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# PREOPERATIVE IMMUNOHISTOCHEMICAL ASSESSMENT OF COMBINATION THERAPY WITH LENTINAN AND INTERLEUKIN-2 FOR COLON CANCER: EXPRESSION OF THE INTERLEUKIN-2R β-CHAIN

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*Abstract*

Twenty-nine patients operated on for colon cancer were preoperatively administered lentinan and recombinant interleukin-2 (rIL-2) in order to study the local immunomodulating effect on the tumor itself. The patients were divided into a control group, group A given 2 mg of lentinan and 100,000 JRU of rIL-2, and group B given 2 mg of lentinan and 400,000 JRU of rIL-2. Groups A and B received 8 doses of each agent from 2 weeks before the operation. Resected tumor tissues were subjected to immunostaining with antibodies for MT-1, CD4 or CD8. Greater local infiltration of CD4-positive T lymphocytes ($p < 0.05$ in group B) and CD8-positive lymphocytes ($p < 0.05$ in group B) was observed in groups A and B than in the control group. This infiltration increased along with the dose of rIL-2. When expression of the IL-2R β-chain was assessed after administration of lentinan and rIL-2, expression was even on the T lymphocytes infiltrating the interstitial around the cancers, and it was enhanced in group A and group B ($p < 0.05$) compared with the controls. Both CD4- and CD8-positive cells expressed the IL-2R β-chain. This study indicated that IL-2R expression on lymphocytes was increased by concomitant administration of lentinan and rIL-2. These lymphocytes subsequently infiltrated tumors and mobilized effector cells.

## *Introduction*

Infiltration of lymphocytes is one of the host defenses against cancer (Miwa, 1984), and the extent of lymphocyte infiltration influences the prognosis (Black, Freeman *et al.*, 1971). Studies on enhancing host antitumor activity by promoting lymphocyte infiltration have resulted in biological response modifiers (BRMs), which are now applied clinically. Many BRMs, such as BCG and OK432, are bacterial components or semipurified mixtures, while lentinan is a high molecular weight neutral polysaccharide purified from

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shiitake mushrooms (Chihara, Maeda *et al.*, 1969). It exhibits an antitumor effect without having any direct cytotoxicity by activating cells such as macrophages, NK cells, B lymphocytes and T lymphocytes (Hamuro, Takarada *et al.*, 1989). Lentinan is especially able to activate T lymphocytes (Hamuro, 1994; Tokuzen, 1971; Ueno, Takizawa *et al.*, 1991), and several reports have appeared on the infiltration of these lymphocytes in patients with gastric cancer. However, we studied patients with colon cancer and found that lentinan enhanced the infiltration of cytotoxic T lymphocytes (Tc) into their tumors (Sakamoto, Koyanagi *et al.*, 1996). Lentinan has also been reported to increase the response to IL-2 (Suzuki, Suga *et al.*, 1990), apparently via the enhancement of IL-2 receptor (IL-2R) expression, and the possibility that the effect of lentinan may be increased by concomitant administration with IL-2 has been suggested.

In the present study, local infiltration of T lymphocytes and T cell subsets (CD4 and CD8) into tumors was analyzed after combination therapy with lentinan and IL-2. IL-2R expression was also examined by immunohistochemistry.

## *Materials and Methods*

### *Subjects and administration*

The subjects were 29 patients who underwent resection of colon cancer at our hospital between May 1997 and June 1998. Informed consent to participation in the study was obtained from all patients. They were then randomized to three groups: an untreated control group (n=10), a group administered 2 mg of lentinan and 100,000 JRU of IL-2 (group A; n=10), and a group administered 2 mg of lentinan and 400,000 JRU of IL-2 (group B; n=9). Groups A and B were given intravenous infusions of lentinan (Ajinomoto Co., Ltd., Tokyo Japan) and recombinant IL-2 (rIL-2, Takeda Chemical Industries, Osaka Japan) dissolved at the specified doses in 200 ml of 5% glucose and administered over 1 hour. Both groups received 2 courses of administration for 4 days with a 4-day washout period from 2 weeks before the operation (Figure 1).

### *Preparation of specimens*

Full-thickness samples of the resected cancers were embedded in OCT compound and frozen rapidly at −73° C to prepare cryosections. The rest of the resected tissue was fixed

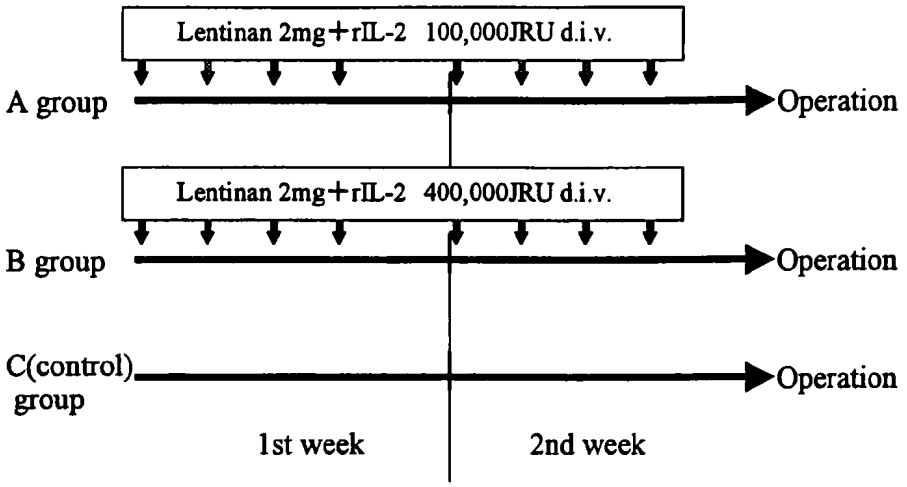


*Figure 1.* Regimen of Lentinan and rIL-2 for each group.

in 20% formalin and embedded in paraffin. Serial sections (3 μm thick) were prepared at the site showing the deepest macroscopic tumor invasion without necrosis. Hematoxylin-eosin (HE) staining and MT-1 staining (an antibody for T lymphocytes; Milab, Malmö, Sweden) were performed by the streptavidin-biotin (SAB) method (Figure 2). T lymphocyte subsets were identified by indirect immunostaining for CD4 (detection of T-helper/inducer lymphocytes; Becton Dickinson, San Jose, USA), CD8 (T-suppressor/cytotoxic lymphocytes; Becton Dickinson, San Jose, USA) and the IL-2R β-chain (IL-2R β-chain/p75; CD122; Coulter, South San Francisco, USA) using cryosections (Figures 3 and 4). In each specimen, five fields were examined, including the deepest site of tumor invasion. Positive cells were counted at a magnification of × 200, and the mean value was calculated.

*Statistical analysis*

Clinical characteristics, such as the age, gender, tumor histology, and Dukes classification, were compared between each group. The F test was used for age, Fisher's test for gender and histological differentiation classification, and the Kruskal-Wallis test for Dukes classification. Intergroup differences of T lymphocytes, CD4 cells, CD8 cells, and IL-2R β-chain expression were analyzed statistically by the Dunnett's t-test, and

$p<0.05$ was taken as significant. Correlations between IL-2R β-chain expression and infiltration of T lymphocytes, CD4 cells, and CD8 cells were determined by the simple regression analysis.

*Results*

*Clinical factors*

No statistical bias was found between any of the groups with regard to age, gender, histological differentiation or Dukes classification (Table I).

**TABLE I**

Clinical characteristics of the subjects

| | C(control) group | Lentinan/IL-2 treated groups | | Statistical analysis |
|---|---|---|---|---|
| | | A group | B group | |
| Number of patients | 10 | 10 | 9 | |
| Age | 58.9±8.3 | 59.4±8.1 | 58.7±12.7 | N.S* p=0.986 |
| Sex Male | 7 | 6 | 4 | N.S** p=0.575 |
| Female | 3 | 4 | 5 | |
| Histological differentiation | | | | |
| Well | 3 | 5 | 3 | |
| Moderately | 7 | 4 | 4 | N.S** p=0.473 |
| Poorly | 0 | 1 | 2 | |
| Dukes classification | | | | |
| A | 5 | 1 | 2 | |
| B | 2 | 5 | 2 | N.S***p=0.312 |
| C | 3 | 4 | 5 | |

*F Test. **Fisher's exact test. ***Kruskall-Wallis test.

*Infiltration of T lymphocytes into tumor tissue*

Figure 5 shows the number of T lymphocytes (MT-1 positive cells) infiltrating into the cancer tissue. Among all of the patients, the highest number of MT-1-positive cells was 215.4 (group B) and the lowest was 17.4 (control group). The number of MT-1-positive cells was significantly greater in group B ($p < 0.05$) than in the control group.

*T lymphocyte subsets*

The T lymphocyte subsets infiltrating into the cancer tissue are shown in Figure 6. Among patients, the highest number of CD4-positive cells was 76.7 (group A) and the

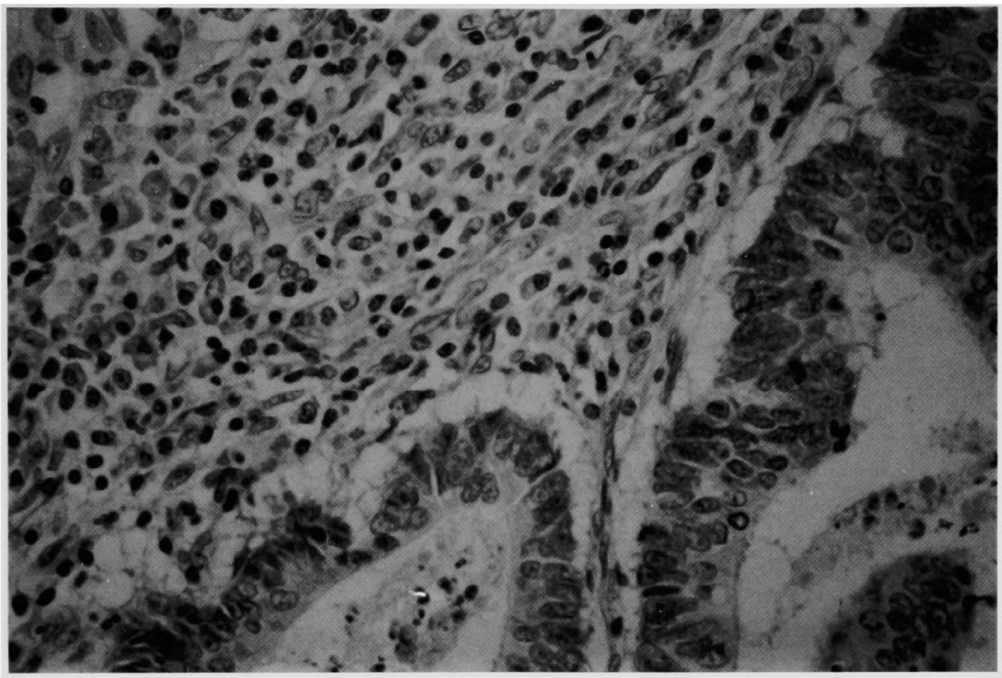

*Figure 2.* T lymphocytes infitrating tumor tissue in patients given Lentinan (2 mg) and rIL-2 (400,000 JRU). Immunostaining with MT-1 (×200)

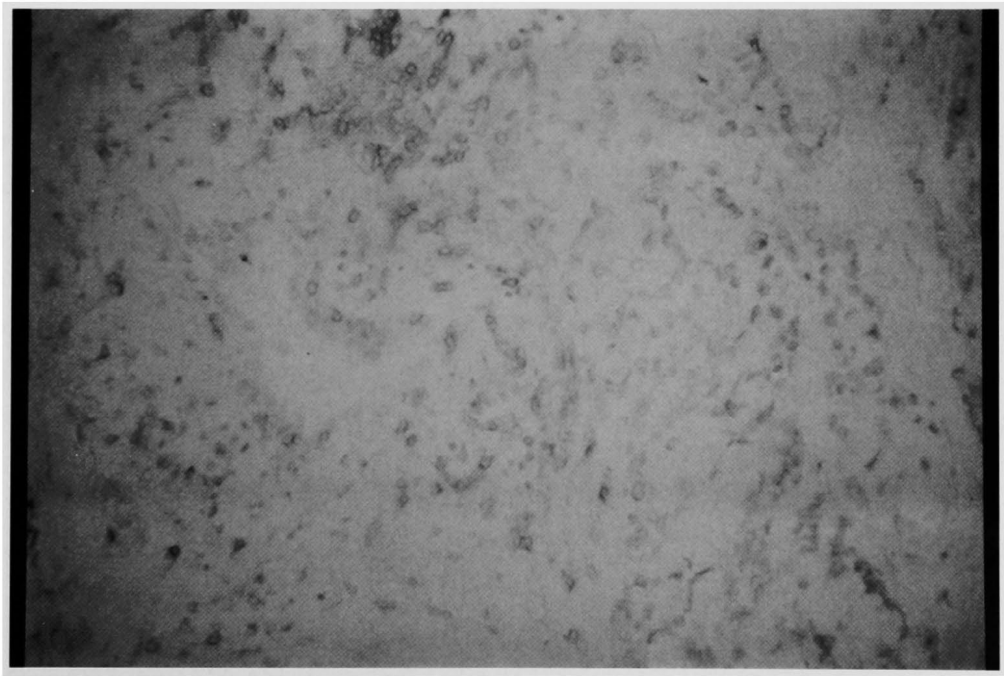

*Figure 3.* T lymphocyte subsets infitrating tumor tissue in patients given Lentinan (2 mg) and rIL-2 (400,000 JRU). a. Immunostaining with CD4 (×200)

*Figure 3.* T lymphocyte subsets infitrating tumor tissue in patients given Lentinan (2 mg) and rIL-2 (400,000 JRU). b. Immunostaining with CD8 (×200)

*Figure 4.* IL-2R expression in cancer tissue from patients given Lentinan (2 mg) and rIL-2 (400,000 JRU).Immunostaining with IL-2Rβ chain (×200)

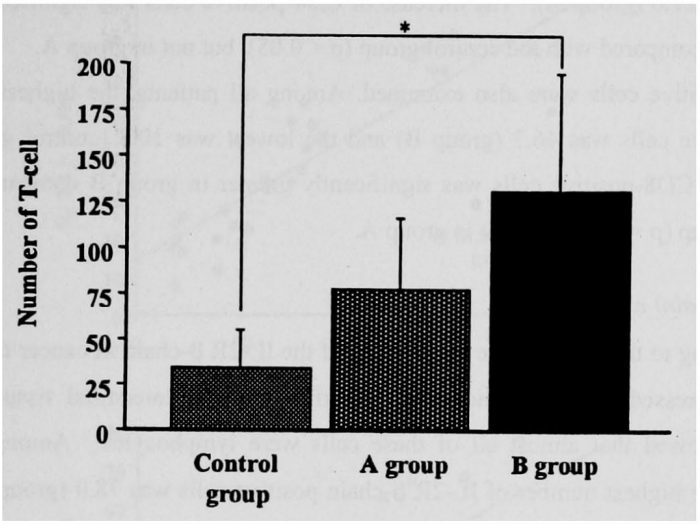


*Figure 5.* Tumor-infiltrating T-lymphocytes. Values are expressed as the mean + SD.
* $p<0.05$ by Dunnett's t-test.

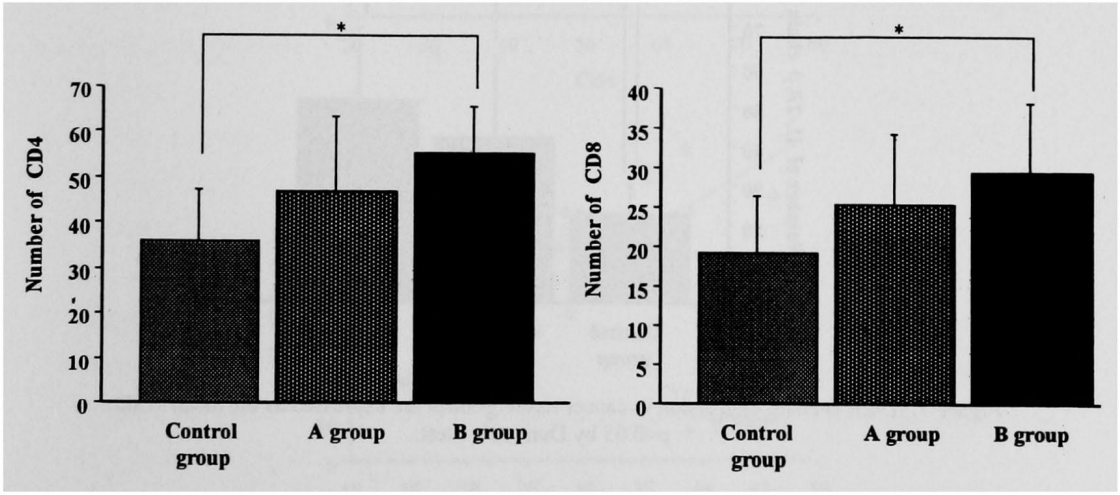


*Figure 6.* Subsets of tumor-infiltrating T-lymphocytes. Values are expressed as the mean + SD.
*$p<0.05$ by Dunnett's t-test.

lowest was 21.6 (group A). The increase of CD4-positive cells was significantly greater in group B compared with the control group ($p < 0.05$), but not in group A.

CD8-positive cells were also examined. Among all patients, the highest number of CD8-positive cells was 46.3 (group B) and the lowest was 10.8 (control group). The increase of CD8-positive cells was significantly greater in group B compared with the control group ($p < 0.05$), but not in group A.

*IL-2R (β-chain) expression*

According to the study of the distribution of the IL-2R β-chain in cancer tissue, it was mainly expressed on the surface of cells infiltrating the interstitial tissues, and HE staining showed that almost all of these cells were lymphocytes. Among all of the patients, the highest number of IL-2R β-chain positive cells was 78.0 (group B) and the lowest was 7.6 (control group). The increase of IL-2R β-chain positive cells was significantly greater in group A and group B ($p < 0.05$) than in the control group (Figure

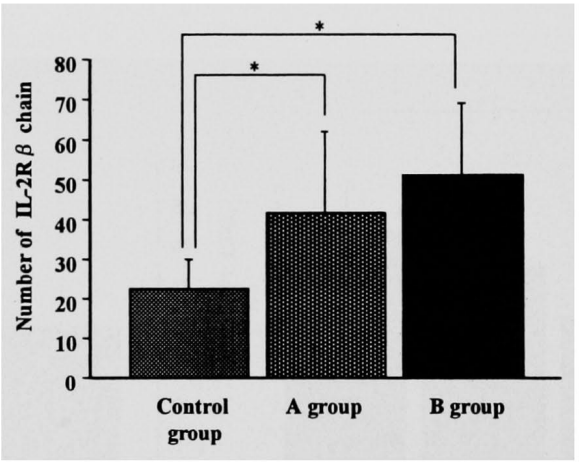


*Figure 7.* IL-2R β-chain expression in cancer tissue. Values are expressed as the mean + SD.
*: $p<0.05$ by Dunnett's t-test.

7). By assessing of the correlation between infiltration of T lymphocytes and IL-2R β-chain positive cells, a strong positive correlation was found (R= 0.891). There was a strong correlation between CD4 and IL-2R β-chain was positively (R= 0.816) as well as between CD8 and IL-2R β-chain positively (R = 0.806) (Figure 8).

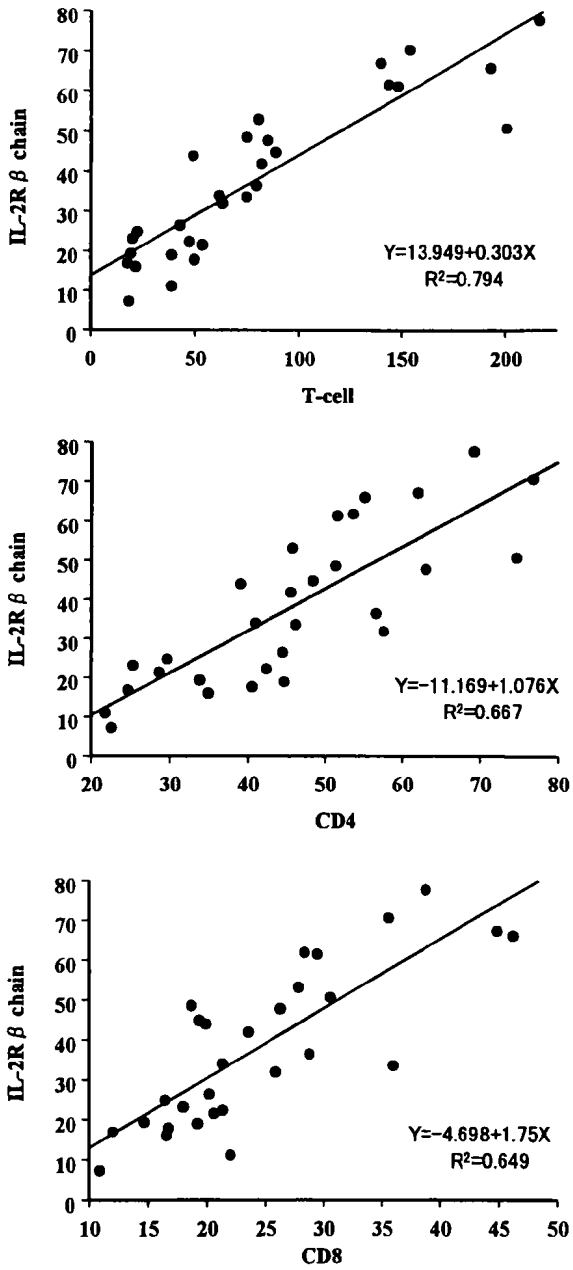


*Figure 8.* Correlation between IL-2Rβ-chain expression and infiltration of T lymphocytes and T cell subsets.

*Discussion*

In cancer-bearing hosts, infiltration of nonspecific inflammatory cells and cytotoxic T-lymphocytes (Tc) is seen as a local response to cancer. This is considered to be a host defense reaction (Miwa, 1984; Ueno, Takizawa *et al.,* 1991) and plays an important role in regulating the host immune response to the tumor. For the cancer to become established in the body, it is necessary for the host immune response to be reduced or for the cancer cells to be able to avoid continuous immune surveillance (Takahashi, Ishii *et al.,* 1989; Urushizaki 1978). Therefore, activation of the local response to a tumor, infiltration of tumor tissue by effector cells such as Tc and macrophages, and secretion of various cytokines after activation of these effector cells are considered to be important in enhancing the host immune response to cancer (Suzuki and Hamuro 1990).

At present, surgery is the treatment of first choice for solid cancer. In recent years, in consideration of QOL, limited operations are often performed in patients without lymph node metastasis and metastasis to other organs, while extended curative operations such as complete resection and dissection are common in patients with extensive primary invasion or lymph node metastasis. This has resulted in marked polarization of cancer surgery. Under current conditions, adjuvant therapy is considered to be important for the prevention of postoperative recurrence and metastasis. At present, the most common treatment methods have a direct action on the tumor, such as antitumor agents or radiotherapy. High doses are used to maintain efficacy and QOL is often impaired because of adverse reactions. Therefore, antitumor activity can be increased without impairing the QOL by continued activation of specific immune mechanism though preoperative immunotherapy to create a local immune response to cancer. We became interested in the pharmacological action of lentinan, *i.e.*, the fact that it has no direct cytotoxic activity but activates the immune defenses of cancer-bearing patients undergoing non-curative resection or patients with recurrence (Chihara, Maeda *et al.,* 1969). Therefore, we studied the possibility that lentinan could inhibit micrometastasis after curative resection. Suga, Izawa *et al.* (1989) found a correlation between the infiltration of neutrophils and macrophages into tumors after lentinan administration and the sensitivity of the tumors to lentinan. They suggested that infiltration of these

nonspecific inflammatory cells play an important role in starting the antitumor immune response that follows such infiltration. Sakamoto, Koyanagi *et al.* (1996) reported that infiltration of cytotoxic T lymphocytes into cancer tissue, especially the interstitium, occurred when lentinan was administered intravenously to patients with advanced colon cancer before surgery. They also found that these infiltrating cytotoxic T lymphocytes adhere to tumor cell membranes or increase the number of emperipoietic T lymphocytes via such adherence. Therefore, lentinan has the following actions. 1) Tumor cells are damaged at an early stage by nonspecific inflammatory cells and the cell fragments are phagocytized by macrophages. This results in the presentation of tumor-specific antigens, *i.e.*, MHC class I antigens and class II antigens, on the cell membrane. 2) Recognition by cells such as helper T lymphocytes (Th) and cytotoxic T lymphocytes (Tc) leads to a tumor antigen-specific immune response. Lentinan enhances the response to IL-2 by increasing IL-2R expression via enhanced accessory cell function (Suzuki and Hamuro 1990). Activated Th and Tc cells appear to play an important role in this response. Activated T lymphocytes promote the secretion of endogenous IL-2, while potentiation with lentinan results in activation of natural killer (NK) cells and lymphokine-activated killer (LAK) cells as well as localized infiltration together with Tc cells into the tumor. Activated cells that infiltrate the tumor secrete various cytokines and finally cause activation of tumor-specific immunity by activating delayed hypersensitivity type T lymphocytes. In 1983, Taniguchi, Matsui *et al.*, successfully cloned the IL-2 gene and obtained rIL-2. When it was used clinically, it appeared that a stronger antitumor effect could be achieved by combination therapy. As mentioned previously, promoting local infiltration of Tc cells into the cancer is the main action of lentinan. In the present study, employing a constant dose of lentinan in both group A and B, localized infiltration of Tc into the tumor showed a dose-dependent increase when rIL-2 was administered, suggesting that rIL-2 is involved in promoting the effective infiltration of T lymphocytes. We also studied T lymphocyte subsets and found infiltration of both CD4 (Th) cells and CD8 (Tc) cells. These results differed from those of Sakamoto, Koyanagi *et al.* (1996), who reported predominant CD8 cell infiltration. Our results suggested that both Tc and Th cells are activated by an autocrine mechanism (Denno, Hirata *et al.*, 1993; Omote, Fujimoto *et al.*, 1990) in association with

enhancement of tumor antigen presenting Th activity and an increase of endogenous IL-2 produced by Th cells when lentinan and rIL-2 are given concomitantly.

The IL-2R is composed of three different subunits (the α-chain, β-chain and γ-chain), and the structures of these sub-units have been determined (Miyazaki, 1998; Taniguchi, 1995). The β-chain and γ-chain play an important role in IL-2 signal transmission. The β-chain and γ-chain heterodimer forms the medium affinity IL-2R, while the α-chain, β-chain, and γ-chain heterotrimer forms the high affinity IL-2R (Miyazaki, 1998). In the β-chain, 286 amino acids form the intracellular domain, and it plays the most important role in IL-2 signal transmission by inducing transcription of the intranuclear proto-oncogenes *c-myc* and *c-fos/c-jun* via the serine-rich domain and serine-rich and acidic domains, respectively, which are intracellular domains of the β-chain. The serine-rich domain is known to be essential for promotion of cell growth by IL-2 (Minami, Oishi *et al.*, 1994; Taniguchi and Minami, 1993). Therefore, we studied expression of the IL-2R β-chain as an index of IL-2R expression. We found that the β-chain was expressed on cells infiltrating the interstitial tissue around the cancer after lentinan therapy. Comparison of the findings after HE and MT-1 staining revealed that the cells with the IL-2R were T lymphocytes. The number of MT-1-positive cells and IL-2R β-chain positive cells showed a strong correlation ($r = 0.891$), and the histological findings supported these results. The same analysis was performed on lymphocyte subsets, revealing that both CD4 and CD8 cells showed expression of the IL-2R β-chain. These results indicated that combination therapy with lentinan and rIL-2 induced infiltration of effector cells into tumor tissue in patients with colon cancer. In the subsequent mechanism of immune activation, immunological memory should be achieved and a potent immune surveillance mechanism against minute recurrent foci should be established. This mechanism would be involved in defending against tumor recurrence.

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*References*

Black, M.M., Freeman, C., Mork, T., Harvei, S. and Gutler, S.J.(1971). Prognostic significance of microscopic structure of gastric carcinomas and their regional lymph nodes. Cancer 27: 703-711.

Chihara, G., Maeda, Y., Hamuro, J., Sasaki, T. and Fukuoka, F.(1969). Inhibition of mouse sarcoma-180 by polysaccharides from lentinus edodes (Berk.) sing. Nature 222: 687-688.

Denno, R., Hirata, K., Yamaguchi, K., Uchiyama, M., Mikami, T., Mukaiya, M. and Minamida, H.(1993). Expression of IL-2 receptor α and β chains after administration of Lentinan to gastric cancer patients. Biotherapy 7: 22-28.

Hamuro, J. (1994) Host Defenses and Cancer. Kodansha, Tokyo Japan pp. 87-90.

Hamuro, J., Takarada, M., Kashima, N. and Mitsugi, K.(1989). Lentinan induced augmented killer T cell generation from thymocytes is due to the elevated susceptibility to helper factor(s). Int. J. Immuno- pharmacology 2: 171.

Minami. Y., Oishi, I. and Taniguchi, T.(1994). Signal transduction mediated by the IL-2 receptor. Biotherapy 8: 943-949.

Miwa, H.(1984). Identification and prognostic implication of lymphocyte infiltration in tumor tissue of cancer patients. Jap. J .Cancer Clin. 30: 753-756.

Miyazaki, T.(1998). Nolecular mechanism of cell proliferative signal mediated by IL-2. Cell Technology 17: 1576-1584.

Omote, K., Fujimoto, T., Mai, T. and Sakai, S.(1990). Analysis of immunological parameters of gastric cancer patients after preoperative administration of BRM. Biotherapy 4: 868-873.

Sakamoto, N., Koyanagi, Y., Nakajima ,A., Kimura, K. and Serizawa, H.(1996). Immunohistochemical analysis of advanced colon cancer after lentinan administration. Ann. Cancer Res. Ther. 4: 91-97.

Suga, T., Izawa, M., Yamashita, A. and Chihara, G.(1989). Localization of host cells in tumors by administration of Lentinan. Proceedings of the 48th general meeting of the Japanese Cancer Association pp. 313.

Suzuki, M. and Hamuro, J.(1990). Anti-tumor polysaccharide Lentinan Its biological function leading to anti-tumor activity. Biotherapy 4: 1114-1126.

Suzuki, M., Suga, T., Kikuchi, T., Takahashi, F., Miyasaka, Y. and Hamuro, J.(1990). Enhancing antitumor activity and analysis of the mechanisms of expression of antitumor activity. Biotherapy 4: 868-873.

Takahashi, T., Ishii, T., Sakai, N., Seki, M., Sato, J., Ochiai, T., Iwaki, K. and Kodaira, S.(1989). Postoperative immunosuppression in cases of gastric and colon cancers produced by surgical operation and the effect of perioperative administration of lentinan. Jpn. J. Gastroenterol. Surg. 22: 53-59.

Taniguchi, T.(1995). Cytokine signaling through nonreceptor protein tyrosine kinases. Science 268: 251-255.

Taniguchi, T., Matsui, H., Fujita, T., Takaoka, C., Kashima, N., Yoshimoto, R. and Hamuro, J.(1983). Structure and expression of a cloned cDNA for human interieukin-2. Nature 302: 305-310.

Taniguchi, T. and Minami, Y.(1993). The IL-2/IL-2 receptor system: A current overview Cell. 73: 5-8.

Tokuzen, R. (1971). Comparison of Local Cellular Reaction to Tumor Graft in Mice Treated with Some Plant Polysaccharides. Cancer Res. 31: 1590.

Ueno, M., Takizawa, C., Kon, Y., Misaka, R., Kawaguchi, M.and Saito, T.(1991). Augmentation of T lymphocyte infiltration in gastric cancer tissues by intravenous injection of lentinan. J. Jpn. Soc. Cancer Ther. 26: 2359-2364.

Urushizaki, I. (1978). Cell-mediated immunity in cancer-patients. Jap. J. Cancer Clin. 24: 378-385.

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