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Synergistic Antimetastatic Effects of Lentinan and Interleukin 2 with Pre- and Post-operative Treatments

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The antimetastatic activity of a combination of lentinan and interleukin 2 (IL-2) was evaluated against spontaneously metastatic 3-methylcholanthrene-induced DBA/2.MC.CS.T fibrosarcoma. Although pre-operative treatment with either IL-2 or lentinan alone exerted little effect on the reduction of lung metastasis colony numbers (7.1% or 28.4% reduction, respectively), the combination exhibited a synergistic effect (85% reduction). Furthermore, 3 of 13 mice given the pre-operative combination treatment achieved complete cure, while no mice given saline did. Although the post-operative combination treatment also reduced the colony number (71% reduction), it caused little prolongation of survival and no mouse achieved complete cure. Synergistic effects were observed between pre- and post-operative treatments with lentinan and IL-2: 8 of 12 mice were completely cured. The antimetastatic activity was abolished in mice treated simultaneously with antibodies to CD4 and CD8 antigens, whereas either CD4, CD8, or NK1.1 antibody alone was ineffective. Analysis of the cellular mechanism involved in the antimetastatic activity revealed the involvement of a tumor-associated antigen-specific delayed-type hypersensitivity response. These data suggest that the life-prolonging effect of the combination of lentinan and IL-2 is mediated by antigen-specific T cells and that the combination of pre- and post-operative therapy with lentinan and IL-2 may be effective to prevent cancer recurrence and metastasis after surgical resection.

Key words: Lentinan — IL-2 — Antitumor effect — Metastasis — Delayed-type hypersensitivity

Immunotherapeutic treatments with recombinant cytokines have resulted in marked reduction or even cure of some advanced chemoresistant tumors of certain histotypes, which are thought to be relatively immunogenic tumors.[1,2] In many advanced tumors, however, immunotherapeutic treatments have very limited effects in terms of both tumor reduction and prolongation of survival of the patients.[3] The limited efficacy of immunotherapy is explained at least in part by the immunosuppression observed in patients with advanced tumors.[4,5] Even in murine tumor model systems, the therapeutic effect of immunotherapy, which is markedly effective against early-stage tumors, decreased with the progression of the tumor stage.[6] These results clearly suggest that immunotherapy may be applied effectively to cancer patients at an early stage of disease. As compared with chemotherapy, one of the most encouraging characteristics of immunotherapy is an augmented acquisition of the immunological memory in patients due to the therapy.[7] Thus, immunotherapy could be valuable in the prevention of cancer recurrence and metastasis after surgery.

Interleukin 2 (IL-2), a lymphokine mainly produced by activated helper T lymphocytes, induces activation of natural killer (NK) cells, lymphokine-activated killer (LAK) cells, and cytotoxic T lymphocytes (CTL).[8,9] Although extensive clinical trials using IL-2 with or without LAK or tumor-infiltrating lymphocytes (TIL) have been conducted against many types of tumors, only a few tumors, such as malignant melanoma, renal carcinoma, and vascular endothelial sarcoma, have responded to the therapy.[10–12] Development of new methods to widen the effective spectrum of the IL-2 therapy is desirable. Lentinan, a fully purified β-1,3-glucan with β-1,6 branches obtained from *Lentinus edodes*, an edible Japanese mushroom, possesses prominent antitumor effects against many murine tumors and has been used clinically in Japan.[7,13] Its antitumor effect is fully host-mediated, and augmentation of responsiveness of immune effector cells, including NK, LAK, and CTL, to lymphokines is thought to be one of the mechanisms by which the antitumor effects are expressed.[13–15] Recently, we demonstrated that the combination of lentinan and IL-2 induced synergistic antitumor effects against IL-2-resistant established murine tumors and showed that the effects were dependent on the CD8-positive T lymphocyte subset.[16] In an attempt to evaluate the usefulness of pre-operative immunotherapy to induce efficient immunological memory, we have investigated the antimetastatic effects and the mechanisms of combined treatment with lentinan and IL-2 in spontaneous metastatic systems using murine fibrosarcoma.

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MATERIALS AND METHODS

Mice C57BL/6 (B6), DBA/2, and C57BL/6×DBA/2F1 (BDF1) mice were purchased from Charles River Japan (Kanagawa). These mice were maintained in specific-pathogen-free conditions and normal female mice, 6–10 weeks of age, were used for the experiments.

Tumor and target cells for cell-mediated cytotoxicity assay The DBA/2-derived, 3-methylcholanthrene-induced DBA/2.MC.CS.T (MCCST) fibrosarcoma was serially passaged subcutaneously in syngeneic DBA/2 mice.[17] The B6-derived B16-BL/6 melanoma and DBA/2-derived P815 mastocytoma were maintained by *in vitro* culture in RPMI-1640 medium supplemented with 10% or 5% fetal bovine serum (FBS), respectively.

Immunomodulators Lentinan (1 mg, Ajinomoto Co., Inc., Tokyo) was dissolved in sterile saline. Human recombinant IL-2 was prepared as described elsewhere; its specific activity was 5×10^7 units/mg and it was dissolved in sterile saline.[18]

Monoclonal antibodies Monoclonal antibody (mAb) against L3T4 (CD4) (from the hybridoma line GK1.5, a gift from Dr. Hiromi-Fujiwara of Osaka University),[19] Lyt2.2 (CD8α) (from hybridoma line 19/178, a gift from Dr. Kagemasa Kuribayashi of Mie University),[20] and NK1.1 (from hybridoma PK136;ATCC-HB191)[21] were prepared as ascites and purified by ammonium persulfate precipitation. For *in vivo* depletion of lymphocyte subsets, BDF1 mice were given two i.p. injections (at an interval of 4 days) of 40 μg of purified mAb diluted in 200 μl of saline. Depletion efficiency was examined 3 days after the final injection of mAb by flow-cytometric analysis of mesenteric lymph node cells for CD4 or CD8 subsets (depletion efficacy was more than 98%), or by the assay of NK and LAK activities of spleen cells for NK1.1 subsets (depletion efficacy was more than 95% or 60%, respectively), as described previously.[16]

Evaluation of antimetastatic activity Experimental metastases were induced by injecting 2×10^5 B16-BL/6 cells i.v. into the tail veins of B6 mice. Fourteen days later, the animals were killed, and the lungs were removed for counting of metastatic tumor nodules. The pre-injection treatment group received 100 μg/mouse of lentinan i.p. (5 mg/kg/day equivalent) on days -7 to -3 (total; 500 μg/mouse) and 2 μg/mouse of IL-2 twice a day i.p. (0.2 mg/kg/day equivalent) on days -4 to -1 (total; 16 μg/mouse) prior to injection of the tumor cells on day 0. The post-injection treatment group received 100 μg/mouse of lentinan i.p. on days 3 to 7 (total; 500 μg/mouse) and 2 μg/mouse of IL-2 twice a day i.p. on days 8 to 11 (total; 16 μg/mouse) after injection of the tumor on day 0.

As a spontaneous metastasis model, MCCST tumor cells were implanted as a single cell suspension into the right footpad of DBA/2 or BDF1 mice at a dose of 2×10^6 cells/25 μl on day -22 or -25. On day 0, legs bearing tumors were amputated. Twenty or 22 days later, the animals were killed, and the lungs were removed for counting of metastatic tumor nodules. In some experiments, lung weight of the mice was also measured. The post-operative treatment group received 40 μg/mouse of lentinan i.p. (2 mg/kg/day equivalent) on days 1 to 5 (total; 200 μg/mouse) and 2 μg/mouse of IL-2 twice a day i.p. on days 4 to 7 (total; 16 μg/mouse), or 50 μg/mouse of lentinan i.p. (2.5 mg/kg/day equivalent) on days 1 to 10 (total; 500 μg/mouse) and 2 μg/mouse of IL-2 twice a day i.p. on days 3 to 9 (total; 28 μg/mouse) after injection of the tumor on day 0. The pre-operative treatment group received 20 μg/mouse of lentinan i.p. on days -18 to -14 and days -11 to -7 (total; 200 μg/mouse) and 2 μg/mouse of IL-2 twice a day i.p. on days -4 to -1 (total; 16 μg/mouse), or 50 μg/mouse of lentinan i.p. on days -9 to -1 (total; 450 μg/mouse) and 2 μg/mouse of IL-2 twice a day i.p. on days -4 to -1 (total; 16 μg/mouse) prior to foot amputation on day 0.

Assay for cell-mediated cytotoxicity Assay for *in vivo* cytolytic activity against MCCST was carried out in 96-well flat-bottomed microplates. Samples of 1×10^6 cells/well of freshly prepared regional lymph node or spleen cells from MCCST-bearing mice were used as effector cells in a 4-h ^{51}Cr-release assay (effector versus target cell ratio; E/T=100) against ^{51}Cr-labeled MCCST targets. Assay for *in vitro*-induced cytolytic activity against MCCST was carried out in 96-well flat-bottomed microplates. Ten, 5 or 2.5×10^5 cells, which were harvested from mixed lymphocytes and tumor culture (MLTC) (5 days) of spleen cells from mice cured by the combined treatment of lentinan and IL-2 and X-ray-inactivated MCCST cells (responder versus stimulator ratio; R/S=300), were used as effector cells. The percentage of specific lysis was calculated by using the standard formula: % specific lysis = (experimental release − spontaneous release)/(maximal release − spontaneous release)×100.

Preparation of 3 *M* KCl-extracted antigen MCCST cells were suspended at 6×10^7 cells/ml in 3 *M* KCl containing 10 m*M* phosphate buffer (pH 7.4). The suspension was incubated overnight at 4°C with gentle shaking. The tumor cell suspension was centrifuged at 24,000*g* for 60 min and the supernatant was dialyzed against distilled water. Insoluble materials were removed by centrifugation at 24,000*g* for 60 min. The supernatant was dialyzed against phosphate-buffered saline and insoluble materials were removed again by centrifugation at 24,000*g* for 60 min. The resultant supernatants were stored at −80°C and used as a 3 *M* KCl-extracted soluble antigen.

Evaluation of DTH activity to tumor-associated antigen A delayed-type hypersensitivity (DTH) response against MCCST was elicited by inoculating a 3 *M* KCl-extracted

soluble antigen from MCCST cells (200 μg) in 25 μl of saline into the hind footpad opposite to the inoculation site of MCCST tumors in MCCST-bearing DBA/2 or BDF1 mice. The footpad swelling was evaluated as the difference between the thickness of the footpad before and 24 h after antigen injection.

RESULTS

Effects of combination therapy with lentinan and IL-2 on experimental metastasis of B16 melanoma in B6 mice To analyze the antimetastatic effect of combination therapy using lentinan and IL-2, we first evaluated the efficacy on the experimental metastasis model using B16-BL/6-bearing mice. Experimental metastases were induced by injecting 2×10^5 B16-BL/6 cells i.v. into the tail veins of B6 mice. The post-tumor injection treatment with lentinan and IL-2 did not significantly reduce the number of metastases when compared to non-treated control mice (Table I). The pre-tumor injection treatment with lentinan alone slightly reduced the number of metastatic nodules, although the reduction was not significant ($P>0.05$) (Table I). On the other hand, the pre-tumor injection treatment with IL-2 alone or the combination of lentinan and IL-2 induced a marked reduction of the colony number ($P<0.05$) (Table I). Furthermore, an *in vivo* lymphocyte subset depletion experiment revealed that the *in vivo* antimetastatic effector in the combination therapy is NK1.1-positive non-specific killer cells, such as NK, or LAK cells and that participation of the antigen-specific T cell population is relatively low (Table I).

Effects of combination immunotherapy with lentinan and IL-2 on a spontaneous metastasis model using MCCST-bearing mice In order to assess the antimetastatic effect of post-operative treatment with the combination of lentinan and IL-2, experiments were performed with the MCCST-fibrosarcoma spontaneous metastasis model. DBA/2 mice were inoculated s.c. into the right footpad with 2×10^6 MCCST tumor cells on day −22, and the legs bearing tumors were amputated on day 0. One day after foot amputation, combined therapy was begun with lentinan and/or IL-2. Two different regimens were examined. As shown in Table II, treatment with lentinan alone, IL-2 alone or the combination, administered i.p. for 5 or 4 consecutive days (exp. 1), failed to reduce significantly the number of metastatic nodules when compared to saline-injected control mice. However, when intensive therapy using lentinan and IL-2, administered for 10 or 7 consecutive days, respectively, was applied (exp. 2), a decrease in the mean number of metastases exceeding 71% ($P<0.01$) and a decrease in lung weight exceeding 78% ($P<0.01$) were observed. Thus, in this spontaneous metastasis model, post-operative treatment with lentinan and IL-2 possesses a significant anti-metastatic effect.

We next investigated the effects of pre-operative treatments with the combination of lentinan and IL-2. The protocol of tumor inoculation and foot amputation

Table I. Antimetastatic Effects of the Combined Administration of Lentinan (LNT) and IL-2 on an Experimental Metastasis Model Using B16-BL/6-bearing Mice[a)]

Exp.	Treatment group	Mice	Number of metastases±SD	Significance[d)]	
1[b)]	Saline	5	650.8±36.8	−	
	pre-LNT/IL-2	7	105.2±74.0	$P<0.01$	
	post-LNT/IL-2	7	573.8±187.7	$P>0.05$	
2[c)]	Saline	9	275.2±237.7	−	
	LNT	9	111.7±86.3	$P>0.05$	
	IL-2	9	23.5±29.2	$P<0.05$	
	LNT/IL-2	10	24.8±35.9	$P<0.05$	−
	LNT/IL-2+c.mAb	10	43.5±51.6	$P<0.05$	$P>0.05$
	LNT/IL-2+anti-CD8	9	14.5±20.7	$P<0.05$	$P>0.05$
	LNT/IL-2+anti-CD4	10	79.5±88.9	$P<0.05$	$P>0.05$
	LNT/IL-2+amti-NK1.1	10	414.4±191.0	$P>0.05$	$P<0.001$

a) B6 mice were injected with 2×10^5 B16-BL/6 cells i.v. into the tail veins on day 0. Fourteen days later, the animals were killed, and the lungs were removed for counting of metastatic tumor nodules.
b) Pre-injected lentinan and IL-2 were administered i.p. with 100 μg/mouse of lentinan (5 mg/kg) on days −7 to −3 and with 2 μg/mouse of IL-2 twice a day on days −4 to −1. Post-injected lentinan and IL-2 were administered i.p. with 100 μg/mouse of lentinan (5 mg/kg) on days 3 to 7 and with 2 μg/mouse of IL-2 twice a day on days 8 to 11.
c) Treatment with lentinan and IL-2 was done pre-injection, as in exp. 1. The mice were given two i.p. injections of 40 μg of anti-CD8, anti-CD4, or anti-NK1.1 on days −8 and −4. Depletion efficiency was more than 98% for CD4 and CD8 subsets and than 95% for NK activity.
d) *P* values are calculated by using Mann-Whitney's U test.

Table II. Therapeutic Effects of the Post-operative Treatment of the Combination of Lentinan (LNT) and IL-2 on a Spontaneous Metastasis Model Using MCCST-bearing Mice

Exp.	Treatment group	Mice	Lung weight (g)	No. of colony	
1[a]	Saline	8	NT[c]	$>50\pm0.0$	($>50\times7$, d24[f])
	LNT	7	NT	$>38.6\pm18.8$[d]	(5, 27, $>50\times4$, d40, d45)
	IL-2	8	NT	$>44.6\pm15.2$[d]	(7, $>50\times7$)
	LNT/IL-2	8	NT	$>46.1\pm10.2$[d]	(23, $>50\times6$, d45)
2[b]	Saline	13	1.00 ± 0.43	$>45.1\pm15.4$	(1, $>50\times9$, d44$\times3$)
	LNT/IL-2	14	0.22 ± 0.06[e]	12.8 ± 14.7[e]	(0$\times3$, 1, 3, 4, 5, 13, 15, 22, 23, 40, 41, d36)

a) DBA/2 mice were injected s.c. into the footpad with 2×10^6 MCCST cells on day -22. On day 0, legs bearing tumors were amputated. Treatment with 40 μg/mouse of lentinan was initiated i.p. on day 1, and continued for 5 days. Treatment with 2 μg/mouse of IL-2 was initiated i.p. twice a day on day 4, and continued for 4 days. Mice were killed on day 24 and lung metastases were counted.
b) DBA/2 mice were injected s.c. into the footpad with 2×10^6 MCCST cells on day -22. On day 0, legs bearing tumors were amputated. Treatment with 40 μg/mouse of lentinan was initiated i.p. on day 1, and continued for 10 days. Treatment with 2 μg/mouse of IL-2 was initiated i.p. twice a day on day 3, and continued for 7 days. Mice were killed on day 22 and lung metastases were counted.
c) NT; not tested.
d) $P>0.05$ compared to group treated with saline alone (Mann-Whitney's U-test).
e) $P<0.01$ compared to group treated with saline alone (Mann-Whitney's U-test).
f) Date of death.

followed the method described in Table II. A dose of 20 μg/mouse of lentinan was administered i.p. for 5 consecutive days starting 4 and 11 days after tumor inoculation. Three days after the final injection of lentinan, 2 μg/mouse of IL-2 was administered i.p. twice a day for 4 days. The mice were killed and their lungs collected 20 days after foot amputation. As shown in Fig. 1, treatment with IL-2 had little effect on lung metastasis (7.1% reduction of colony number) when compared to saline-injected control mice. Treatment with lentinan alone reduced the number of metastases by 28.4% when compared to saline control mice, but the reduction was not statistically significant. On the other hand, a decrease in the mean number of metastases exceeding 85% was observed ($P<0.01$) in mice given both lentinan and IL-2. Furthermore, 6 of 10 mice given both of them were found to have no sign of lung metastasis.

As pre- and post-operative treatments with the combination of lentinan and IL-2 both exhibited marked antimetastatic effects in terms of reduction of lung metastasis colony number, the prolongation of survival by the pre- and/or post-operative treatments with lentinan and IL-2 in combination was examined. The schedules of the pre- or post-operative combination treatments were as described in Fig. 2. Mean survival times (MST) of the non-treated group and the group which underwent only foot amputation were 40.3 ± 8.9 days and 55.6 ± 7.6 days, respectively (exp. 1). The post-operative treatment induced a slight prolongation of survival times (MST = 64.1 ± 10.5 days) although this was not significant ($P>0.05$) (exp.1). On the contrary, 3 of 13 mice given pre-operative combination treatment survived even 210 days after tumor inoculation, and a significant prolon-

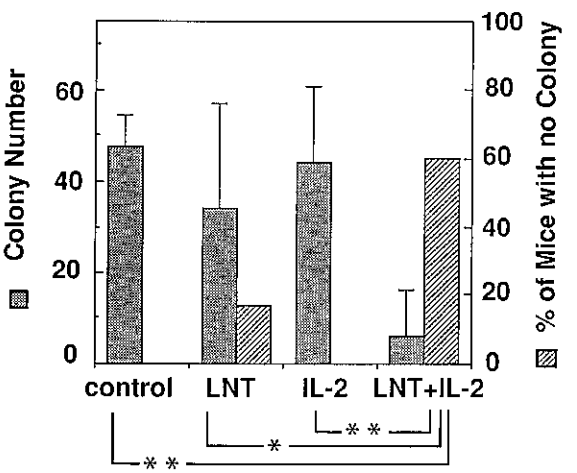

Fig. 1. Antimetastatic effects of pre-operative treatment with the combination of lentinan (LNT) and IL-2 on a spontaneous metastasis model using MCCST-bearing mice. DBA/2 mice were inoculated s.c. into the footpad with 2×10^6 MCCST cells on day -22. Then 20 μg/mouse (1 mg/kg) of lentinan was injected i.p. on days -18 to -14 and days -11 to -7, and 2 μg/mouse of IL-2 was injected i.p. twice a day on days -4 to -1. On day 0, legs bearing tumors were amputated. Mice were killed on day 20 and lung metastases were counted. P values are calculated by using Mann-Whitney's U test. *; $P<0.05$, **; $P<0.01$.

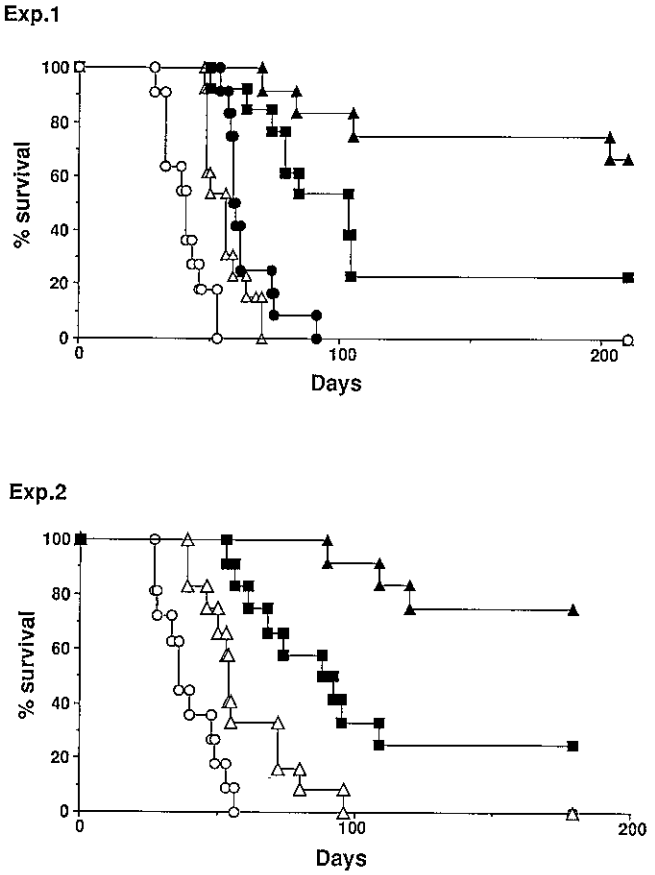

Fig. 2. Life-prolonging effects of the combination pre- and post-operative treatments with lentinan and IL-2 in a spontaneous metastasis model using MCCST-bearing mice. DBA/2 mice were inoculated s.c. into the footpad with 2×10^6 MCCST cells on day -22 and legs bearing tumors were amputated on day 0. In experiment 1, the pre-operative treatment group received 20 μg/mouse of lentinan i.p. on days -18 to -14 and days -11 to -7 and 2 μg/mouse of IL-2 i.p. twice a day on days -4 to -1. The post-operative treatment group received 50 μg/mouse of lentinan i.p. on days 1 to 10 and 2 μg/mouse of IL-2 i.p. twice a day on days 3 to 10. In experiment 2, the pre-operative treatment group received 50 μg/mouse of lentinan i.p. on days -9 to -1 and 2 μg/mouse of IL-2 i.p. twice a day on days -4 to -1. The post-operative treatment group received 50 μg/mouse of lentinan i.p. on days 1 to 10 and 2 μg/mouse of IL-2 i.p. twice a day on days 3 to 10. The statistical significance of differences was evaluated by Kaplan-Meier's methods. Exp. 1: $P>0.05$; operation (op) only vs. op+post-operative treatment. $P<0.001$; op only vs. op+pre-operative treatment. $P<0.001$; op only vs. op+pre- and post-operative treatment. $P<0.05$; op+pre-operative treatment vs. op+pre- and post-operative treatment. Exp. 2: $P<0.05$; op only vs. op+pre-operative treatment. $P<0.001$; op only vs. op+pre- and post-operative treatment. $P<0.01$; op+pre-operative treatment vs. op+ pre- and post-operative treatment. ○; Control, △; op only, ■; op+lentinan/IL-2 (pre), ●; op+lentinan/IL-2 (post), ▲; op+lentinan/IL-2 (pre+post).

gation of survival was observed ($P<0.001$) (exp. 1). Furthermore, addition of post-operative treatment to the pre-operative treatment dramatically augmented the therapeutic effects ($P<0.001$ vs. saline, and $P<0.05$ vs. pre-treatment alone); 8 of 12 mice survived even at 210 days after tumor inoculation (exp. 1). Similar results were obtained in experiment 2.

Effects of *in vivo* lymphocyte subset depletion on the efficacy of the combination of lentinan and IL-2 To clarify which lymphocyte subsets contribute to the anti-metastatic effect of the combination therapy, anti-CD8, anti-CD4, or anti-NK1.1mAb was injected i.p. 15 and 19 days after tumor inoculation. As shown in Fig. 3, either anti-CD4, anti-CD8, or anti-NK1.1 mAb treatment did not influence the therapeutic effects in terms of reduction of lung metastasis colony number. Treatment with anti-CD4 and anti-CD8 simultaneously, however, completely eliminated the antimetastatic effects of the pre-operative combination treatment (Fig. 3). We next examined whether specific immunity against MCCST was acquired in mice after the combination therapy. DBA/2 mice cured by the pre- and post-operative treatment were re-challenged with MCCST or P815 mastocytoma, another DBA/2-derived tumor. All of 6 mice rejected 2×10^6 MCCST cells, but none of 6 mice rejected 2×10^6 P815 cells.

Analysis of the cellular mechanism of the antimetastatic effect of the combination of lentinan and IL-2 Since tumor-associated antigen-specific memory activity was acquired in the mice cured by the combination of lentinan and IL-2, we further analyzed the contributions of CD8 and CD4 T cells in detail. Spleen cells from cured mice were co-cultured with X-ray-inactivated MCCST cells for 5 days (R/S=300). Cytolytic activity of the resultant cells against MCCST tumor was determined by 4-h ^{51}Cr-release assay. As shown in Table III, only a marginal level of killing activity was observed in the cells after MLTC. Furthermore, only a marginal level of *in vivo* killer activity of freshly prepared regional lymph node cells was detected when these cells were collected the day after the pre-operative combination treatment (data not shown).

Next, to examine the role of DTH responses against tumor-associated antigen in the acquisition of tumor-associated antigen-specific memory activity, 200 μg of 3 *M* KCl-extracted soluble antigen from MCCST cells or saline was injected into the footpads of normal or cured mice, and the footpad swelling 24 h after the injection was measured to evaluate DTH response. DTH response against solubilized MCCST antigen was observed only in cured mice (Fig. 4).

Role of tumor-associated antigen-specific DTH response in the antimetastatic effect of lentinan and IL-2 The effects of pre-operative treatment with lentinan or IL-2

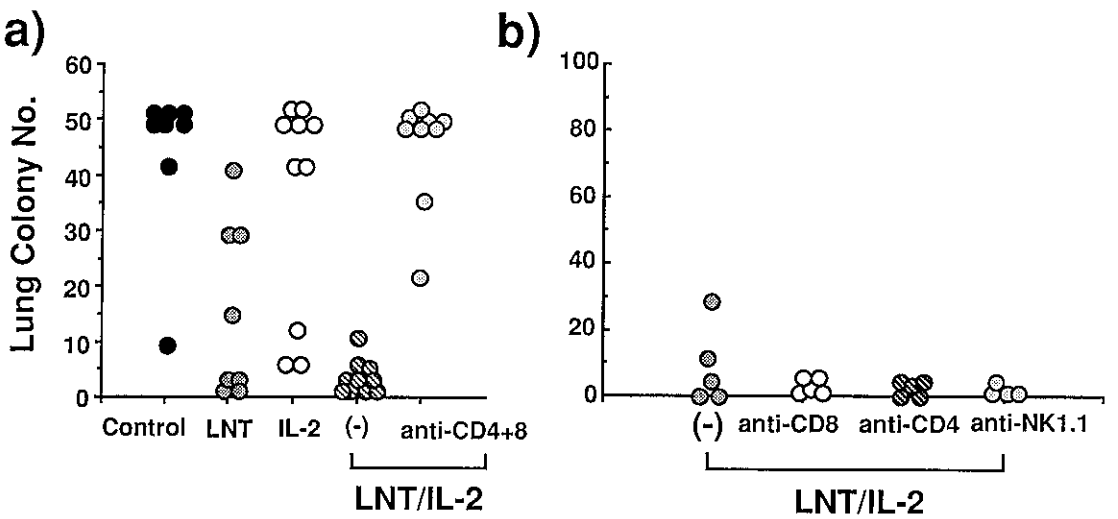

Fig. 3. Abrogation of the antimetastatic effects of lentinan (LNT) and IL-2 against MCCST by *in vivo* depletion of $CD4^+$ and $CD8^+$ cells. BDF1 mice were inoculated s.c. into the footpad with 2×10^6 MCCST cells on day -25. Then 50 μg/mouse of lentinan was injected i.p. on days -9 to -1, and 2 μg/mouse of IL-2 was injected i.p. twice a day on days -4 to -1. On day 0, legs bearing tumors were amputated. Mice were killed on day 20 and lung metastases were counted. The mice were given two i.p. injections of 40 μg of purified anti-CD4, anti-CD8, anti-NK1.1, or both anti-CD4 and anti-CD8 on days -10 and -6. Depletion efficiency of the treatments was more than 98% for CD4 and CD8 subsets and more than 95% for NK activity.
a) Exp. 1. b) Exp. 2

Table III. CTL Activity of Spleen Cells from Mice Cured by the Combination of Lentinan and IL-2 after *in vitro* MLTC[a)]

Mice	Stimulator	E/T		
		100	50	25
Cured	+	3.1±0.4[b)]	0.6±0.8	1.2±0.9
	−	5.4±0.6	2.9±0.9	1.4±0.8
Normal	+	4.0±0.6	1.7±0.7	0.9±0.2
	−	1.2±0.4	1.7±0.7	0.9±0.2

a) DBA/2 mice were inoculated s.c. with 2×10^6 MCCST tumor cells. Foot amputation and treatment with lentinan and IL-2 were performed as described for experiment 1 in the legend to Fig. 2. Spleen cells from the mice cured by the treatments were cultured with X-ray-inactivated MCCST cells for 5 days (responder/stimulator ratio=300). Killing activity of the resultant cells against MCCST were measured by using the 4-h ^{51}Cr-release assay using MCCST target cells.
b) Percent specific lysis against MCCST±SD.

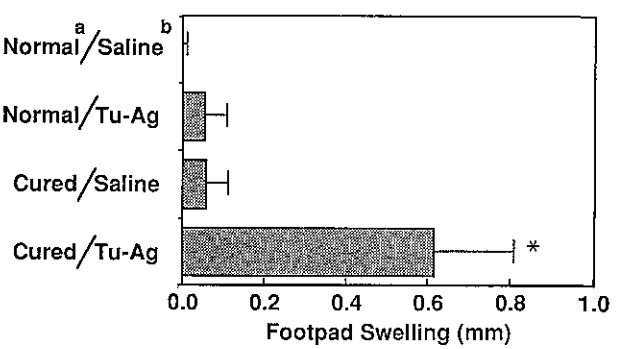

Fig. 4. DTH response against tumor-associated antigens of the cured mice by the combination of lentinan and IL-2. DBA/2 mice were inoculated s.c. with 2×10^6 MCCST tumor cells. Foot amputation and treatment with lentinan and IL-2 were performed as described for experiment 1 in the legend to Fig. 2. The cured or normal mice were injected with 200 μg/25 μl of 3 *M* KCl soluble antigen from MCCST or saline (25 μl) into the footpad. The footpad swelling 24 h after the injection was measured by using a micrometer. The statistical significance of differences was evaluated by using Mann-Whitney's U test. a; mice inoculated with antigen (Tu-Ag) or saline, b; reagents inoculated into footpad. *; $P<0.01$ vs. normal/saline, normal/Ag and cured/saline.

on the induction of a tumor-associated antigen-specific DTH response in tumor-bearing mice were examined. When DTH response was evaluated 6 days after foot amputation, either lentinan or IL-2 alone could augment the DTH response, and the combination of the two induced additive augmentation of the DTH response (Fig. 5). However, augmentation of the DTH response by pre-operative treatment of lentinan and IL-2 was not observed when the DTH response was evaluated 13 days after foot amputation. For the longer-term maintenance of the augmented DTH response, the combination of pre-

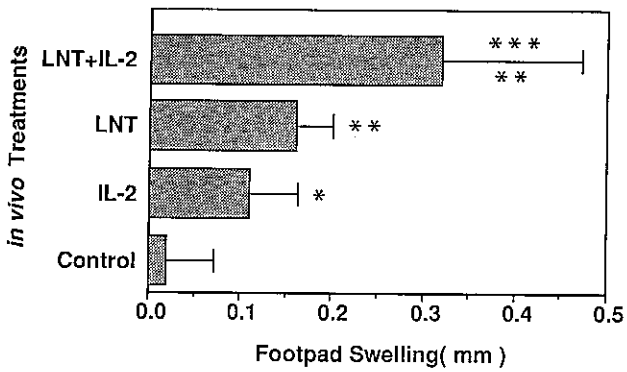

Fig. 5. Effects of pre-operative treatment with lentinan (LNT) and IL-2 on induction of DTH response against tumor-associated antigens. DBA/2 mice were inoculated s.c. with 2×10^6 MCCST cells into the footpad on day -25 and legs bearing tumors were amputated on day 0. The pre-operative treatment with lentinan and IL-2 was perfomed as described for experiment 1 in the legend to Fig. 2. The mice were tested for DTH response by inoculating 3 *M* KCl soluble antigen from MCCST into the footpad on day 6. The statistical significance of differences was evaluated by using Mann-Whitney's U test. *; $P<0.05$ vs. control, **; $P<0.01$ vs. control, ***; $P<0.05$ vs. LNT.

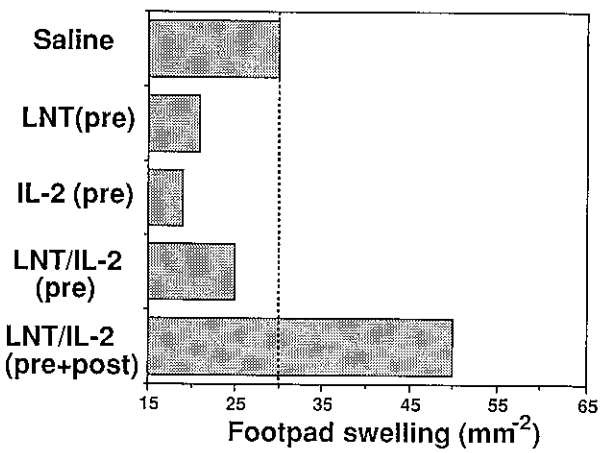

Fig. 6. Effects of the combination of pre- and post-operative treatments with lentinan (LNT) and IL-2 on long-term maintenance of the augmented DTH response. DBA/2 mice were inoculated s.c. with 2×10^6 MCCST cells into the footpad on day -25 and legs bearing tumors were amputated on day 0. The pre-operative and post-operative treatments with lentinan and IL-2 were performed as described for experiment 1 in the legend to Fig. 2. The mice were tested for DTH response by inoculating 3 *M* KCl soluble antigen from MCCST into the footpad on day 13.

and post-operative treatments with lentinan and IL-2 was required (Fig 6). Previous reports demonstrated that lentinan possesses the ability to augment the responsiveness of macrophages to macrophage activating factor (MAF) and to augment DTH response induced by a CD4-positive T cell clone.[13, 22] To clarify whether the augmented DTH response induced by lentinan was caused only by augmentation of the responsiveness to MAF or by augmentation of tumor-associated antigen-specific sensitization, a cell transfer experiment was performed. Lymph node cells from lentinan-treated MCCST-bearing mice exerted an augmented DTH response compared to cells from saline-treated mice when the cells were injected into either saline-treated (footpad swelling; LNT 0.27 mm vs. saline 0.03 mm) or lentinan-treated (footpad swelling; LNT 0.21 mm vs. saline 0.11 mm) recipient mice. These results clearly demonstrate that pre-operative treatment with lentinan augments the tumor-associated antigen-specific sensitization in tumor-bearing mice.

DISCUSSION

In the present study, we have demonstrated that the combination of lentinan and IL-2 exhibits synergistic antimetastatic effects (evaluated in terms of reduction of lung colony numbers) as compared with treatment with either lentinan or IL-2 alone in a spontaneous metastatic system using MCCST fibrosarcoma. Synergy was also observed between pre- and post-operative adjuvant therapy with lentinan and IL-2 when the efficacy was evaluated in terms of complete cure and survival period, which are key indicators of the effectiveness of therapy in cancer patients.

Synergistic antitumor effects have been described in a variety of murine tumor models with combinations of IL-2 and other cytokines.[23–25] In many cases, synergistic augmentation of NK/LAK or tumor-specific CD8-positive CTL activities was suggested to explain the expressed antitumor activities.[23–25] Yamasaki *et al.* reported that synergistic antimetastatic activity and synergistic augmentation of LAK activity by treatment with lentinan and IL-2 were observed in the Lewis-lung carcinoma/DBA/2 system.[26] In this study, we also observed that antimetastatic effects exerted by lentinan and IL-2 injection before tumor inoculation were completely abrogated by anti-NK1.1 mAb in the experimental metastatic model using B16-BL/6 (Table I). When injected after tumor inoculation, the combination did not exert any antimetastatic effects, suggesting that non-specific killer cells such as NK and LAK cells do not have the potential to kill tumor cells already established in metastatic sites, although they do have the potential to kill tumor cells in the circulation. In contrast, the finding that *in vivo* depletion of the NK1.1-positive population did

not affect the antimetastatic activity in the MCCST/BDF1 spontaneous metastasis model demonstrated that participation of NK/LAK in the synergistic antimetastatic activity is minor in the spontaneous metastasis system (Fig. 3). The fact that the lentinan/IL-2-induced antimetastatic effect was completely abrogated in mice injected simultaneously with antibodies to CD4 and CD8 (Fig. 3) suggests that T cells are involved in the induction of this antimetastatic response. Furthermore, the fact that the mice cured after lentinan/IL-2 therapy rejected rechallenge with MCCST tumor indicates that the cured mice had acquired immunological memory against MCCST tumor. To elucidate the cellular mechanism of the acquired memory activity against MCCST, CTL- or DTH-inducing activity in the cured mice was examined. CTL activity against MCCST tumor in spleen cells of the cured mice could not be observed after MLTC (Table III). In contrast, marked DTH response against solubilized MCCST antigen was observed in the cured mice after rechallenge using MCCST tumor (Fig. 4). Furthermore, additive sugmentation of DTH response was observed with the combination of lentinan and IL-2 (Fig. 5). The augmentation of DTH response by lentinan and IL-2 was consistent with synergistic antimetastatic effects, suggesting that DTH response against tumor-associated antigens plays a crucial role in the synergy between lentinan and IL-2. The potential of DTH-induced T cells to induce the reduction of established tumors without any requirement of cytotoxic T cells has been described by other investigators.[27,28] It is well recognized that the Thl-subtype of $CD4^+$ T cells is a main inducer of DTH response and that IL-2 functions as the major factor to activate and proliferate the Thl subpopulation of $CD4^+$ T cells.[29] Furthermore, Zinkernagel reported that the $CD8^+$ T cells also induced antigen-specific DTH response.[30] Activation and proliferation of tumor-associated antigen-specific DTH-induced T cell population (which may include both CD4-and CD8-positive populations) is one of the possible mechanisms by which IL-2 enhances the DTH response and antimetastatic effects in this MCCST/DBA/2 system. On the other hand, it was reported that increased vascular permeability is important in the infiltration of cells into DTH sites[31] and that injection of lentinan induces vascular dilatation and hemorrhage, designated as a VDH response, in a T cell-dependent manner.[32] In an immunotherapy model employing the FBL-3 system, we have also shown that the increase of vascular permeability at the tumor site by lentinan was one of the mechanisms contributing to the enhancement of DTH response and antitumor effects.[22] The augmentation of two distinct steps, activation of tumor-associated antigen-specific T cells by IL-2 and increase of vascular permeability of tumor vessels by lentinan, may participate in the induction of synergistic augmentation of the DTH response and antimetastatic activity.

One of the most important and promising areas for immunotherapy is the prevention of recurrence and metastasis. Although post- and pre-operative treatments with lentinan/IL-2 both induced marked reduction of the lung tumor colony number in this MCCST spontaneous metastases model (Table II and Fig. 1), increase of life span (ILS) of the mice given the post-operative therapy reached only 115.2% compared to the mice that underwent only surgical resection (Fig. 2). On the other hand, ILS of the mice given pre-operative therapy with lentinan and IL-2 reached more than 207% and 3 of 13 mice (23%) showed complete cure and long survival (>210 days) (Fig. 2), indicating that the pre-operative treatment is far better as adjuvant therapy for surgical resection than the post-operative treatment. Furthermore, we found that the combination of pre- and post-operative therapy with lentinan and IL-2 exerted marked synergistic curative effects; 8 of 12 mice (66.7%) showed complete cure and long survival (>210 days) (Fig. 2). The possibility that the augmented curative effects of the combined pre- and post-operative treatments were caused by the increased amount of lentinan or IL-2 may be ruled out, because increase in the injected lentinan dose in pre-operative treatments did not augment the curative effects (Fig. 2 exp. 1 vs. exp. 2, total LNT dose; exp. 1: 200 μg/mouse vs. exp. 2: 450 μg/mouse). Similar results were obtained for IL-2 dose (data not shown). Recently, the efficacy of pre-operative lentinan treatments was confirmed in the Madison lung 109 carcinoma system. By histological studies of regional lymph nodes at the time of operation, it was confirmed that pre-operative administration greatly inhibited regional lymph node metastasis and augmented infiltration of host infammatory cells into the primary tumor site (Suga *et al.*, unpublished results). These results provide hints us to the mechanisms of synergistic antimetastatic effects of the pre- and post-operative therapy with lentinan and IL-2. The putative mechanisms are as follows: 1) pre-operative treatment triggers partial destruction of tumors by host inflammatory cells such as neutrophils and macrophages that infiltrate into the primary tumor site accompanied with DTH responses, and induces tumor antigen-specific memory cells; 2) NK/LAK cell activities augmented by the pre-operative treatments may participate in the tumor cell killing mainly in the blood; 3) post-operative treatment induces clonal expansion and activation of tumor antigen-specific memory cells; 4) memory cells exposed to IL-2 may be easily activated[33] and function against established ecotopic micrometastasis and as effector cells of systemic immune surveillance. Further studies, especially of the organ-specific effector mechanisms responsible for inhibition of metastasis by the combination

therapy, are necessary before the *in vivo* synergy can be fully understood. Regardless of the mechanism, however, the synergy of combined pre- and post-operative therapy with lentinan and IL-2, together with surgical resection, may provide a new method of increasing the potency of IL-2 therapy in clinics.

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