)	(-)
6	76 F	C Min	Borr III	R ₃	n_0	1, 3	(-)	(-)	
7	34 F	M Min	II c	R ₃ +16	n_0	3, 4sd, 4d	7, 9	12p, 14V	16
8	42 F	M Post	II c	R ₃ +16	n_0	3, 4d, 5, 6	(-)	(-)	(-)
9	48 M	M Min	Borr IV	R ₃	n_3	(-)	(-)	(-)	
10	49 M	M Min	Borr III	R ₃	n_1	3, 4sb, 4d	7, 8, 9	12p	
11	57 M	M Min	II c	R ₃	n_0	3, 4d	(-)	(-)	
12	71 M	M Post	II c+III	R ₃	n_0	3, 4d, 6	(-)	(-)	
13	37 M	A Maj	Borr V	R ₃	n_3	4sb, 4d	(-)	(-)	
14	40 M	A Ant	Borr IV	R ₂	n_2	(-)	(-)		
15	57 M	A Maj	Borr II	R ₃	n_1	3, 4d	9	(-)	
16	64 F	AMC Circ	Borr IV	R ₃ +16	n_4	(-)	(-)	(-)	(-)
17	72 F	A Maj	Borr II	R ₂	n_1	4sb, 4d	7		
18	76 F	A Min	Borr I	R ₂	n_0	3, 4d	7, 8, 11		

a: C, cardiac area and fundus; M, bulk of the corpus; A, antral area; Min, lesser curvature; Maj, greater curvature; Ant, anterior wall; Post, posterior wall; Circ, circular.

b: II c, depressed type; II c+III, depressed type with an excavation.

c: R₂: gastrectomy with dissection of group 1 and 2 lymph nodes, R₃: extended radical gastrectomy with dissection of group 1, 2 and 3 lymph nodes.

d: Grade of lymph node involvement identified histologically.

*: Regional lymph nodes defined and grouped according to the general rules for the gastric cancer study (see Ref. 7).

such BRMs as OK-432 and PSK has been demonstrated by the fact that the cytotoxicity of lymphocytes derived from regional lymph nodes is enhanced (3) and that the number of OKT4⁺ cells, a T-cell subset, is increased (4). Nevertheless, the developmental process has not yet been studied closely.

In the present study, we obtained interesting findings with regard to the pharmacokinetics of PSK infused intratumorally by immunohistological staining using anti-PSK antibody. The injected PSK appeared to be phagocytized by histiocytes in the tumor and its periphery or directly transported to regional lymph nodes by lymphatic flow. In the lymph nodes, the histiocytes that have phagocytized PSK were seen abundantly in the follicular marginal zones and marginal sinuses which were thought to be the sites of immune responses. It appeared that ANAE positive lymphocytes of the paracortical area were partially activated T-cells. This finding suggests the possibility that histiocytes that have phagocytized PSK may play a major role in the expression of the immune response.

The onset of a variety of immune responses requires, in addition to lymphocytes, the presence of non-lymphocytic cells termed antigen-presenting cells which have been considered to be Ia⁺ macrophages (8, 9). Recently, however, the presence of dendritic cells has drawn special attention (10). Cells with antigen-presenting ability in the lymph nodes include macrophages, follicular dendritic cells (FDC, *i. e.* one type of dendritic cells), lymphoid dendritic cells (LDC) and interdigitating cells (IDC). Among these cells, Ia⁺ macrophages, LDC and IDC are OKIa1⁺, and IDC is S-100 protein⁺ and OKT6⁺ (11, 12). Macrophages react with OKM1. The fact that there were a large number of these positive cells in the regional lymph nodes positive for anti-PSK antibody seems to indicate that histiocytes (or macro-

phages) took on the characters of IDC through phagocytization of PSK or that PSK induced an increase in IDC in the lymph nodes.

In particular, the double staining with anti-PSK antibody and anti-S-100 protein antibody revealed that many of the anti-PSK antibody-positive cells were positive for S-100 protein, which may be proof that the histiocytes that phagocytized PSK act as antigen-presenting cells. The antitumor effect would be expected to exist if these antigen-presenting cells cause activated T-cells to increase (13-18). From these results, we may make a series of speculations: (a) Administration of PSK into the tumor site produces an increase in the number of local infiltrative lymphocytes. (b) Recognition of tumor antigen might occur in a locally growing tumor. (c) Subsequently, the lymphocytes flow into regional lymph nodes by virtue of the drainage effect, where the immune response is activated. (d) The regional lymph nodes serve as the site of presentation and memory of antigen. (e) Consequently, local and systemic antitumor activities might be enhanced.

PSK was present in group 1 lymph nodes in the periphery of tumors in almost all cases, but, when compared with the n(+) group, the n(-) group displayed a more extensive distribution of locally infused PSK in the regional lymph nodes. Especially in n₀ cases, PSK was distributed even in group 4 lymph nodes. In contrast, there were some n₃ cases where no distribution of PSK was seen in the excised lymph nodes; it was thus considered that the transfer of PSK was blocked by tumor infiltration within the stomach wall and tumor metastasis into the lymphatic vessels. These findings indicate that PSK should be administered into the vicinity of tumors where intrawall lymphatic flow is relatively normal. In particular, in gastric cancer patients in whom lymphatic metastasis is not marked, the pos-

sibility was suggested that intratumoral administration of PSK contributes not only to enhancement of antitumor activity in the remaining lymph nodes despite complete dissection during the operation, but also to the formation of barriers against tumors. We believe that this therapeutic modality will become a more effective preoperative adjuvant immunotherapy with more careful selection of patients and an improved mode of administration.

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