Immunohistochemical analysis of advanced colon cancer after lentinan administration

— Emperipolesis of tumor-infiltrating lymphocytes —

ORIGINAL ARTICLE Annals of Cancer Research and Therapy

Nobuaki Sakamoto · Yasuhisa Koyanagi · Atsushi Nakajima
Kozaburo Kimura*1), Hiromi Serizawa*2)

We examined the effects of a biological response modifier, lentinan, on tumor-infiltrating lymphocytes in advanced colon cancer. The subjects were 57 curatively resected patients with Dukes' B or C colon cancer, which was histologically diagnosed as well differentiated or moderately differentiated adenocarcinoma. The control group (n=29) did not receive any preoperative treatment. In the lentinan-treated group, lentinan was administered intravenously at 2 weeks and 1 week before surgery at a dose of 2mg (n=10) , 4mg (n=8) , or 8mg (n=10) each time.

The number of tumor-infiltrating lymphocytes increased depending on the dose of lentinan, and there was a significant difference between the control group and the patients given 4mg or 8mg of lentinan ($P<0.05$). Comparison of the subsets of tumor-infiltrating lymphocytes showed no difference in $CD4^+$ cells between the groups. However, $CD8^+$ cells showed a dose-dependent increase with lentinan treatment and there was a significant difference between the control group and patients given 8mg of lentinan ($P<0.05$). In addition, the number of emperipoletic lymphocytes detected by light microscopy at all lentinan doses was significantly higher than in the control group ($P<0.05$). Emperipoletic lymphocytes were mainly $CD8^+$ T cells, but a few were $CD4^+$, with the latter being almost all detected around ducts lined with cancer cells. The emperipoletic $CD8^+$ cells were confirmed to be cytotoxic T lymphocytes by double staining with CD8 and CD11b.

When cytotoxic T lymphocytes, natural killer cells, and other killer cells attack cancer cells, they must make contact with the tumor cell membrane. Detection of emperipoletic lymphocytes at the light microscopic level means that these lymphocytes are in contact with the cell membrane or have penetrated into cancer cells.

Therefore, the finding that emperipolesis was significantly enhanced by lentinan supports the role of killer cells in the antitumor effect of this agent.

Ann Cancer Res Ther 4 (2) : 91~97, 1995/Received 11 Sept 1995, Accepted 31 Oct 1995
Key words : tumor-infiltrating lymphocytes, emperipolesis at the light microscopic level, HLA-class I and HLA-DR, advanced colon cancer, lentinan

There have been many reports on the correlation between prognosis and the number of lymphocytes infiltrating the primary tumor in patients with breast cancer, gastric cancer, and other malignancies[1~5]. Tumor-infiltrating lymphocytes are considered to be T cells and may play an important role in immunological surveillance[6].

Lentinan is a polysaccharide biological response modifier (BRM) that has an indirect antitumor effect through activation of the host immune system rather than having a direct cytotoxic effect[7~9]. It has been demonstrated in animal models that lentinan enhances the response to interleukin-2 (IL-2) of various antitumor effector cells, such as cytotoxic T lymphocytes (CTL), natural killer (NK) cells, and lymphokine-activated killer (LAK) cells. These antitumor effector cells are activated to infiltrate lesions by lentinan, which modifies the tumor microenvironment[10, 11].

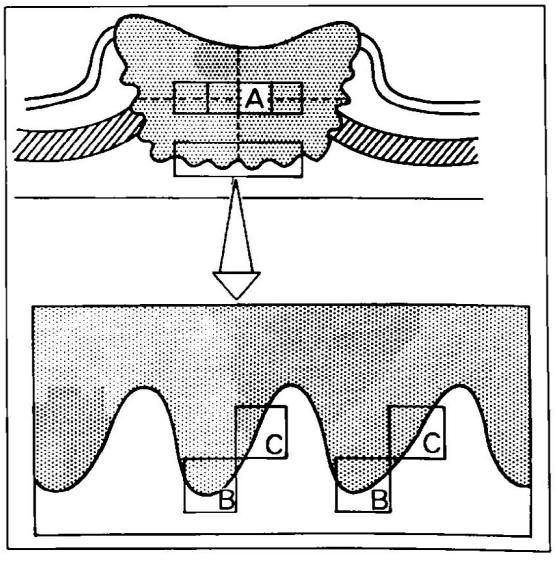

Fig. 1 Observation fileds for tumor-infiltrating lymphocytes and emperipoletic lymphocytes

*1) Department of Surgery, Tokyo Medical College
*2) Department of Pathology, Tokyo Medical College
Correspondence to : Nobuaki Sakamoto, Department of Surgery, Tokyo Medical College, 6-7-1, Nishishinjuku Shinjuku-ku, Tokyo 160, Japan
TEL 03-3342-6111, FAX 03-3340-4575

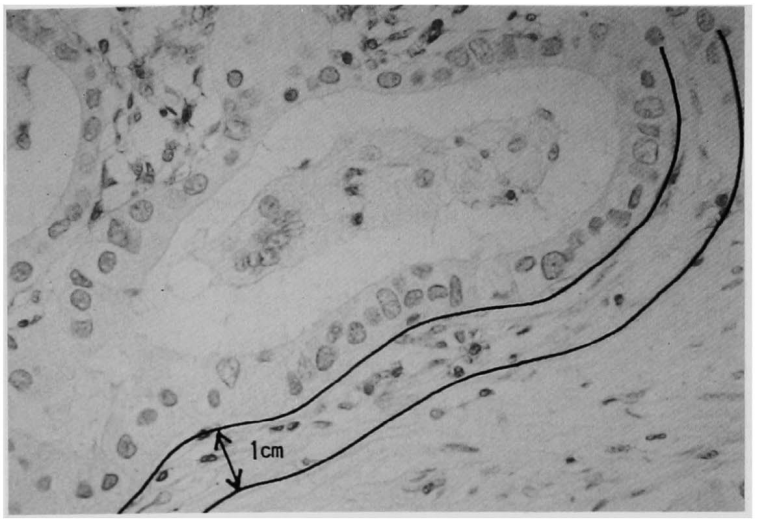

Fig. 2 The method of counting tumor-infiltrating lymphocytes
Note that 1 cm = 25 μm actual length (×200×0.79)

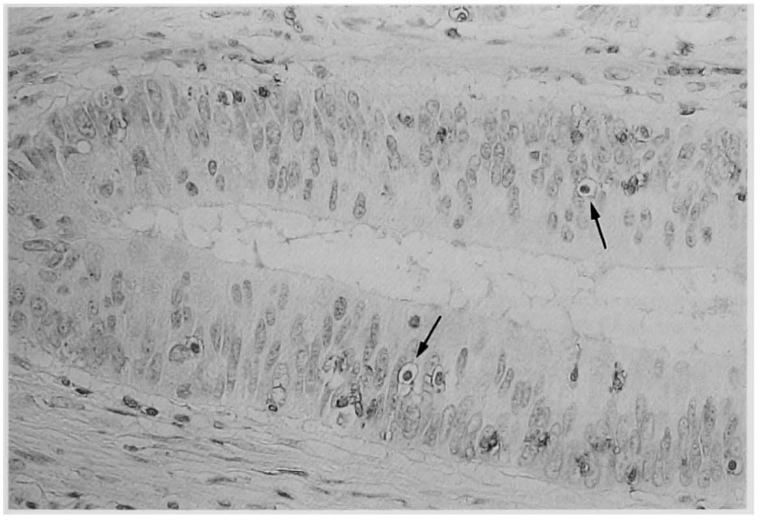

Fig. 3
Light microscopic detection of emperipoletic T lymphocytes (arrows) in the tumor of a patient given lentinan at 8 mg (× 200 × 0.79)

In addition, a clinical trial of lentinan in patients with gastric cancer has confirmed that preoperative administration enhances the immune response at the tumor site[12].

In the present study, lentinan was administered preoperatively to patients with advanced colon cancer by intravenous infusion, and the tumor expression of HLA-class I and HLA-DR antigens as well as lymphocyte infiltration were investigated. In addition, emperipolesis of infiltrating lymphocytes was observed by light microscopy.

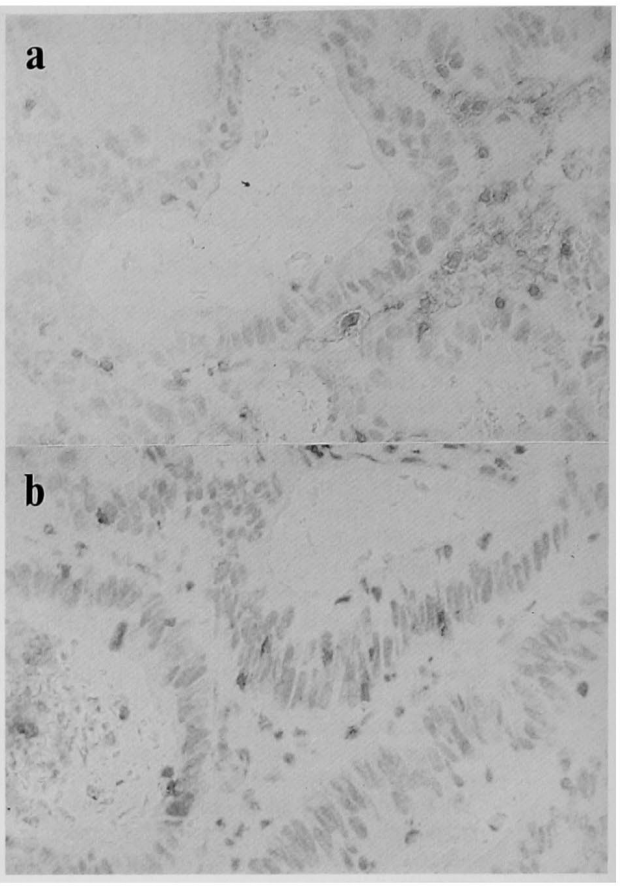

Fig. 4 Emperipoletic T lymphocyte subset in the tumor of a patient given lentinan (8 mg)
a : Immunostaining with Leu 3a (CD4)
b : Immunostaining with Leu 2a (CD8) (both×200×0.65)

Materials and methods

Patients

The subjects were 57 patients with histologically well differentiated or moderately differentiated Dukes' B or C adenocarcinoma of the colon, who underwent curative resection at our department from September 1993 to December 1994. The control group (n=29) did not receive any preoperative treatment. The 2mg group (n=10) received 2mg of lentinan twice preoperatively by intravenous infusion at 2 weeks and 1 week before the operation, while the 4 mg group (n=8) received 4 mg twice preoperatively and the 8mg group (n=10) received 8mg twice preoperatively.

Immunomodulator

Lentinan (Lentinan, Ajinomoto Co. Ltd., Tokyo, Japan) was dissolved in 500ml of 5% glucose for intravenous administration.

Table 1 Clinical characteristics of the subjects

		Control group	Lentinan-treated groups			Statistical analysis
			2mg group	4mg group	8mg group	
No. of patients		29	10	8	10	
Age	Mean	63	61	58	62	P=0.650 ns*1)
	(sd)	(10.5)	(6.7)	(15.0)	(10.2)	
Sex	Male	15	5	7	6	P=0.318 ns*2)
	Female	14	5	1	4	
Histological differentiation						
	Well	2	2	1	1	P=0.659 ns*2)
	Moderately	27	8	7	9	
Dukes' classification						
	B	16	7	4	8	P=0.451 ns*3)
	C	13	3	4	2	

*1) F test. *2) Fisher's exact test. *3) Kruskal-Wallis test

Tissue processing and monoclonal antibodies

Sections of formalin-fixed specimens were obtained at the site of deepest invasion of each lesion. Immunostaining was done with an antibody for T lymphocyte detection (MT-1; Milab, Malmö, Sweden) and with an antibody for HLA-DR detection (LN-3; Nichirei Co. Ltd., Tokyo, Japan).

In addition, the absence or minimal infiltration of B cells was confirmed by immunostaining with an anti-B cell antibody (L-26; Dakopatts, Copenhagen, Denmark).

To detect CD4, CD8, and HLA-class I antigens, a wedge was cut from the anal side of each freshly resected tumor. Frozen sections of the tissue wedge were stained with Leu 3a for detection of CD4 and Leu 2a for detection of CD8 (Becton Dickinson Immunocytometry Systems, California, USA), as well as with an anti-human HLA-class I antibody (Dakopatts) for detection of HLA-class I (A, B, and C) antigens.

Immunohistochemistry

Paraffin sections were obtained from formalin-fixed specimens. Thin sections (3 μm) were deparaffinized with xylene, immediately immersed in ethanol, and washed in phosphate-buffered saline (PBS). Then the sections were incubated overnight with monoclonal antibodies at 4℃, and those for indirect immunostaining were incubated with the secondary antibody plus HRPO-labeled goat anti-mouse IgG (gamma $^{+}$L) for another 1 hour at 37℃.

After excess reagent was washed off with PBS, the sections were reacted with a diaminobenzidine solution for 5 min. Nuclei were counterstained with methylgreen. Fresh specimens were embedded in OCT-compound, frozen in N-hexane (-80℃), and cut into serial sections 3 μm thick on a cryostat microtome. Frozen sections were fixed in acetone for 5 min at 4℃.

After washing, the cryostat sections were incubated with monoclonal antibodies for 1 hour at 37℃, and those for indirect immunostaining were incubated with the secondary antibody and HRPO-labeled goat anti-mouse IgG for another 1 hour at 37℃. After excess antibody was washed off with PBS, the sections were reacted with a diaminobenzidine solution for 5 min and the nuclei were counterstained with methylgreen.

Observation and immunohistochemical analysis

(1) Tumor-infiltrating lymphocytes and lymphocyte subsets(CD4^{+} and CD8^{+})

Tumor-infiltrating lymphocytes were counted using a 200 power microscope. Eight sites in each of 3 sections (24 in all) were photographed, as shown in **Fig.1**. Eight sites were within the main body of the tumor (A), 8 were at the points of deepest invasion (B), and 8 were diagonally superior to the points of deepest invasion (C). Sites (B) and (C) were observed separately because of counting errors at (B) due to frequent collapse of cancer cell nests. The T lymphocytes located within 1cm (actual length: 25 μm) from the edge of ducts lined with cancer cells on the photographs were counted in each photograph to determine the number of T lymphocytes per 100 cm^2 (actual area: 0.0625 mm^2) (**Fig. 2**).

For CD4 and CD8 positivity, 3 sections within each tumor were selected randomly and all lymphocytes in each section were counted with a 200 power microscope to determine the number of positive lymphocytes per field (0.95 mm^2).

(2) Expression of HLA-class I and HLA-DR antigens

HLA-class I expression was categorized as strong (90% or more class I-positive cancer cells lining ducts in all observation fields) or as weak (less than 90% class I-positive cancer cells lining ducts).

HLA-DR expression was graded as follows:

Grade 0 meant no HLA-DR-positive cancer cells lining the

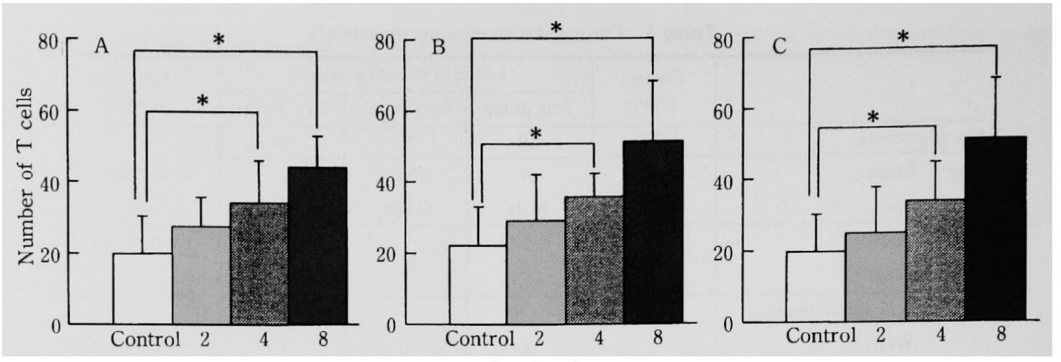

Fig. 5 Tumor-infiltrating lymphocytes
Values are expressed as the mean + SD. *P < 0.05 by Dunnett's t-test. LNT : lentinan

ducts in all observation fields, while Grade Ⅰ was up to 10% HLA-DR-positive cancer cells, Grade Ⅱ was 10～30%, and Grade Ⅲ was 30% or more.

(3) Emperipoletic lymphocytes

To confirm the presence of emperipolesis with absolute accuracy, it should be distinguished from phagocytosis by electron microscopy[13]. However, T lymphocytes penetrating into the ducts lined with cancer cells were defined as emperipoletic in this study when they remained intact without any changes, such as karyolysis or pyknosis, at a magnification of 200 under light microscopy (**Fig. 3**). The number of emperipoletic T lymphocytes per approximately 3,000 cancer cells was counted in the 24 observation fields mentioned above.

Statistics

Uniformity of patient characteristics was evaluated with the F test, Fisher's exact test, and the Kruskal-Wallis test, while differences were analyzed with Dunnett's t-test. A P value less than 0.05 was considered significant. Differences in the expression of HLA-class I and HLA-DR antigens were compared between the control group and the lentinan-treated groups using Wilcoxon's test.

Results

(1) Clinical and histological findings

The groups were identical with respect to age, sex, histological differentiation, and pathological stage according to Dukes classification (**Table 1**).

(2) Tumor-infiltrating lymphocytes

The number of tumor-infiltrating lymphocytes increased depending on the dose of lentinan, with significant differences between the untreated control group (20 ± 10) and the 4 mg (34 ± 12) and 8 mg groups (44 ± 9) (**Fig. 5**).

(3) Subsets of tumor-infiltrating lymphocytes

No difference in CD4 positivity was seen among the different groups, but $CD8^+$ cells showed a dose-dependent increase with lentinan treatment. There was a significant difference between the control group (55 ± 22) and the 8 mg group (123 ± 51) (**Fig. 6**).

(4) HLA-class I expression

About 80% of both the control group and the lentinan-treated patients showed strong expression of HLA-class I antigens by tumor cells and there were no differences among the groups (data not shown).

(5) HLA-DR expression

A tendency for an increase of HLA-DR expression by tumor cells was noted in the lentinan-treated patients when compared to the control group (**Table 2**).

(6) Emperipoletic lymphocytes

The lentinan-treated patients showed an increase of emperipoletic lymphocytes when compared to the control group, with significant differences among the control groups (17 ± 11), each of the 2 mg (43 ± 30), 4 mg (42 ± 21), and 8 mg groups (76 ± 40) (**Fig. 7**).

(7) Emperipoletic lymphocyte subsets

Most of the emperipoletic lymphocytes were $CD8^+$ T cells, but a few were $CD4^+$ T cells, although the latter were almost all detected around ducts lined with cancer cells (**Fig. 4a・b**). However, it must be remembered that this analysis only assessed CD4 and CD8 positivity, and that serial sections were employed.

We confirmed that the emperipoletic $CD8^+$ cells were cytotoxic T lymphocytes by double staining with CD8 and CD11b.

Discussion

Lentinan is a neutral polysaccharide and a biological response modifier (BRM)[14]. This drug has an indirect antitumor effect by activating T-cell dependent host immunity, rather than having a direct cytotoxic effect. It enhances the sensitivity of effector cells to lymphokines

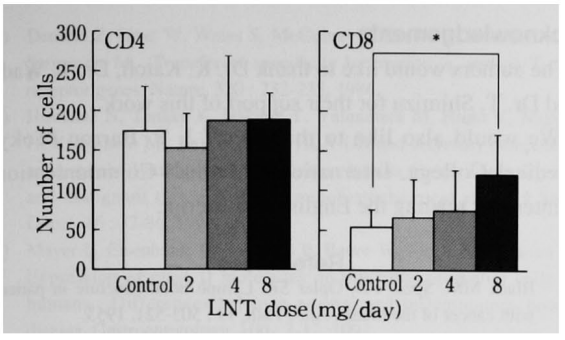

Fig. 6 Subsets of tumor-infiltrating lymphocytes
Values are expressed as the mean+SD.
*P<0.05 by Dunnett's t-test. LNT : lentinan

Table 2 HLA-DR expression

	Control group	Lentinan-treated groups	Wilcoxon test
Grade 0	14	6	Z=−2.234 *P=0.026
Grade I	9	12	
Grade II	2	3	
Grade III	3	7	

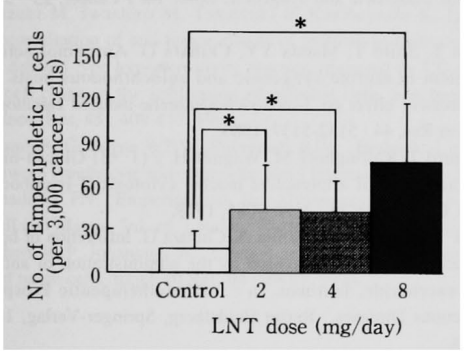

Fig. 7 Emperipoletic T lymphocytes
Values are expressed as the mean+SD.
*P<0.05 by Dunnett's t-test. LNT : lentinan

produced by T cells like IL-2, leading to increased activity of antitumor effector cells such as CTL, NK cells, LAK cells, and macrophages[7~9]. In addition, lentinan is reported to alter the tumor microenvironment by promoting the development of cellular fibers, which enhances the ability of various effector cells to infiltrate lesions[15]. This mechanism is supported by the finding that the infiltration of effector cells is correlated with the therapeutic effect of lentinan. In the present study, lentinan was administered preoperatively to patients with advanced colon cancer, and we noted an increase of T cell infiltration into tumors, especially $CD8^+$ cells.

Several modifications of the tumor microenvironment are necessary for antitumor effector cells to infiltrate successfully, such as production of chemotactic factors, promotion of the binding of effector cells to tumor vessels, and an increase of vascular permeability in the lesion.

It was recently reported that lentinan induces production of a chemotactic factor, macrophage inflammatory protein-1 α (MIP-1 α, LD 78)[16]. The fact that MIP-1 α selectively promotes the migration of activated $CD8^+$ cells[17] supports the possibility that the increase of $CD8^+$ T cells in tumor tissue which we noted was induced by an increase of a chemotactic factor like MIP-1 α.

It is also necessary for T cells to recognize HLA antigens and tumor antigens in order to cause tumor cell damage. HLA-class I and HLA-class II antigens are associated with the recognition of foreign antigens by cytotoxic T cells and helper T cells, respectively[18, 19], and there is a correlation between HLA antigen expression and lymphocyte infiltration[20].

We therefore investigated the changes of HLA antigen expression by tumor cells after administration of lentinan.

HLA-class I antigens were generally expressed strongly in most tumors of both the control and lentinan-treated groups, while HLA-DR antigen expression was increased in the latter compared to the former group.

Since previous studies of colon cancer cell lines have shown that HLA-DR expression is induced by cytokines like IFN γ and is enhanced in inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis[21], the increase of HLA-DR expression observed in the present study could have been induced by cytokines produced within the tumor in response to the increased infiltration of $CD8^+$ T cells after lentinan administration. $CD8^+$ cells have been reported to play an important role in the induction of HLA-DR antigen expression on intestinal epithelial cells[22], a finding that supports our hypothesis. Some investigators have reported a correlation between HLA-DR expression by colon cancer cells and the prognosis[23], but others did not confirm this association[24]. HLA-DR expression has also been reported to correlate with the effect of immunotherapy[25, 26], so the significance of HLA-DR antigen expression has not yet been fully explained.

Concerning the antitumor action of lentinan, it has been shown to enhance delayed-type hypersensitivity (DTH) to tumor cells, and this action is correlated with its therapeutic effect[27]. There is a possibility that the increase of HLA-DR antigen expression on cancer cells produced by lentinan activates $CD4^+$ cells around the tumor and has a beneficial therapeutic effect through the enhancement of regional DTH. Therefore, HLA-DR antigen expression, and its correlation with prognosis should be studied further in relation to lentinan therapy.

In this study, we also observed emperipolesis by light microscopy. Emperipolesis is a term used by Humble et al.[28] to describe the phenomenon of lymphocytes "wandering around inside" cultured malignant cells. In a wider sense, this term indicates that one cell penetrates another, but remains intact[29].

When CTL, NK cells, and other killer cells injure cancer cells, they must come into contact with the tumor cell membrane. The modes of contact have been described based on electron microscopic findings as point contact, broad contact, and peripolesis, which can be all expressed as peripolesis or as emperipolesis[30~35].

Cancer cells are destroyed by cytotoxic substances, such as perforin, that are released from lymphocytes following contact with the tumor cell membrane[36, 37]. Although another pathway that induces apoptosis has been reported[38, 39], contact with the tumor cell membrane is also required.

Although it should be strictly determined whether a basement membrane is present, if contact of lymphocytes with tumor cells is examined by light microscopy, those lymphocytes which appear to be migrating into cancer cells can be said to be in contact with the tumor cell membrane (peripolesis) or to have entered the tumor cells (emperipolesis). Therefore, the significant increase in emperipoletic T lymphocytes that we observed by light microscopy following lentinan administration provides important information on the antitumor mechanism of this agent.

When Itoh et al.[40] isolated tumor-infiltrating lymphocytes from human tumors, such as melanoma, they found that a few of the cells showed strong binding to autologous tumor cells (tumor-binding cells), and that most of these cells were $CD8^+$. We postulate that these tumor-binding cells correspond to the emperipoletic lymphocytes we observed by light microscopy.

Thus, our study on modification of the tumor microenvironment by lentinan administration in patients with advanced colon cancer suggests some mechanisms for the action of this agent. However, lentinan itself does not alter IL-2 production, although it can increase the sensitivity of IL-2 receptors on antitumor effector cells. Tumor-bearing hosts may show a decrease in responsiveness to IL-2 or this may be combined with a decrease in IL-2 production. Although a therapeutic effect can be expected from lentinan alone in the former case, concomitant administration of both lentinan and IL-2 may be necessary in the latter case. Itoh et al.[40] reported an increase in tumor-binding lymphocytes after culture with rIL-2. Provided that these cells are identical to the emperipoletic lymphocytes observed in this study, it can be inferred that combination therapy with lentinan and IL-2 would increase emperipoletic T lymphocytes, and augment the antitumor effect. In fact, it has been demonstrated that combined administration of lentinan and IL-2 has an augmented antitumor effect in mice[41, 42].

Therefore, it is possible that combined administration of lentinan and IL-2, or lentinan/IL-2 plus chemotherapy agents, may provide effective immunotherapy or immunochemotherapy for advanced colon cancer.

Acknowledgements

The authors would like to thank Dr. K. Katoh, Dr. T. Wada, and Dr. T. Shimizu for their support of this work.

We would also like to thank Prof J. P. Barron (Tokyo Medical College, International Medical Communications Center) for reading the English manuscript.

References

1) Black MM, Speer FD, Opler SR. Lymph node structure in patients with cancer of the breast. Am J Path, 19 : 505-521, 1953.
2) Black MM, Freeman C, Mork T, Harvei S, Gutler SJ. Prognostic significance of microscopic structure of gastric carcinomas and their regional lymph nodes. Cancer, 27 : 703-711, 1971.
3) Bennett SH, Futrell JW, Roth JA, Hoye RC, Ketcham AS. Prognostic significance of histologic host response in cancer of the larynx or hypopharynx. Cancer, 28 : 1255-1265, 1971.
4) Watanabe H, Enjoji M, Imai T. Gastric carcinoma with lymphoid stroma. Its morphologic characteristics and prognostic correlations. Cancer, 38 : 232-243, 1976.
5) Hasumi K, Sugano H, Sakamoto G, Masubuchi K, Kubo H. Circumscribed carcinoma of the uterine cervix, with marked lymphocytic infiltration. Cancer, 39 : 2503-2507, 1977.
6) Shimokawara I, Imamura M, Yamanaka N, Ishii Y, Kikuchi K. Identification of lymphocyte subpopulations in human breast cancer tissue and its significance. Cancer, 49 : 1456-1464, 1982.
7) Zákány J, Chihara G, Fachet J. Effect of lentinan on tumor growth in murine allogeneic and syngenetic hosts. Int J Cancer, 25 : 371-376, 1980.
8) Suga T, Shiio T, Maeda YY, Chihara G. Antitumor activity of lentinan in murine syngeneic and autochthonous hosts and its suppressive effect on 3-methylcholanthrene-induced carcinogenesis. Cancer Res, 44 : 5132-5137, 1984.
9) Hamuro J, Röllinghoff M, Wagner H. $\beta(1\rightarrow3)$ Glucan-mediated augmentation of alloreactive murine cytotoxic T-lymphocytes *in vivo*. Cancer Res, 38 : 3080-3085, 1978.
10) Suga T, Izawa M, Yamashita A, Chihara G. Infiltration of host cells in local tumor sites triggered by the administration of anti-tumor polysaccharide, lentinan. In : Immunotherapeutic Prospects of Infectious Diseases. Berlin Heidelberg, Springer-Verlag, 189-193, 1990.
11) Hamuro J, Takatsuki F, Suga T, Kikuchi T, Suzuki M. Synergistic antimetastastic effects of lentinan and interleukin 2 with pre- and post-operative treatments, Jpn J Cancer Res, 85 : 1288-1297, 1994.
12) Ueno M, Takizawa C, Kon Y, Misaka R, Kawaguchi M, Saito T. Augmentation of lymphocyte infiltration in gastric cancer tissues by intravenous injection of lentinan. J Jpn Soc Cancer Ther, 26 : 2359-2364, 1991. (In Japanese)
13) Shamoto M. Emperipolesis of hematopoietic cells in myelocytic leukemia. Electron microscopic and phase contrast microscopic studies. Virchows Arch. B (Cell Pathol), 35 : 283-290, 1981.
14) Chihara G, Maeda Y, Hamuro J, Sasaki T, Fukuoka F. Inhibition of mouse sarcoma 180 by polysaccharides from lentinus edodes (Berk.) sing. Nature, 222 : 687-688, 1969.
15) Suzuki M, Yamashita A, Hamuro J. Histopathological changes of tumor sites triggered by the combined administration of lentinan and IL-2. Biotherapy, 6 : 409-410, 1992. (In Japanese)
16) Hazama S, Oka M, Shimoda K, Suzuki M, Iizuka N, Wadamori K, Yamamoto K, Hirasawa K, Suzuki T. Cytokine (LD 78) induction from whole blood culture with lentinan: For evaluation of the response to lentinan. Biotherapy, 9 : 615, 1995. (In Japanese)
17) Taub DD, Conlon K, Lloyd AR, Oppenheim JJ, Kelvin DJ. Preferential migration of activated $CD4^+$ and $CD8^+$ T cells in response to MIP-1 α and MIP-1 β. Science, 260 : 355-358, 1993.
18) Saito T, Weiss A, Miller J, Norcross MA, Germain RN. Specific antigen-Ia activation of transfected human T cell expressing murine Ti $\alpha\beta$-human T3 receptor complexes. Nature, 325 : 125-130, 1987.

19) Dembic Z, Hass W, Weiss S, McCubrey J, Kiefer H, Boehmer HV, Steinmetz M. Transfer of specificity by murine α and β T-cell receptor genes. Nature, 320 : 232-238, 1986.
20) Hirozane N, Tanaka K, Nakane Y, Yamamura M, Hioki K, Nagura H, Yamamoto M. Expression of HLA-DR and secretory component antigens and lymphocyte infiltration in human gastric nonmalignant and malignant tissues : An immunohistochemical study. J Surg Oncol, 46 : 77-86, 1991.
21) Mayer L, Eisenhardt D, Salomon P, Bauer W, Plous R, Piccinini L. Expression of class II molecules on intestinal epithelial cells in humans. Differences between normal and inflammatory bowel disease. Gastroenterology, 100 : 3-12, 1991.
22) Mayer L, Shlien R. Evidence for function of Ia molecules on gut epithelial cells in man. J Exp Med, 166 : 1471-1483, 1987.
23) Tanaka K, Morita M, Okusa T, Nakane Y, Takada H, Okamura A, Hioki K. The effect of local immune reaction on tumor bearing host of colorectal carcinoma: Correlation with HLA-DR expression and TIL. Biotherapy, 8 : 201-204,1994. (In Japanese)
24) Möller P, Momburg F, Koretz K, Moldenhauer G, Herfarth C, Otto HF, Hämmerling GJ, Schlag P. Influence of major histocompatibility complex class I and II antigens on survival in colorectal carcinoma. Cancer Res, 51 : 729-736, 1991.
25) Ransom JH, Pelle B, Hanna MG Jr. Expression of class II major histocompatibility complex molecules correlates with human colon tumor vaccine efficacy. Cancer Res, 52 : 3460-3466, 1992.
26) Rubin JT, Elwood LJ, Rosenberg SA, Lotze MT. Immunohistochemical correlates of response to recombinant interleukin-2-based immunotherapy in humans. Cancer Res, 49 : 7086-7092, 1989.
27) Suzuki M, Iwashiro M, Takatsuki F, Kuribayashi K, Hamuro J. Reconstitution of anti-tumor effects of lentinan in nude mice: Roles of delayed-type hypersensitivity reaction triggered by CD-4 positive T cell clone in the infiltration of effector cells into tumor. Jpn J Cancer Res, 85 : 409-417, 1994.
28) Humble JG, Jayne WHW, Pulvertaft RJV. Biological interaction between lymphocyte and other cells, Brit J Haematol, 2 : 283-294, 1956.
29) Ghadially FN. Emperipolesis. In : Ultrastructual Pathology of the Cell and Matrix. 3rd. ed. London : Butterworths, 1148-1153, 1988.
30) Biberfeld P, Johansson A. Contact areas of cytotoxic lymphocytes and target cells. Exp Cell Res, 94 : 79-87, 1975.
31) Bykovskaja SN, Rytenko AN, Rauschenbach MO, Bykovsky AF. Ultrastructual alteration of cytolytic T lymphocytes following their interaction with target cells. Ⅲ. Plasmalemma "membranosomas". Cell Immunol, 42 : 197-207, 1979.
32) Sanderson CJ, Glauert AM. The mechanism of T-cell mediated cytotoxicity. VI. T-cell projections and their role in target cell killing. Immunol, 36 : 119-129, 1979.
33) Roder JC, Kiessling R, Biberfeld P, Andersson B. Target-effector interaction in the natural killer (NK) cell system. II. The isolation of NK cells and studies on the mechanism of killing. J Immunol, 121 : 2509-2517, 1978.
34) Burns ER, Zucker-Franklin D, Valentine F. Cytotoxicity of natural killer cells. Correlation with emperipolesis and surface enzymes. Lab Invest, 47 : 99-107, 1982.
35) Munakata Y. Ultrastructual differences in three types of lymphocytic cytotoxities against Chang liver cells and a human hepatoma cell line *in vitro*. Gastroenterol Jpn, 79 : 808-819, 1982. (In Japanese)
36) Podack ER, Hengartner H, Lichtenheld MG. A central role of perforin in cytolysis. Ann Rev Immunol, 9 : 129-157, 1991.
37) Yagita H, Nakata M, Kawasaki A, Sinkai Y, Okumura K. Role of perforin in lymphocyte-mediated cytolysis. In:Advances in Immunology, San Diego, Academic Press, 51 : 215-242, 1992.
38) Ashwell JD, Berger NA, Cidlowski JA, Lane DP, Korsmeyer SJ. Coming to terms with death : Apoptosis in cancer and immune development. Immunol Today, 15 : 147-151, 1994.
39) Liu C-C, Walsh CM, Young J D-E. Perforin : Structure and function. Immunol Today, 16 : 194-201, 1995.
40) Itoh K, Tilden AB, Balch CM. Interleukin 2 activation of cytotoxic T-lymphocytes infiltrating into human metastatic melanomas. Cancer Res, 46 : 3011-3017, 1986.
41) Suzuki M, Higuchi S, Taki Y, Miwa K, Hamuro J. Induction of endogenous lymphokine-activated killer activity by combined administration of lentinan and interleukin 2. Int J Immunopharmacol, 12 : 613-623, 1990.
42) Suzuki M, Kikuchi T, Takatsuki F, Hamuro J. Curative effects of combination therapy with lentinan and interleukin-2 against established murine tumors, and the role of CD-8 positive T cells. Cancer Immunol Immunother, 38 : 1-8, 1994.