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Curative effects of combination therapy with lentinan and interleukin-2 against established murine tumors, and the role of CD8-positive T cells

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Abstract. The antitumor activity of a combination of an antitumor polysaccharide, lentinan (a β1-3 glucan with β1-6 branches), and interleukin-2 (IL-2) was evaluated against established MBL-2 lymphoma and S908.D2 sarcoma at i.d. sites. Treatment of the MBL-2-tumor-bearing BDF1 mice with lentinan and IL-2 induced complete regression of tumor in 87.5% of mice treated. In contrast, treatments using either lentinan or IL-2 alone failed to induce complete regression of tumor, although temporal growth inhibition of tumor was observed about in half of the mice treated. Improvements of antitumor effects by the combination of lentinan and IL-2 were also observed in the MBL-2/B6 and S908.D2/B10.D2 systems. Expression of the antitumor effects of lentinan/IL-2 treatments required the intact T cell compartment, because the effects were not observed when nude mice were used. In the MBL-2/B6 system, the antitumor action of lentinan/IL-2 treatment was abolished in mice treated with antibody to CD8 antigen, whereas antibodies to CD4 or NK1.1 were ineffective. Furthermore, augmented tumor-specific cytotoxic T lymphocyte (CTL) activity was observed in regional lymph node cells of the mice after lentinan and IL-2 administration. These data indicate that the antitumor effects of lentinan/IL-2 are mediated by $CD8^+$ CTL but not by $CD4^+$ T cells or $NK1.1^+$ NK/LAK cells, and suggest that this combined therapy may be effective against even established tumors that are resistant to IL-2 therapy.

Key words: IL-2 – Lentinan – Synergistic antitumor effects – Tumor-specific CTL

Introduction

It has been suggested that host resistance, mediated by several distinct immune effector cells, is critical in preventing tumor growth [4, 24]. Immunotherapy aims at controlling tumor growth through augmentation of the activities of these immune effector cells [8, 24]. Interleukin-2, a lymphokine produced by activated helper T lymphocytes, plays a central role in augmentation of natural killer (NK) activity, induction of cytotoxic T lymphocytes (CTL) and lymphokine-activated killer (LAK) activities, and production of diverse lymphokines, such as interferon γ and granulocyte/macrophage-colony-stimulating factor, from T cells [17, 29]. A large amount of highly purified recombinant IL-2 (rIL-2) has become available for in vivo studies through cloning of the complementary DNA for IL-2 and its expression in *E. coli* [28, 35]. Although therapy using rIL-2 alone or in combination with LAK cells has been extensively investigated in animal models and in clinical studies, IL-2 therapy has not been fruitfully applied in clinics because of its limited antitumor spectrum and the detrimental side-effects of the application of a large amount of rIL-2 [19, 26]. Furthermore, substantial improvement of the therapeutic efficacy has not been observed in clinics even when IL-2 was applied in combination with either LAK cells or chemotherapeutics [21, 25]. In order to circumvent these problems, a new approach to induce therapeutic efficacy with lower doses of IL-2 may be required.

By contrast, lentinan, a fully purified β1–3 glucan with β1–6 branches obtained from *Lentinus edodes* (Berk.sing), has been used as an anticancer drug in clinics in Japan [8]. Lentinan is a true biological response modifier in the sense that it lacks direct cytotoxic effects against tumor cells and its antitumor effects are mediated through the host defense/immune systems [8]. Previous reports have demonstrated that lentinan augments responsiveness of CTL and NK cells to IL-2 [9, 10]. Recently we found that combined administration of lentinan and IL-2 was able to induce in vivo LAK activity efficiently both in normal and in tumor-bearing mice [33]. These data suggest that the

Abbreviations: B6, C57BL/6; BDF1, C57BL/6 × DBA/2 F1; Lyt2, murine CD8α, Lyt2.1, allele of murine CD8; Lyt2.2, allele of murine CD8; Lyt3, murine CD8β; L3T4, murine CD4

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combination of lentinan with IL-2 may be one of the new ways to increase the therapeutic efficacy of IL-2 treatment against tumors.

In the present study, we have investigated the efficacy of combination therapy of IL-2 with lentinan in three distinct murine tumor/host systems.

Materials and methods

Mice. C57BL/6 (B6), and C57BL/6 × DBA/2F1(BDF1) mice were purchased from Charles River Japan (Kanagawa, Japan). C57BL/6 nude mice or B10.D2 mice were purchased from Clea Japan Inc., (Tokyo) or Shizuoka Laboratory Animal Center (Shizuoka) respectively. 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 B6-derived Moloney-virus-induced MBL-2 lymphoma, Friend-virus-induced FBL-3 erythroleukemia, and B16 melanoma were maintained by in vitro culture in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). The B6-derived EL-4 thymoma, B10.D2-derived Rous-sarcoma-virus-induced S908.D2 sarcoma, C3H-derived MM102 mammary adenoma, DAB/2-derived P815 mastcytoma, and A/Sn-derived Moloney-virus-induced Yac-1 lymphoma were maintained by in vitro culture in RPMI-1640 medium supplemented with 5% FBS. These cells were used as target cells for cell-mediated cytotoxicity assays. Samples containing 2×10^6 MBL-2, B16, or S908.D2 tumor cells in 0.1 ml saline were inoculated intradermally (i.d.) into syngeneic or semi-syngeneic hosts in antitumor testings.

Immunomodulators. Lentinan [Lentinan (Ajinomoto 1 mg), Ajinomoto Co. Ltd., Tokyo] was dissolved in 1 ml sterile saline. Human recombinant IL-2 (rIL-2) was prepared as described elsewhere; its specific activity was 5×10^7 units/mg and it was dissolved in sterile saline at a final concentration of 20 μg/ml [28].

Monoclonal antibodies. Monoclonal antibody (mAb) against L3T4 (CD4) (from the hybridoma line GK1.5, a gift from Dr. Hiromi Fujiwara of Osaka University) [7], Lyt2.1 (CD8α) (from the hybridoma line 116-13.1; ATCC-HB129, a gift from Dr. Tomio Tada of University of Tokyo), Lyt2.2 (CD8α) (from hybridoma 19/178, a gift from Dr. Eiichi Nakayama of Nagasaki University and Dr. Kagemasa Kuribayashi of Kyoto University) [20], and NK1.1 (from hybridoma PK136; ATCC-HB191) [13] were prepared as ascites. In some experiments, protein-A purified mAb were used. mAb against Thy1.1 (from hybridoma F7D5) and Lyt3.2 (CD8β) were purchased from Olac (Oxon, UK) and Meiji-Nyugyo Co. (Tokyo) respectively.

Evaluation of antitumor activity. Samples containing 2×10^6 MBL-2 or S908.D2 were inoculated i.d. into B6, BDF1, B6 nude, or B10.D2 mice, and 100 μl (100 μg/shot) lentinan (corresponding to 5 mg/kg) or saline was administered intraperitoneally (i.p.) to the mice daily for 4 days, starting 7 days (for MBL-2) or 10 days (for S908.D2) after tumor transplantation. On the day following the final injection of lentinan, 100 μl (2 μg/injection) IL-2 (corresponding to 0.1 mg/kg) or saline was administered i.p. twice a day for 4 days. Therapeutic activity was evaluated by the tumor growth inhibition and prolongation of survival. Tumor growth was monitored from days 7 to 60 after tumor inoculation. The size of each tumor was taken to be the product of the largest tumor diameters and the shortest tumor diameters (mm^2).

Depletion of lymphocyte subsets in vivo and in vitro. For in vivo depletion of lymphocytes subsets, B6 mice were given two i.p. injections (at an interval of 4 days) of 50 μg purified anti-NK1.1, 40 μg purified anti-L3T4, 25 μl ascites fluid of anti-Lyt2.2, or 25 μl ascites fluid of control anti-Lyt2.1 diluted in 200 μl saline. Depletion efficiency was examined 3 days after the final injection of mAb by flow-cytometric analysis of mesenteric lymph node cells for L3T4 or Lyt2 subsets, or by the assay of NK and LAK activities of spleen cells for NK1.1 subsets.

For in vitro depletion of lymphocyte subsets, 10^7 cells/ml were incubated with a 1 : 2000 dilution of anti-Thy1.2 ascites, a 1 : 500 dilution of anti-Lyt2.2 (CD8α) ascites, or a 1 : 500 dilution of anti-Lyt3.2 (CD8β), anti-L3T4 (CD4), or anti-NK1.1 ascites for 40 min on ice. Resultant cells were incubated with a 1 : 10 dilution of low-toxicity rabbit complement (Cederlane, Ontario, Canada) at 37° C for 40 min. Viable cells were purified by sedimentation on Lympholyte-M (Cederlane).

Assay for cell-mediated cytotoxicity. Assay for cytolytic activity against MBL-2 was carried out in 96-well flat-bottom microplates. Samples of 1×10^6 or 0.25×10^6 cells/well of freshly prepared regional lymph node or spleen cells from MBL-2-bearing mice were used as effector cells in a 4-h ^{51}Cr-release assay (E/T = 100 or 25) against ^{51}Cr-labeled MBL-2 targets. The percentage specific lysis was calculated by the standard formula: specific lysis (%) = 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release).

For a "cold"-target inhibition assay, 3, or 10×10^4 cells/well of unlabeled target cells were added in the 4-h ^{51}Cr-release assay.

Assays for NK and LAK activities were carried out in 96-well flat-bottom microplates. Samples containing 1×10^6 cells/well spleen cells were cultured with or without 250 units/ml IL-2 in 5% FBS/RPMI-1640 medium for 1 day (for NK activity) or 3 days (for LAK activity). At the end of culture, cells were tested for NK activity in a 4-h ^{51}Cr-release assay (E/T ratio = 100, 50 or 25) using Yac-1 cells as target cells, or for LAK activity in a 4-h ^{51}Cr-release assay (E/T ratio = 100, 50 or 25) using P815 cells as target cells.

Immunofluorescence staining and flow-cytometry analysis. For analysis of lymphocyte subpopulations, aliquots of cells (1×10^7/ml) were stained with fluorescein-isothiocyanate(FITC)-conjugated anti-Lyt2 mAb and with phycoerythrin(PE)-conjugated anti-L3T4 (Becton Dickinson, Mountain View, Calif., USA). The stained cells (1×10^4 cells) were analyzed with a FACScan cell sorter.

Results

Antitumor effects of combined administration of lentinan and IL-2

Lentinan and IL-2 were injected either alone or in combination into mice bearing MBL-2 lymphoma to assess the efficacy of the combination. B6 mice were challenged i.d. with 2×10^6 cells/animal of syngeneic MBL-2 lymphoma and were divided into four groups at random on the 7th day after tumor inoculation. A dose of 0.1 mg/injection of lentinan or saline was administered i.p. for 4 consecutive days starting 7 days after tumor implantation. Following the day of the final injection of lentinan, 2 μg/injection of IL-2 or saline was administered i.p. twice a day for 4 days. Inasmuch as synergistic induction of immune effector activities, including NK, LAK, and CTL was observed only when the animals received lentinan prior to IL-2 [8, 33] (and unpublished observation), the administration schedules were selected on the basis of the data as mentioned above. As shown in Fig. 1, all mice treated with saline died of local tumor growth within 40 days. Three of ten mice and five of nine mice were induced to achieve complete regression at the original site by the treatment of either lentinan or IL-2 alone respectively. However, one of the mice that achieved complete regression following the IL-2 treatment had metastases to the regional lymph node. Furthermore, the combination of lentinan with IL-2 exerted

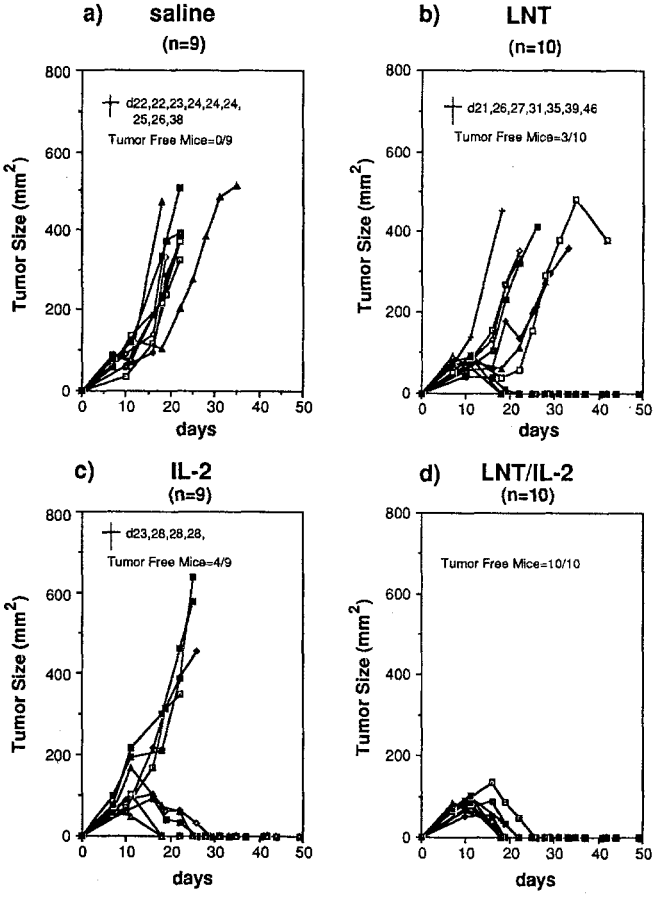

Fig. 1 a–d. Antitumor effects of lentinan and/or interleukin-2 against MBL-2 tumor in C57BL/6 mice. Samples containing 2×10^6 cells of MBL-2 tumor were inoculated i.d. on day 0. The growth curves of the individual tumor in mice administered saline (i.p.) on days 7–14 (**a**), mice treated with lentinan (*LNT*, 0.1 mg/injection, i.p.) on days 7–10 (**b**), mice treated with interleukin-2 (*IL-2*, 2 μg/injection × 2/day, i.p.) on days 11–14 (**c**), and mice treated with both lentinan and interleukin-2 (**d**). Tumor size was taken to be the product of the largest tumor diameters and the shortest tumor diameters (mm^2)

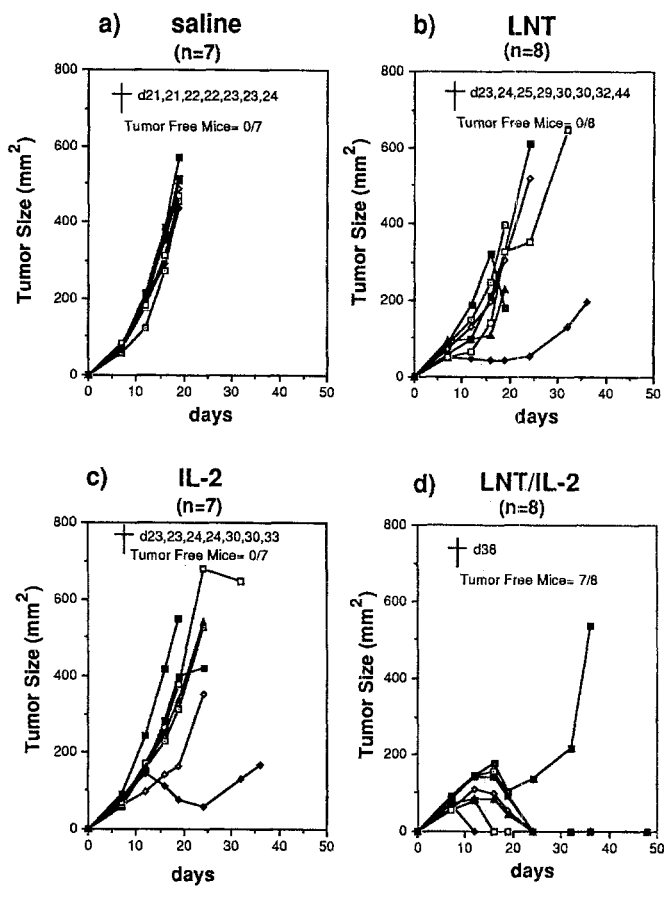

Fig. 2 a–d. Antitumor effects of lentinan and/or IL-2 against MBL-2 tumor in BDF1 mice. Samples containing 2×10^6 cells of MBL-2 tumor were inoculated i.d. on day 0. The growth curves of the individual tumor in mice administered saline on days 7–14 (**a**), mice treated with lentinan (*LNT*, 0.1 mg/injection, i.p.) on days 7–10 (**b**), mice treated with interleukin-2 (*IL-2*, 2 μg/injection × 2/day, i.p.) on days 11–14 (**c**), and mice treated with both lentinan and interleukin-2 (**d**)

augmented antitumor effects in this system and resulted in complete regression of tumors in all mice (ten of ten).

The efficacy of combined therapy with lentinan and IL-2 against MBL-2 in a semi-syngeneic BDF1 host was also examined. The protocol for therapy of MBL-2/BDF1 was identical to that in the MBL-2/B6 system. All mice treated with saline died of local tumor growth within 25 days (Fig. 2). Although substantial prolongation of survival was observed following treatment with either lentinan or IL-2 alone (lentinan: 135%, IL-2: 139%) no mice showed complete regression and all of them died of local tumor growth within 40 days. In contrast, seven of eight (87.5%) mice that received both lentinan and IL-2 achieved complete tumor regression.

Next, to examine the efficacy of combined therapy with lentinan and IL-2 against another S908.D2 tumor, Rous-sarcoma-virus-induced fibrosarcoma raised in B10.D2 mice, B10.D2 mice were challenged i.d. with 2×10^6 cells/animal S908.D2. The protocol was identical to that mentioned above except that drug treatments were started 10 days after tumor implantation. Neither tumor growth inhibition nor increase in survival rate was observed in the mice treated with IL-2 alone. Five of ten mice treated with lentinan alone showed complete regression. The combination of lentinan with IL-2 again exerted the augmented antitumor effects and resulted in the complete regression of tumors in all mice (nine of nine) (Fig. 3).

CD8-positive T cells mediated regression of MBL-2 by combined administration of lentinan and IL-2

To clarify whether the intact T cell compartment is required for the manifestation of antitumor effects in the combination therapy against MBL-2, B6 nude mice were challenged with MBL-2 under the conditions shown in Fig. 1. Neither temporal tumor growth inhibition (data not shown) nor significant improvement in survival rate was observed in B6 nude mice even when both lentinan and IL-2 were administered (Fig. 4). These results indicate the necessity of intact T cell compartments for the antitumor effects caused by lentinan and/or IL-2.

To evaluate further which subpopulation of T lymphocytes, $CD8^+$ or $CD4^+$, mainly participates in the eradica-

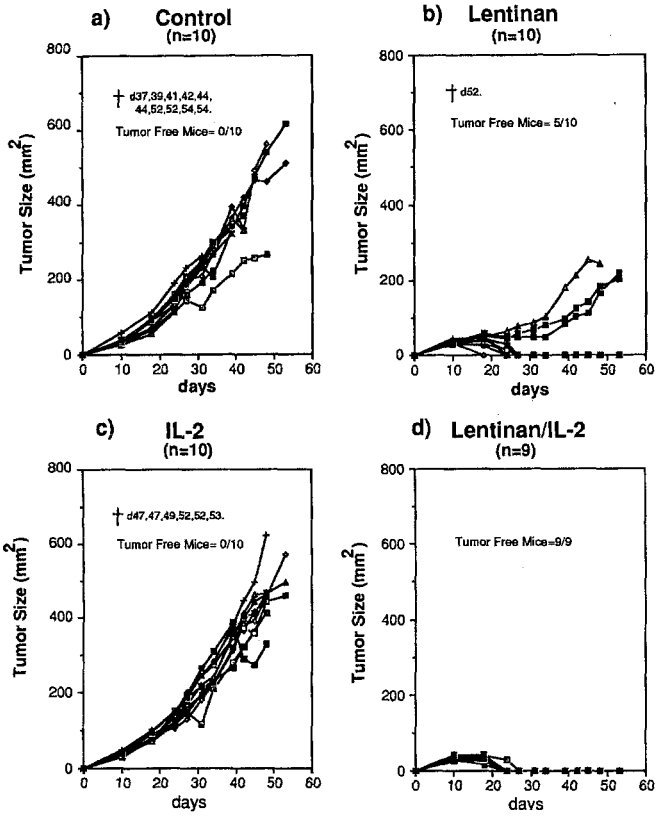

Fig. 3 a–d. Antitumor effects of lentinan and/or IL-2 against S908.D2 tumor in B10.D2 mice. Samples containing 2×10^6 cells of S908.D2 tumor were inoculated i.d. on day 0. The growth curves of the individual tumor in mice administered saline on days 10–17 (**a**), mice treated with lentinan (*LNT*, 0.1 mg/injection, i.p.) on days 10–13 (**b**), mice treated with interleukin-2 (*IL-2*, 2 μg/injection × 2/day, i.p.) on days 14–17 (**c**), and mice treated with both lentinan and interleukin-2 (**d**)

tion of MBL-2 lymphoma and whether NK/LAK cells are involved or not, anti-CD8α, anti-CD4, or anti-NK1.1 mAb was injected i.p. 5, 9, 13, and 19 days after tumor inoculation. On day 12, the depletion efficiency of CD4 or CD8 populations was determined by flow-cytometric analysis of the mesenteric lymph node cells using a FITC-labeled anti-CD8 and a PE-labeled anti-CD4 mAb. The depletion efficiency of the NK1.1 population was determined by assay of NK and LAK activities of the spleen cells on day 12. In

Table 1. Effects of anti-NK1.1 mAb administration on induction of natural killer and lymphokine-activated killer activities

mAb[a]	E/T	Specific lysis (%)			
		NK activity[b]		LAK activity[c]	
		Medium	IL-2[d]	Medium	IL-2
None	100	1.0	29.1	1.9	58.6
	50	0.0	15.9	0.3	36.7
	25	0.0	10.1	2.2	20.7
Anti-NK1.1	100	0.0	0.9	0.0	23.9
	50	0.0	0.0	0.0	14.3
	25	0.0	0.0	0.7	10.9

[a] 40 μg/200 μl purified anti-NK1.1 mAb or saline was injected twice i.p. into B6 mice (four mice per group) at an interval of 4 days; spleen cells were collected 3 days after the final injection of the antibody and subjected to the NK and LAK assay

[b] NK activity was examined in a 4-h ^{51}Cr-release assay using Yac-1 target cells after 24 h culture with or without IL-2

[c] LAK activity was examined in a 4-h ^{51}Cr-release assay using P815 target cells after 72 h culture with or without IL-2

[d] 250 U/ml

the anti-CD4-treated group, the CD4-positive cells were reduced to 0.9%, representing a specific depletion of at least 98% of CD4-positive cells compared with normal mice (53.7%) (data not shown). In the same way, the CD8-positive cells were reduced to 0.1% in the anti-CD8-treated group, representing a specific depletion of at least 98% of CD8-positive cells compared with normal mice (23.2%) (data not shown). The NK activity from the spleen cells of the anti-NK1.1-treated group was reduced to 0.9%, representing a specific depletion of at least 96% compared with the activity in normal mice (29.1%) (Table 1). On the other hand, the LAK activity in the spleen cells of the anti-NK1.1-treated group was partially reduced to 23.9%, representing a specific depletion of only 60% compared with the activity in normal mice (58.6%) (Table 1). Five of six mice injected with control mAb showed complete regression of tumor and prolongation of survival (more than 80 days) when treated with lentinan and IL-2. Anti-CD4 or anti-NK1.1 mAb treatments did not influence the therapeutic effects in terms of either tumor growth inhibition or prolongation of survival rate. By contrast, anti-CD8 mAb treatment eliminated the therapeutic effects completely. In

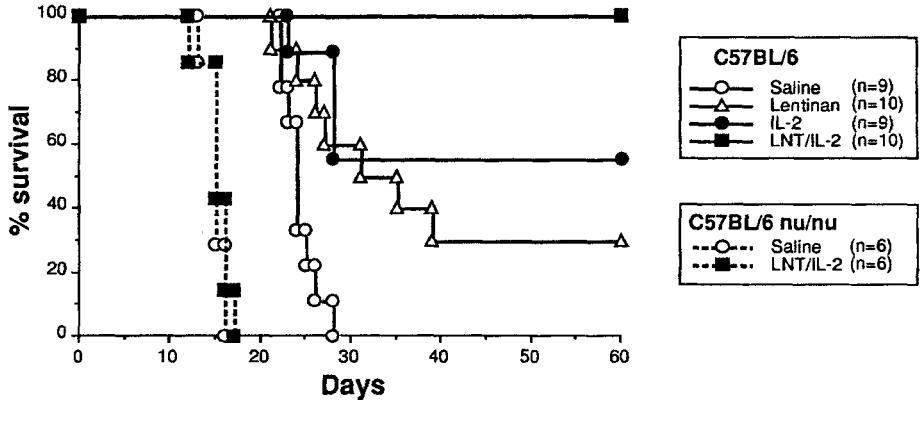

Fig. 4. Life-prolongation effect of lentinan and/or IL-2 against MBL-2 in C57BL/6 normal or nude mice. Survival curves of MBL-2-bearing C57BL/6 normal (———) or nude (– – – –) mice treated with saline (○), lentinan (△), IL-2 (●), or both lentinan and IL-2 (■). The treatment protocol was identical to that in Fig. 1. The experiments shown in Fig. 1 and Fig. 4 were done simultaneously and the C57BL/6 mice shown in Fig. 4 were the same as those in Fig. 1

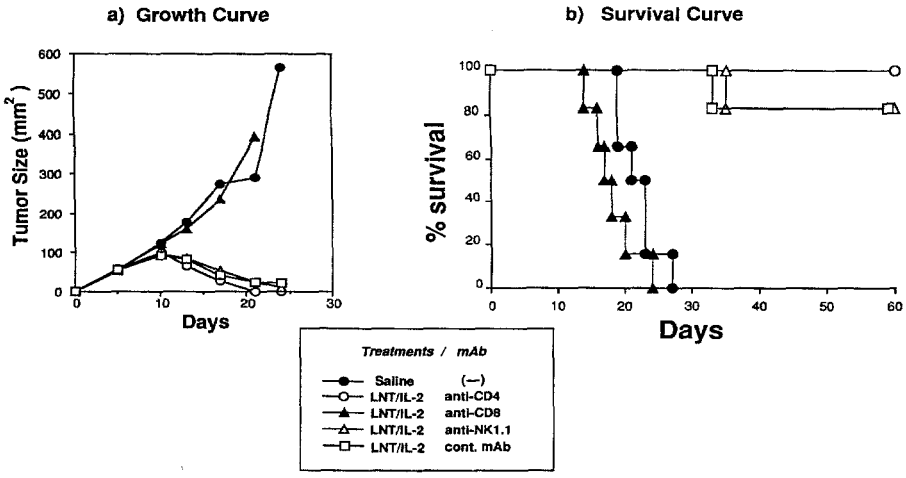

Fig. 5 a, b. Abrogation of the antitumor effect of the combined administration of lentinan and IL-2 against MBL-2 by in vivo depletion of $CD8^+$ cells (**a** growth curve; **b** survival curve). C57BL/6 mice (six mice per group) were inoculated i.d. with 2×10^6 MBL-2 tumor cells on day 0. The mice were given four i.p. injection of 40 μg purified anti-CD4 (○), 25 μl ascites fluid of anti-CD8 (▲), 50 μg purified anti-NK1.1 (△), or 25 μl ascites fluid of control mAb (anti-Lyt2.1) (□) diluted in 200 μl saline on days 5, 9, 13 and 17. The treatment protocol was identical to that in Fig. 1. The tumor size is the average of that from six mice treated

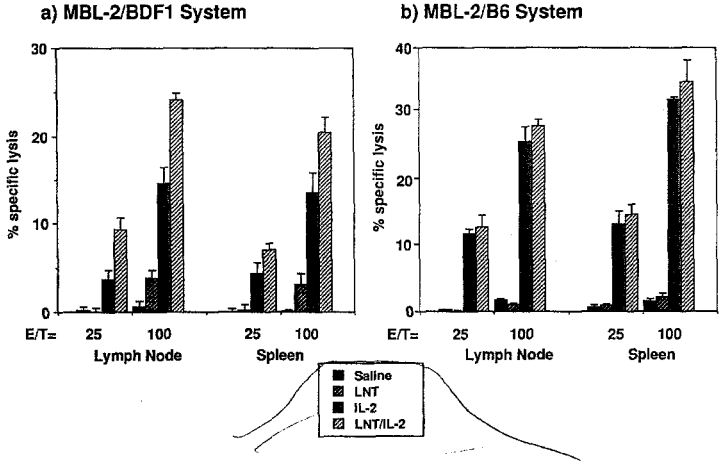

Fig. 6 a, b. Effects of combined administration of lentinan and IL-2 on induction of killer activity against MBL-2 in tumor-bearing host. BDF1 (**a**) or C57BL/6 (**b**) mice (four mice per group) were inoculated i.d. with 2×10^6 MBL-2 tumor cells on day –7. Samples containing 0.1 mg/0.1 ml lentinan were injected i.p. on days 0, 1, 2 and 3, and/or 2 μg/injection IL-2 was injected twice on day on days 4, 5, 6 and 7. On the day after the final injection of IL-2, killer activity against MBL-2 of freshly prepared spleen or regional lymph node cells was measured by 4-h ^{51}Cr-release assay (E/T = 25, 100) against 1×10^4 cells/well MBL-2. ■ Saline; ▨ LNT; ▒ IL-2; ▨ LNT/IL-2

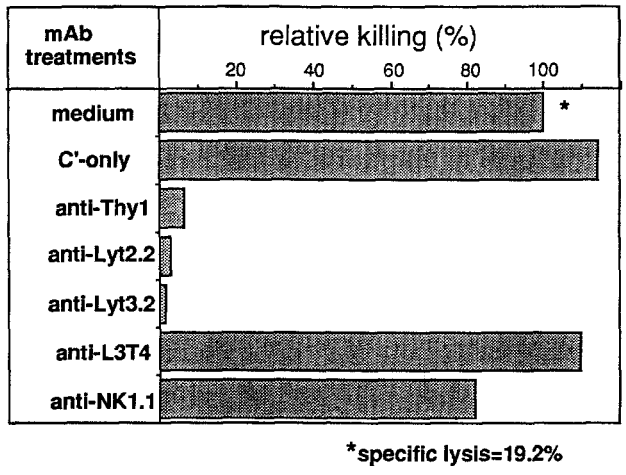

Fig. 7. Surface phenotype of killer cells against MBL-2 induced by lentinan and IL-2 in tumor-bearing C57BL/6 mice. C57BL/6 mice (four mice per group) were inoculated i.d. with 2×10^6 MBL-2 tumor cells on day –7. Samples containing 0.1 mg/0.1 ml lentinan were injected i.p. on days 0, 1, 2 and 3, and 2 μg/injection IL-2 was injected twice a day on days 4, 5 and 6. On the day after the final injection of IL-2, the killer cells from regional lymph node cells were incubated with the antibodies for 40 min on ice, and then treated with low-toxicity rabbit complement at 37° C for 40 min. The killer activity of the resultant cells was measured by 4-h ^{51}Cr-release assay (E/T = 100) against 1×10^4 cells/well MBL-2

anti-CD8-treated mice, no significant inhibition of tumor growth was observed (Fig. 5).

Induction of tumor-specific CD8-positive CTL in vivo during combination therapy with lentinan and IL-2

To examine the immunomodulatory effects of lentinan and/or IL-2 on the induction of cytotoxic effector cells against MBL-2, lentinan (0.1 mg/injection, 1/day for 4 days) and/or IL-2 (2 μg/injection, 2/day for 4 days) were administered to MBL-2-bearing B6 or BDF1 mice. One day after the final injection of IL-2, spleen and lymph node cells were excised and cytolytic activities of freshly prepared spleen or regional lymph node cells were assayed for their ability to kill MBL-2. In BDF1 mice, no significant cytotoxic activities could be detected in either spleen or lymph node cells of mice treated with saline (Fig. 6a), while lentinan or IL-2 augmented cytolytic activities. Furthermore, the combination of lentinan and IL-2 exerted synergistic augmentation of cytolytic activities of lymph node cells. In B6 mice, no significant level of cytolytic activities could be observed in spleen and lymph node cells of mice treated either with saline or lentinan alone (Fig. 6b). Although administration of IL-2 alone aug-

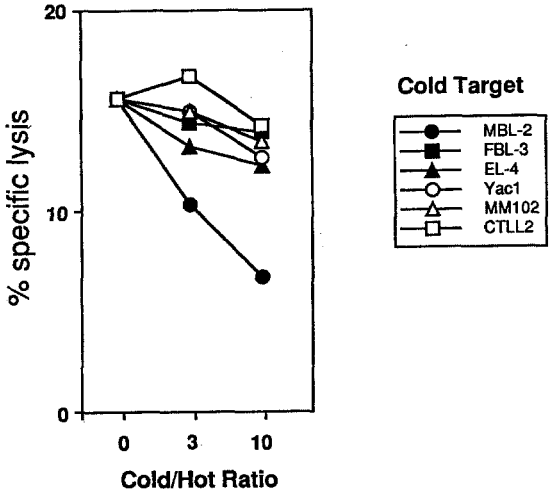

Fig. 8. "Cold" target inhibition assay of killer cells induced by lentinan and IL-2 in tumor-bearing C57BL/6 mice. Induction of killer cells against MBL-2 was performed by the method described in Fig. 7. Samples containing 1×10^6 effecter cells from four mice receiving lentinan and IL-2 were mixed with 1×10^4 ^{51}Cr-labeled MBL-2 and the indicated ratio of non-labeled (*Cold*) tumor cells. After 4 h incubation, the amount of ^{51}Cr released was measured. The percentage specific lysis was calculated as described in Materials and methods

Table 2. Specificity of protective immunity induced by combined treatment with lentinan and IL-2

Expt.	Mice	Ratio of mice rejecting tumor/mice challenged, after challenge with[a]	
		MBL-2	B16-BL6
1	Cured[b]	5/ 5	0/ 5
	Normal[c]	0/ 5	0/ 5
2	Cured	7/ 7	0/ 7
	Normal	0/ 7	0/ 7
Total	Cured	12/12	0/12
	Normal	0/12	0/12

[a] Mice were challenged i.d. with 2×10^6 MBL-2 or B16-BL-6 cells
[b] B6 mice that rejected MBL-2 tumor following treatment with lentinan and IL-2
[c] Normal B6 mice

mented cytolytic activities of spleen and lymph node cells, synergistic augmentation was not observed, contrasting with the presumed synergy in BDF1.

The cell-surface phenotype of the cytotoxic cells of regional lymph node cells from the B6 mice treated with lentinan and IL-2 was analyzed (Fig. 7). Depletion of subpopulations expressing Thy1, CD8α, or CD8β eliminated almost all of the cytolytic activity. On the other hand, depletion of CD4$^+$ or NK1.1$^+$ subpopulations resulted in only marginal reduction in the cytotoxic activity against MBL-2. Almost the same results were obtained in the cell-surface phenotype analysis of cytotoxic cells of B6 mice treated with IL-2 alone (data not shown). The cytotoxic cells induced by lentinan and IL-2 are specific for autologous tumor because only unlabeled MBL-2 cells inhibited the cytolytic activity, but other H-2 compatible or incompatible tumor cells did not (Fig. 8). These results indicate that the cells cytotoxic to MBL-2 induced by lentinan and IL-2 were autologous tumor-specific CD8-positive CTL rather than anomalous killer cells such as NK and LAK cells.

Finally, to test whether specific immunity against MBL-2 was acquired in the mice cured by the combination therapy, the mice were rechallenged with MBL-2 or B16 melanoma, another B6-derived tumor. These mice rejected 2×10^6 MBL-2 cells, but were not able to reject B16. Inoculation of 2×10^6 B16 cells i.d. showed tumor progression resulting in the death of all mice (Table 2).

Discussion

The antitumor effect of IL-2 is considered to be mediated by at least the following three steps: augmentation of cytolytic activity of antitumor effector cells, infiltration of these cells into the sites of tumor progression, and eradication of tumor burdens by the activated effector cells [11, 27]. However, suppression of responsiveness of antitumor effector cells to IL-2 in tumor-bearing hosts [3] and reduced infiltration of these effector cells into tumor sites are thought to be some of the causes of the limited efficacy of IL-2 therapy against tumors [11, 15]. In order to circumvent these critical problems, the combined use of cytokines, such as interferons, tumor necrosis factor or IL-1, with IL-2 has been attempted to provide tools to increase the potency of IL-2 immunotherapy [2, 5, 6, 16]. On the other hand, we and others demonstrated that an antitumor polysaccharide, lentinan, augments the responsiveness of tumor-specific CTL, NK, and LAK precursor cells to IL-2 in vivo and the infiltration of host cells into a tumor site [9, 10, 31, 33]. These data suggest that the combined use of lentinan with IL-2 is the alternative rational way to improve the potency of IL-2 immunotherapy. The current studies have demonstrated that combined treatments with lentinan and IL-2 of mice bearing syngeneic or semi-syngeneic MBL-2 lymphoma or S908.D2 fibrosarcoma resulted in augmented curative antitumor effects when compared with the treatment with either lentinan or IL-2 alone (Figs. 1–3). It should be kept in mind that the combination of lentinan with IL-2 has exerted augmented antitumor effects not only against tumors that are resistant against IL-2 treatment (S908.D2/B10.D2 system), but also against MBL-2/BDF1, which is resistant against either lentinan or IL-2 treatment alone.

The augmented antitumor effects of lentinan and IL-2 are likely to be T-cell-mediated, because the effects against MBL-2 or S908.D2 were completely abrogated in B6 nude mice (Fig. 4) or in Balb/c nude mice (data not shown), H-2-compatible to S908.D2. Possible T cell involvements in tumor cell eradication have so far been suggested: (a) CD8-positive tumor-specific CTL directly mediate tumor cell killing; (b) CD4-positive T cells intervene in a tumor-specific DTH response and in macrophage activation or (c) CD4-positive T cells function as helper cells for NK/LAK activation. Augmentation of tumor-specific CTL induction, responsiveness of macrophages to macrophage-activating-factor, and responsiveness of NK/LAK cells to

IL-2 by in vivo administration of lentinan have previously been reported [9, 10, 33]. To evaluate further which of the mechanisms mainly participate in the eradication of MBL-2 lymphoma in the combination therapy, selective in vivo depletion of each subpopulation, CD4+, CD8+ T cells, or NK1.1-positive NK/LAK cells, was attempted by injection of the specific mAb. CD4-positive T cells or NK1.1-positive NK/LAK cells appeared not to play a substantial role in the antitumor effects of lentinan/IL-2, because the injection of mAb to CD4 or NK1.1 did not affect the antitumor properties. In contrast, CD8-positive T cells may be essential, because complete abrogation of the effects was observed in mice injected with mAb to CD8 (Fig. 5). Although anti-NK1.1 mAb injection was able to induce almost complete abrogation of NK activity, partial abrogation of LAK activity (60%) was observed simultaneously. Previous reports have demonstrated that LAK cells are divided into at least two populations according to their surface markers, NK1.1-positive, T-cell-receptor(TcR)-negative NK-type LAK cells and TcR-positive, NK1.1-negative T-type LAK cells [12, 23]. The remaining LAK activity in our experiments is likely to be due to the presence of T-type LAK cells. Furthermore, recent reports have demonstrated that some of the T-type LAK cells express CD8α (Lyt2) antigen but do not express CD8β (Lyt3) antigen [1]. To evaluate further whether in vivo antitumor effector cells were antigen-specific CTL or anomalous killer cells (including T-type LAK cells) with broad specificity, surface-phenotype and target-specificity analyses were performed. The killer cells against MBL-2 induced by lentinan and IL-2 expressed Thy1, CD8α, and also CD8β but not CD4 and NK1.1. Furthermore, cold-target inhibition assays demonstrated that the killer cells induced were specific for an autologous tumor, because other H-2-compatible tumors (FBL-3, EL-4) or H-2-incompatible lymphoma (Yac-1) induced by the same virus could not block the killing activity against MBL-2 in cold-target inhibition assays. These results suggest that CD8-positive CTL, but not CD4-positive T cells, anomalous killer cells, or NK/LAK cells, are likely to play critical roles in antitumor effects manifested by lentinan and IL-2 in the MBL-2/B6 system. However, CD4-positive T cell requirements in CD8-positive CTL activation in the induction phase can not be ruled out, because mAb treatment was started on 5 day after tumor inoculation.

Previous studies also attempted to identify in vivo antitumor effector cells induced by IL-2 alone or in combination with other cytokines through the in vivo depletion of lymphocyte subsets using mAb [2, 6, 18, 22]. Brunda et al. and Mule et al. demonstrated the importance of NK/LAK populations as in vivo effector cells on pulmonary or liver metastasis [2, 18]. In these studies, however, a roles for CTL in the regression of metastatic tumor can not be ruled out, because antiserum against asialo-GM1 was used to deplete the NK/LAK populations. Asialo-GM1 was also demonstrated to be expressed on some populations of activated CTL [32]. On the other hand, Peace and Cheever and Ciolli et al. reported that antitumor effects exerted by IL-2 or by its combination with IL-1 against established tumor were mediated by CD8-positive CTL but not by NK/LAK cells [6, 22], which is consistent with our current results using lentinan and IL-2. These results suggest the possibility that different types of in vivo antitumor effector cells play roles in the inhibitory effects against the metastatic or primary tumors.

In the MBL-2/BDF1 system, augmentation of IL-2-induced CTL activity by combined use of lentinan was consistent with the augmentation of antitumor effects. Our previous studies suggested that augmentation by lentinan of responsiveness of CTL-precursor to IL-2 was due to augmentation of accessory cell functions, especially through IL-6 production, of macrophages [34]. It is known that IL-6 possesses the activity to induce IL-2 receptor in CTLp [30]. These results demonstrated that combined use of an agent improving responsiveness to IL-2 with IL-2 clearly potentiates the antitumor effect of IL-2 even against tumors that are resistant to IL-2 therapy. On the other hand, the combination of lentinan with IL-2 exerted augmented antitumor effects in the MBL-2/B6 system although marked augmentation of CTL activity was not observed, while this antitumor effect was completely abrogated by the depletion of the CD8+ CTL population, as discussed above. The discrepancy may suggest the possible participation of other antitumor mechanisms by lentinan rather than the augmentation of CTL activities. Further studies are needed, especially on the effects of lentinan on the infiltration of host inflammatory cells into tumor burdens, because it has been demonstrated that lentinan enhanced the vascular permeability in mice [14].

In this paper, we have shown that the combination of lentinan and IL-2 improved the therapeutic effects of IL-2, not only against susceptible tumors but also against tumors resistant to IL-2 therapy. These results suggest that the combination therapy will offer a new tool to improve the limited antitumor spectrum of IL-2 therapy.

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