Jpn. J. Cancer Res. 85, 409–417, April 1994

Reconstitution of Anti-tumor Effects of Lentinan in Nude Mice: Roles of Delayed-type Hypersensitivity Reaction Triggered by CD4-positive T Cell Clone in the Infiltration of Effector Cells into Tumor

Manabu Suzuki,[1,3] Michihiro Iwashiro,[2] Fumihiko Takatsuki,[1] Kagemasa Kuribayashi[2] and Junji Hamuro[1]

[1]Basic Research Laboratories, Central Research Laboratories, Ajinomoto Co. Inc., 1-1 Suzuki-cho, Kawasaki 210 and [2]Institute for Immunology, Faculty of Medicine, Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606

Lentinan, an antitumor polysaccharide used clinically in Japan, requires the intact T cell compartment to manifest its antitumor effects. The aim of the current study was to clarify the mechanisms playing crucial roles in the T cell requirement in the expression of antitumor effects of lentinan. Lentinan treatment of BDF1 mice transplanted intradermally with FBL-3 induced complete tumor regression and a marked increase in survival time. The antitumor action of lentinan was abolished in mice treated simultaneously with antibodies to CD4 and CD8 antigens, whereas antibody to CD4, CD8 or NK1.1 alone was ineffective. The natural killer, cytotoxic T lymphocyte, and helper T cell activities were already augmented in this FBL-3/BDF1 system and thus further augmentation of these activities by lentinan was not observed. These activities did not correlate with the antitumor activity of lentinan, as was confirmed in lymphocyte subset depletion experiments. On the contrary, the delayed-type hypersensitivity (DTH) response against tumor-associated antigens was triggered by lentinan and was abrogated only in mice treated simultaneously with antibodies to CD4 and CD8 antigens. Furthermore, a non-cytolytic tumor-associated antigen-specific CD4$^+$ T cell clone able to induce the DTH response in concert with lentinan reconstituted the antitumor effects in B6 nude mice when administered with lentinan. These results suggest that, in addition to the augmentation of immune effector cell activity against tumors, infiltration of these cells into the tumor burden initiated by the DTH responses at tumor sites may be involved in eradication of tumors by lentinan.

Key words: Lentinan — Antitumor effect — DTH — CD4 T cell clone — Effector infiltration

Many mechanisms may contribute to the failure of a tumor-bearing host to reject tumor cells,[1-4] including immunosuppression due to the presence of the tumor and weak immunogenicity of tumor cells.[3,4] The failure of the adoptive transfer of activated immune effector cells shows that activation of immune effector cells itself is not sufficient to reject tumor cells.[5,6] These observations raised the possibility that infiltration of the effector cells into the tumor burden is important in the rejection of tumors. Small numbers of non-cytolytic T cells capable of inducing DTH[4] against tumor antigen were shown to enhance the antitumor activity of adoptively transferred cytotoxic effector cells.[7] We speculate that DTH responses at tumor sites may play crucial roles in regulating the infiltration of immune effector cells into the tumor burden.

Lentinan, an antitumor polysaccharide used clinically in Japan, is a true biological response modifier in the sense that it lacks direct cytotoxic effects against tumor cells; its antitumor effects are mediated through the host defense-immune systems.[8,9] Expression of antitumor effects of lentinan requires the intact T cell compartment, because the effects were abolished in newborn thymectomized mice and in nude mice.[8,10] Augmentations of NK, LAK, and CTL activities and DTH responses against tumor antigen were observed after administration of lentinan.[8,11,12] These augmented immune effector activities were thought to participate in the antitumor effects of lentinan. Although the requirement of intact T cells for activity was confirmed, it is not known what kinds of mechanisms are involved.

In this report, we provide evidence that non-cytolytic tumor antigen-specific CD4-positive T cell clones are able to reconstitute in nude mice both the antitumor effects and the DTH response of lentinan against FBL-3.

[3] To whom correspondence should be addressed.

[4] Abbreviations used: DTH, delayed-type hypersensitivity; B6, C57BL/6; BDF1, C57BL/6×DBA/2 F1; CTL, cytotoxic T lymphocyte; FBS, fetal bovine serum; IL-2, interleukin 2; IL-4, interleukin 4; γ-IFN, γ-interferon; LAK, lymphokine-activated killer cell; mAb; monoclonal antibody; MLTC, mixed lymphocyte and tumor culture; NK, natural killer cell; TIL, tumor-infiltrating lymphocyte; i.d., intradermally; i.p., intraperitoneally; i.v., intravenously.

MATERIALS AND METHODS

Mice C57BL/6×DBA/2 F1 (BDF1) mice were purchased from Charles River Japan (Kanagawa). C57BL/6 nude (B6 nude) mice were bred in the animal facility of Kyoto University. These mice were maintained under 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 Friend virus-induced FBL-3 erythroleukemia was maintained by *in vitro* culture in RPMI-1640 supplemented with 10% FBS. The A/Sn derived Moloney virus-induced Yac-1 lymphoma was maintained by *in vitro* culture in RPMI-1640 supplemented with 5% FBS. These cells were used as target cells for cell-mediated cytotoxicity assays. Ten million (1×10^7) or 5×10^5 FBL-3 tumor cells in 0.1 ml of saline were inoculated i.d. in BDF1 or B6 unde mice, respectively, for antitumor testing.

T cell clone An FBL-3-specific $CD4^+$ T cell clone (BL4L-23) was established from MLTC and limiting dilution cultures as described previously.[13] In brief, 5×10^6 immune spleen cells from B6 mice were mixed with 1×10^5 X-ray-inactivated FBL-3 and incubated for 5 days. A limiting dilution culture of MLTC cells was carried out on a 1 cell per well basis in the presence of 5 ng/ml rIL-2. Each growing colony was expanded, recloned on a 0.2 cell per well basis, and passed every 7 day by transferring 1×10^5 cells to a new well. The antigen specificity of BL4L-23 is the envelope protein of Friend virus plus $I\text{-}A^b$.[13] This clone produces IL-2 and γ-IFN but not IL-4 after antigenic stimulation.

Immunomodulators Lentinan is a polysaccharide with an average molecular weight of 600,000 isolated from the Japanese edible mushroom *Lentinus edodes*. It is composed exclusively of glucose units with a β (1-3) linkage as the main chain and a β (1-6) linkage as the branched chain.[14] Lentinan (1 mg, Ajinomoto Co., Ltd., Tokyo) was dissolved in 1 ml of sterile saline (1 mg/ml).

mAbs mAb against L3T4 (CD4) (from the hybridoma line GK1.5, a gift from Dr. Hiromi Fujiwara of Osaka University),[15] Lyt2.2 (CD8) (from the hybridoma line 19/178, a gift from Dr. Eiichi Nakayama of Okayama University),[16] and NK1.1 (from the hybridoma PK136; ATCC-HB191)[17] were prepared as ascites. In some experiments, protein-A-purified mAbs were used.

Evaluation of *in vivo* and *in vitro* killer activity Assay for *in vivo* killer activity against FBL-3 was carried out in 96-well flat-bottomed microplates. One×10^6 cells/well of freshly prepared regional lymph node or spleen cells from FBL-3-bearing mice were used as effector cells in a 4-h ^{51}Cr-release assay (E/T=100) against ^{51}Cr-labeled FBL-3 targets. Assay for *in vitro* killer activity against FBL-3 was carried out in 96-well flat-bottomed microplates. Cells harvested from MLTC (5 days) of spleen cells from FBL-3-bearing mice and X-ray-inactivated FBL-3 (responder/stimulator ratio=300) were used as effector cells (1×10^6 cells/well). The percentage of specific lysis was calculated by use of the standard formula: % specific lysis = (experimental release − spontaneous release)/(maximal release − spontaneous release)×100.

Assay for NK activity was carried out in 96-well flat-bottomed microplates. Spleen cells (1×10^6 cells/well) were cultured with 250 units/ml of IL-2 in 10% FBS RPMI1640 medium for 1 day. At the end of culture, cells were tested for NK activity in a 4-h ^{51}Cr-release assay (E/T=100) against ^{51}Cr-labeled Yac-1 cells as targets.

Production of IL-2 and assay for IL-2 activity Spleen cells (3×10^3/ml) from FBL-3-bearing BDF1 mice were cultured with X-ray-inactivated FBL-3 (1×10^4/ml) in 10% FBS RPMI1640. After incubation for 5 days, culture supematants were harvested and stored at −20°C until use. Supernatants were assayed for IL-2 activity according to their ability to support the proliferation of the IL-2-dependent T cell line, CTLL-2. CTLL-2 (4×10^3/well) were cultured with the supernatants for 24 h. Proliferation was assessed in terms of the uptake of [^{3}H]thymidine during 4 h pulsing with 18.5 kBq [^{3}H]thymidine/well.

Evaluation of DTH activity A DTH response against FBL-3 was elicited by inoculating X-ray-inactivated FBL-3 (5×10^5) or 3 *M* KCl-extracted soluble antigen from FBL-3 (200 μg) in 25 μl of saline into the footpads of FBL-3-bearing BDF1 or B6 nude mice. The footpad swelling 24 h after the injection was measured by using a micrometer.

Evaluation of antitumor activity Ten million (1×10^7) or 5×10^5 FBL-3 tumor cells in 0.1 ml of saline were inoculated i.d. in BDF1 or B6 nude mice, respectively. Then 100 μg/100 μl (corresponding to 5 mg/kg) of lentinan or saline was administrated i.p. into mice daily for 5 consecutive days starting 7 days after tumor transplantation. In B6 nude mice, 1×10^7 FBL-3-specific BL4L-23 cells ($CD4^+$ T cell clone) were injected i.v. on the day of tumor transplantation. Therapeutic activity was evaluated in terms of tumor growth inhibition and prolongation of survival days. The size of each tumor was represented as the product of the largest tumor diameter by the shortest tumor diameter (mm^2).

***In vivo* depletion of lymphocyte subsets** For *in vivo* depletion of lymphocyte subsets, BDF1 mice were given three i.p. injections (at intervals of 4 days) of 50 μg of purified anti-NK1.1, 40 μg of purified anti-L3T4 (CD4), or 25 μl of ascites fluid of anti-Lyt2.2 (CD8) diluted in 200 μl of saline. Three days after the second injection of mAb, depletion efficiency was examined by the flow-cytometric analysis of lymph node cells for CD4 or CD8

subsets, or by the assay of NK activity of spleen cells for NK1.1 subsets. Depletion efficiencies of CD4 or CD8 subsets were more than 98% and that of NK1.1 was more than 95%.

RESULTS

Identification of lymphocyte subsets required for antitumor effects of lentinan against FBL-3 The antitumor effect of lentinan was examined against FBL-3 erythroleukemia. BDF1 mice were inoculated i.d. with 1×10^7 viable FBL-3 tumor cells. These mice bore solid tumors approximately 10 mm in diameter on average at 1 week after the tumor inoculation. Lentinan (0.1 mg/shot) or saline was administered i.p. for 5 consecutive days starting 7 days after tumor inoculation. As shown in Fig. 1, all mice treated with saline died of local tumor growth within 50 days. In contrast seven of eight mice (87.5%) which received lentinan showed complete tumor regression. The antitumor effects of lentinan were T cell-dependent, because the antitumor effects induced by lentinan were abolished by the administration of antibodies against both CD4 and CD8 antigens (Fig. 2).

To clarify which subpopulation of T lymphocytes, $CD8^+$ or $CD4^+$ T cells, mainly participates in the eradication of FBL-3 and whether NK/LAK cells are involved or not, anti-CD8, anti-CD4, or anti-NK1.1 mAb was injected i.p. before the onset of the therapy. Eight of nine mice injected with control mAb showed complete tumor regression and prolongation of survival (>80 days) when treated with lentinan. Either anti-CD4 or anti-CD8 mAb treatment did not influence the therapeutic effect in terms of either tumor growth inhibition (data not shown) or prolongation of survival rate. Treatment with anti-NK1.1 caused only a marginal inhibition of the therapeutic effects. Combined administration of anti-CD4 and anti-CD8 completely eliminated the therapeutic effects as described above (Fig. 2).

Effects of lymphocyte subset depletion on the immune effector cells function We next examined the influence of mAb administration on the immune effector cell function after lentinan treatment. Spleen or regional lymph node cells were collected from FBL-3-bearing BDF1 mice on the day after final injection of lentinan. Freshly prepared spleen cells or lymph node cells were assayed for killing activity against FBL-3 (*in vivo* killer activity). A significant level of cytolytic activity was observed in the saline-treated mice, and augmentation of the cytolytic activity by lentinan was not observed (Fig. 3). Administration of anti-CD4 or anti-CD8 had little influence on the cytolytic activity. In contrast, administration of anti-NK1.1 completely eliminated the cytolytic activity. These results indicate that *in vivo* killer activity observed in the tumor-bearing host can be ascribed to the NK cells, and the *in vivo* killer activity does not correlate with the antitumor effects of lentinan in this tumor/host system.

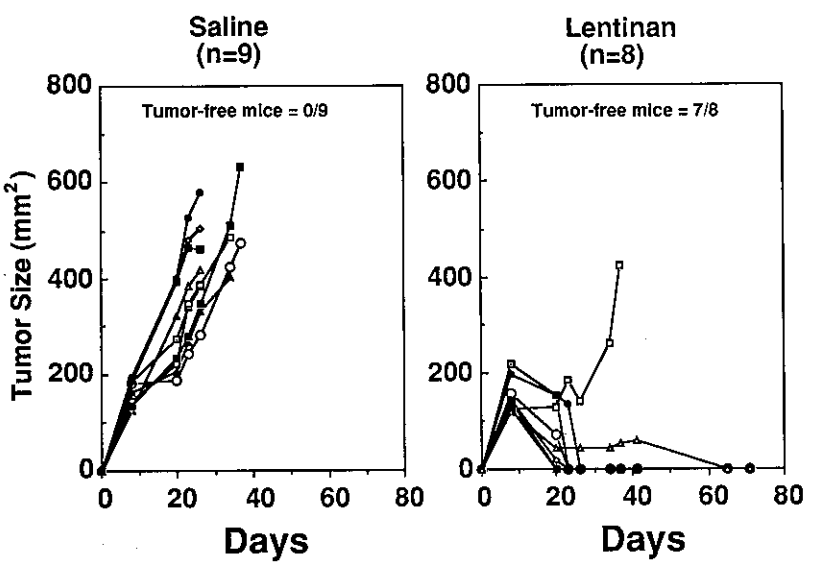

Fig. 1. Antitumor effects of lentinan against FBL-3 tumor in BDF1 mice. FBL-3 (1×10^7 cells) was inoculated i.d. on day 0. The growth curves of the individual tumors in mice given saline (i.p.) on days 7–11 (a), and mice treated with lentinan (5 mg/kg, i.p.) on days 7–11 are shown. Tumor size was represented as the product of the largest tumor diameter by the shortest tumor diameter (mm^2).

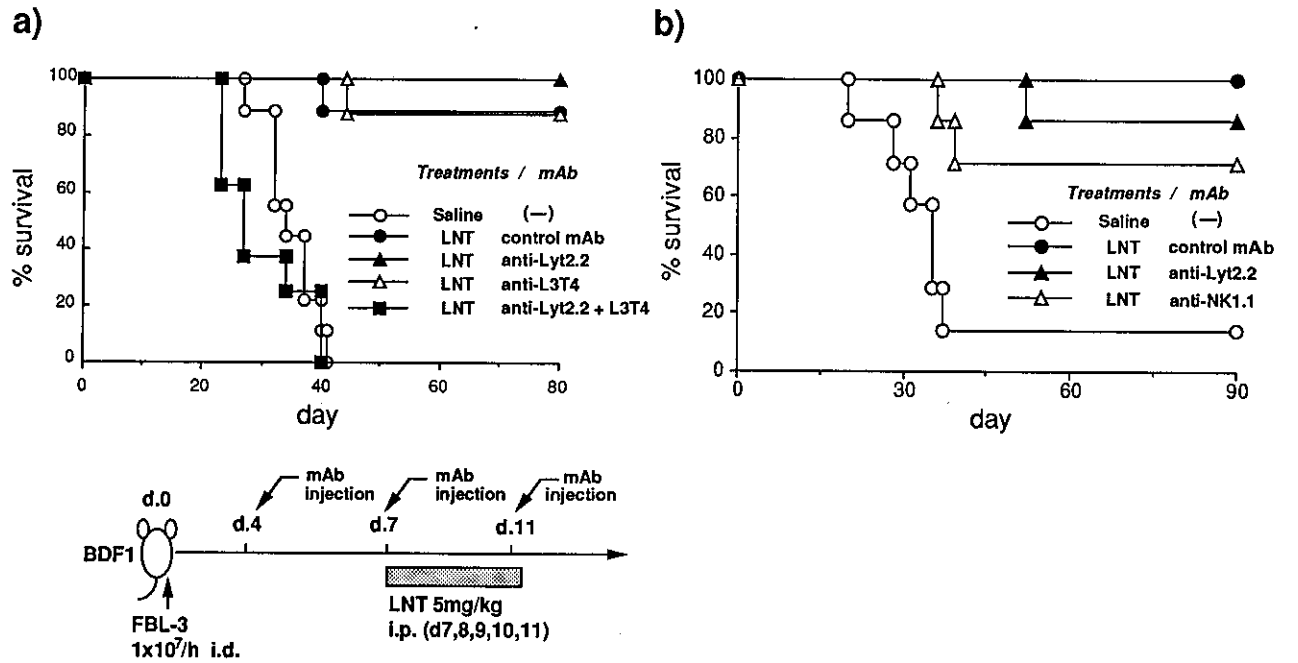

Fig. 2. Abrogation of the antitumor effects of lentinan (LNT) against FBL-3 by *in vivo* depletion of $Lyt2^+$ and $L3T4^+$ cells. BDF1 mice were inoculated i.d. with 1×10^7 FBL-3 tumor cells on day 0. (a) The mice (n=9, except the group given anti-Lyt2.2+anti-L3T4 (n=8)) were given three i.p. injections of 25 μl of ascites fluid of anti-Lyt2.2 (▲), 40 μg of purified anti-L3T4 (△), both anti-Lyt2.2 and anti-L3T4 (■) or 25 μl of ascites fluid of control mAb (anti-human IL-6) (●) diluted in 200 μl of saline on days 4, 7, and 11. (b) The mice (n=7) were given three i.p. injections of 25 μl of ascites fluid of anti-Lyt2.2 (▲), 50 μg of purified anti-NK1.1 (△), or 25 μl of ascites fluid of control mAb (●) diluted in 200 μl of saline on days 4, 7, and 11. The treatment protocol was identical with that in Fig. 1. The statistical significance of differences was evaluated by Kaplan-Meier's method. (a) $P>0.05$, saline vs. LNT with anti-Lyt2.2+anti-L3T4, LNT with control mAb vs. LNT with anti-Lyt2.2 or LNT with anti-L3T4. $P<0.001$; saline vs. LNT with control mAb, LNT with anti-Lyt2.2, or LNT with anti-L3T4. (b) $P>0.05$; LNT with control mAb vs. LNT with anti-Lyt2.2 or LNT with anti-NK1.1. $P<0.001$; saline vs. LNT with control mAb or LNT with anti-Lyt2.2, $P<0.01$; saline vs. LNT with anti-NK1.1.

In vitro killer (Fig. 4) and IL-2-producing activity (Fig. 5) of spleen cells, collected from FBL-3-bearing BDF1 mice on the day after final injection of lentinan, were examined after *in vitro* sensitization to FBL-3. Marked augmentation of the activity by the lentinan treatment was not observed in either assay. The results shown in Fig. 5 indicate that the lymphocyte subpopulation producing IL-2 is mainly the CD4-positive T cells and the IL-2-producing capacity does not correlate with the antitumor activity of lentinan. *In vitro* killer activity was partially inhibited by either anti-CD8 or anti-NK1.1 injection (Fig. 4). The killer activity was almost completely eliminated by anti-CD4 (Fig. 4). In lymphocyte subset depletion experiments, the *in vitro* killer activity did not correlate with the antitumor activity.

In contrast, DTH response against FBL-3 was observed only in FBL-3 tumor-bearing hosts treated with lentinan (Fig. 6). Furthermore, the DTH responses induced by lentinan were inhibited only when both anti-CD4 and anti-CD8 mAbs were injected at the same time; any one of anti-CD4, anti-CD8 and anti-NK1.1 alone was not inhibitory. The absence and presence of DTH responses induced by lentinan in lymphocyte subset-depleted mice were consistent with those of antitumor effects. These results suggest that the DTH responses against tumor-associated antigens induced by lentinan play a crucial role in the antitumor effect of lentinan.

Reconstitution of tumor-specific DTH response and antitumor effect in nude mice by tumor-specific $CD4^+$ T cell clone To confirm the role of DTH responses against tumor-associated antigens in the antitumor effects of lentinan, we used an FBL-3-specific CD4-positive T cell clone, BL4L-23. This clone proliferates on stimulation with FBL-3 and syngeneic spleen cells in the presence of IL-2.[13] The antigen specificity of this clone is the envelope protein of Friend virus plus $I\text{-}A^b$. This clone does not possess cytolytic activity against FBL-3 tumor cells detected by the 4-h or 16-h ^{51}Cr-release assay (data not shown). After an antigenic stimulus, this clone produces IL-2 and γ-IFN but not IL-4. This profile of cytokine

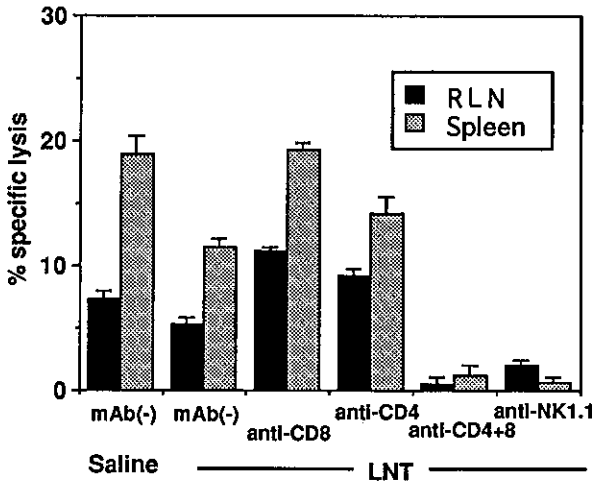

Fig. 3. Effects of *in vivo* depletion of lymphocyte subsets on the induction of *in vivo* killer activity against FBL-3. BDF1 mice were inoculated i.d. with 1×10^7 FBL-3 tumor cells on day 0. *In vivo* depletion of lymphocyte subsets was performed as described in Fig. 2. Lentinan (LNT, 5 mg/kg) or saline was injected i.p. on days 7–11. On the day after the final injection of LNT, the spleen and regional lymph node (RLN) cells were collected and examined for cytolytic activity against FBL-3 by 4-h ^{51}Cr-release assay (E/T=100).

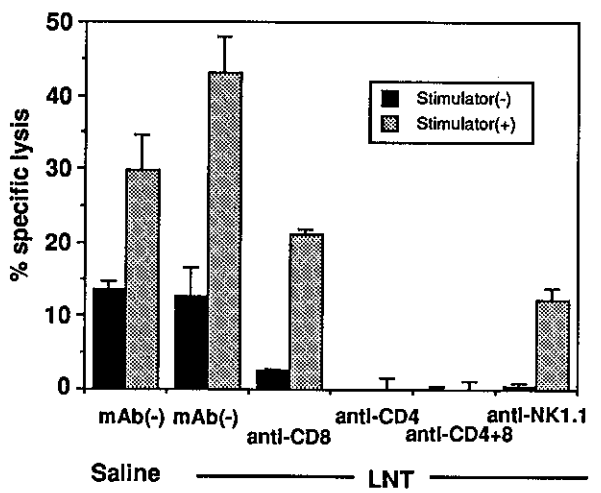

Fig. 4. Effects of *in vivo* depletion of lymphocyte subsets on the induction of *in vitro* killer activity against FBL-3. BDF1 mice were inoculated i.d. with 1×10^7 FBL-3 tumor cells on day 0. *In vivo* depletion of lymphocyte subsets was performed as described in Fig. 2. Lentinan (LNT, 5 mg/kg) or saline was injected i.p. on days 7–11. On the day after the final injection of LNT, the spleen cells were collected and cultured with or without X-ray irradiated FBL-3 (responder/stimulator ratio = 300) for 5 days. The cytolytic activity of the cells against FBL-3 was measured by 4-h ^{51}Cr-release assay (E/T =100).

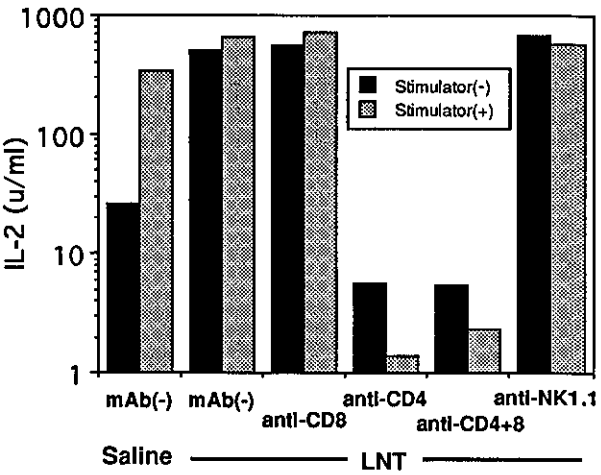

Fig. 5. Effects of *in vivo* depletion of lymphocyte subsets on the production of IL-2 after *in vitro* culture. BDF1 mice were inoculated i.d. with 1×10^7 FBL-3 tumor cells on day 0. *In vivo* depletion of lymphocyte subsets was performed as described in Fig. 2. Lentinan (LNT, 5 mg/kg) or saline was injected i.p. on days 7–11. On the day after the final injection of lentinan, the spleen cells were collected and cultured with or without X-ray-irradiated FBL-3 (responder/stimulator ratio = 300). After culture for 5 days, the culture supernatant was collected and tested for ability to support the growth of CTLL-2 cells (IL-2 assay).

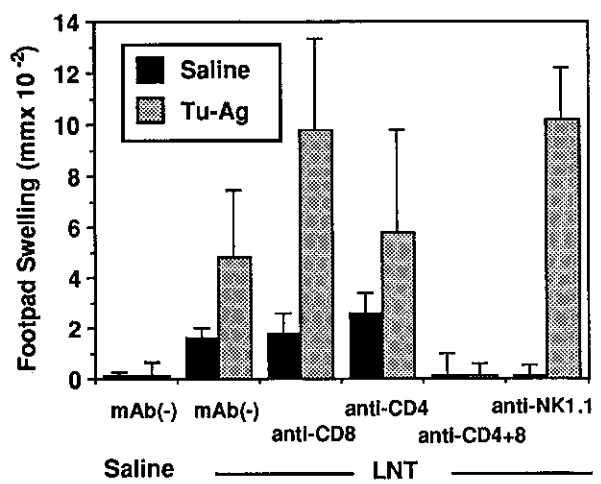

Fig. 6. Effects of *in vivo* depletion of lymphocyte subsets on the induction of DTH response against FBL-3 antigens. BDF1 mice were inoculated i.d. with 1×10^7 FBL-3 tumor cells on day 0. *In vivo* depletion of lymphocyte subsets was performed as described in Fig. 2. Lentinan (LNT, 5 mg/kg) or saline was injected i.p. on days 7–11. On the day after the final injection of LNT, the mice were tested for DTH response (footpad swelling) by inoculating 200 μg of 3 *M* KCl-extracted soluble antigen from FBL-3 or saline into footpads.

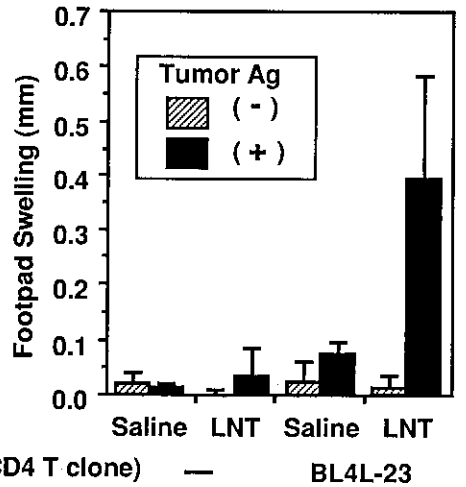

Fig. 7. Reconstitution of DTH response in nude mice by the tumor-specific $CD4^+$ T cell clone. B6 nude mice were inoculated i.d. with 5×10^5 FBL-3 tumor cells on day 0. One$\times10^7$ FBL-3 specific $CD4^+$ T cell clone (BL4L-23) was injected i.v. on the day of tumor transplantation. Lentinan (LNT, 5 mg/kg) or saline was injected i.p. on days 7–11. On the day after the final injection of LNT, the mice were tested for DTH response (footpad swelling) by inoculating 5×10^5 X-ray-inactivated FBL-3 or saline into footpads.

Table I. Reconstitution of Antitumor Effects of Lentinan in Nude Mice by the Tumor-specific $CD4^+$ T Cell Clone

Treatment	Injected CD4 T cells	No. of survivors/total number of mice		
		Exp. 1	Exp. 2	Total
Saline	(−)	0/5	0/6	0/11
	BL4L-23	0/4	0/4	0/8 *
Lentinan	(−)	0/5	0/4	0/9
	BL4L-23	2/5	3/5	5/10**

B6 nude mice were inoculated i.d. with 5×10^5 FBL-3 tumor cells on day 0. FBL-3-specific $CD4^+$ T cell clone (BL4L-23; 1×10^7 cells) was injected i.v. on the day of tumor transplantation. Lentinan (5 mg/kg) or saline was injected i.p. on days 7–11. Survival ratio of each group was examined at day 80 after tumor inoculation.
*, Not significant vs. saline alone.
**, $P<0.05$ vs. lentinan alone.

production is thought to be of Th-1 type. This clone induced a significant DTH response against FBL-3 antigen when injected i.v. into FBL-3-bearing B6 nude mice (Fig. 7). Although lentinan alone induced a marginal DTH response against FBL-3 antigen in FBL-3-bearing B6 nude mice, synergistic augmentation of the DTH responses was observed when nude mice infused previously with BL4L-23 were injected with lentinan (Fig. 7).

We next examined whether the antitumor effect of lentinan, absent in nude mice, could be reconstituted by BL4L-23. When 1×10^7 FBL-3 tumor cells were inoculated into B6 nude mice (similar conditions to Fig. 1 and Fig. 2), the combination of BL4L-23 and lentinan had no antitumor effect (data not shown). As shown in Table I, however, the tumor-specific $CD4^+$ T cell clone (BL4L-23) is able to reconstitute partly the antitumor effects of lentinan in B6 nude mice inoculated with 1×10^5 FBL-3 tumor cells. Lentinan treatment alone failed to inhibit tumor growth or to prolong the life span of FBL-3-bearing B6 nude mice. Although significant growth inhibition of FBL-3 tumor in B6 nude mice was observed when BL4L-23 alone was injected, all mice tested died of local tumor growth within 40 days (data not shown). In contrast, six of twelve mice (50%) showed complete tumor regression by the combination therapy of lentinan and BL4L-23, indicating synergy between lentinan and $CD4^+$ T cells (Table I). On the other hand, Friend gag protein-specific $CD8^+$ CTL clone, which is able to kill FBL-3 tumor *in vitro* and which does not induce DTH response against FBL-3 even when administered together with lentinan, could not reconstitute the antitumor effects in B6 nude mice when administered with lentinan (data not shown).

DISCUSSION

The present study demonstrates that a non-cytolytic, tumor-associated antigen-specific $CD4^+$ T cell clone is able to reconstitute both the lentinan-induced antitumor effects and DTH responses against FBL-3 in nude mice which lack them. Moreover, the intensities of the DTH responses induced by lentinan are consistent with the antitumor effects of lentinan in mice deprived of certain subsets of lymphocytes.

Different processes are thought to be responsible for tumor rejection of solid and disseminated tumors.[18] In a disseminated tumor, immune effector cells may have easy access to tumor cells, and thus high activity of immune effector cells is the main hallmark of successful immunotherapy. In contrast, activated immune effector cells are required to infiltrate into the tumor burden to eradicate solid tumors. One reason for the failure of adoptive immunotherapy with cultured LAK or TIL cells might be poor ability of the cells to infiltrate into the tumor burden (extravascularization and infiltration).

Recent observations have revealed that both $CD4^+$ and $CD8^+$ T cells possess the ability to reject established tumors.[19-22] $CD8^+$ T cells function as cytotoxic T lymphocytes and their direct cytolytic activity is required to induce tumor destruction.[19,20] On the other hand, some possible roles of $CD4^+$ T cells in tumor rejection have been proposed. $CD4^+$ T cells function as helper T cells

for CTL against tumor cells.[21] $CD4^+$ T cells have been considered to function also as a DTH reaction inducer, and tumor-associated antigen-specific DTH responses at tumor sites may induce activation and infiltration of non-specific effector cells, such as macrophages, NK cells, and so on.[22, 23] Furthermore, $CD4^+$ T cells may also function as cytotoxic T lymphocytes.[24] Although it has not been clarified whether either of these mechanisms is critical for tumor rejection, cooperative interactions among these multiple cells may be required to achieve complete tumor rejection. In a disseminated leukemia model using FBL-3 (ascites form), Greenberg *et al.* demonstrated that the combination of chemotherapy and adoptive transfer of $CD4^+$ or $CD8^+$ T cells from immunized mice was capable of inducing therapeutic effects.[18, 25] On the other hand, Nakayama *et al.* reported that $CD4^+$ T cells function as CTL for class II-positive mutant FBL-3 in a solid tumor model.[26, 27]

Although lentinan is known to augment NK and CTL activity, augmentation of *in vivo* or *in vitro* killer activity of lentinan is probably not so important in this model system, because the induced activities did not correlate well with the antitumor effects in lymphocyte subset depletion experiments. NK, CTL and helper T cell (tested in terms of IL-2-producing activity) were already augmented in tumor-bearing mice (Figs. 3–5). This seems to be a typical example of the situation that activation of immune effector cells is not enough to induce tumor rejection. On the contrary, the DTH response against FBL-3 was triggered by lentinan and correlated well with the antitumor effects in lymphocyte subset depletion experiments. In this experiment, in addition to $CD4^+$ T cell subset, $CD8^+$ T cell subset exerted DTH-inducing activity against FBL-3. These results are consistent with the observation by Zinkernagel,[28] who indicated that the $CD8^+$ T cells induced antigen-specific DTH response. Furthermore, in reconstitution experiments using nude mice, a non-cytolytic tumor-associated antigen-specific $CD4^+$ T cell clone able to induce the DTH response in concert with lentinan reconstituted the antitumor effects of lentinan. Therefore, the DTH responses at tumor sites induced by lentinan and the subsequent infiltration of immune effector cells into the tumor burden appear to be important in the antitumor action of lentinan.

Fujiwara *et al.* have proposed that secretion of γ-IFN/MAF from $CD4^+$ T cells and subsequent activation of macrophages by these factors was one of the critical pathways leading to tumor destruction.[21, 22] Involvement of CTL and NK cells in DTH reactions induced tumor destruction was also suggested.[29] In our model of solid FBL-3, NK and CTL also play partial roles in tumor destruction, although participation of activated macrophages was not evaluated, because the antitumor effects of lentinan were abolished when both anti-CD8 and anti-NK1.1 mAb were injected simultaneously (data not shown). These results suggest that augmented infiltration of NK and antigen-specific CTLs into tumor sites by the lentinan-induced DTH reaction against tumor-associated antigen is the major mechanism underlying the antitumor effects of lentinan against FBL-3. Although both NK and CTLs function as effector cells in BDF1 mice, only NK cells function as effector cells in B6 nude mice. The absence of the antigen-specific CTLs may explain the weak antitumor effects of the combination of the DTH-inducible $CD4^+$ tumor-associated antigen-specific T cell clone and lentinan in B6 nude mice.

Increased vascular permeability is important in the infiltration of cells into DTH sites.[30, 30] It was reported that cooperation of early DTH-mediating T cells, enhancing vascular permeability, with cultured effector cells, mediating the late phase of DTH response (that is, cellular infiltration) was required to reject tumors.[7] Askenase *et al.* demonstrated that T cell-dependent secretion of vasoactive amines from cutaneous mast cells played a role in increased vascular permeability in DTH responses.[30, 31] Maeda *et al.* demonstrated that injection of lentinan induced vascular dilatation and hemorrhage at the ears of mice, designated as a VDH response, in a T cell-dependent manner.[32, 33] Lentinan has been reported to increase the susceptibility of mice to histamine or serotonin.[34] Furthermore, we observed enhanced leakage of fluorescein isothiocyanate-dextran at tumor sites around 12 h after lentinan injection in the same tumor model in a T cell-dependent manner, indicating an increase of vascular permeability at the tumor site by lentinan in this tumor model (Takatsuki *et al.*, unpublished results). These results suggest that enhanced vascular permeability is crucial for the augmentation of the DTH response against tumor-associated antigen, and lentinan-induced DTH responses at tumor sites play a role in the eradication of tumors.

Our present data support the notion that, in addition to the augmentation of immune effector activity against tumors, regulation of extravascularization and infiltration of immune effector cells into the tumor burden is crucial to eradicate solid tumors.

ACKNOWLEDGMENTS

We thank Dr. H. Fujiwara and Dr. E. Nakayama for providing us with monoclonal antibody and monoclonal antibody-producing cell lines.

(Received September 21, 1993/Accepted January 5, 1994)

REFERENCES

1) Heidelberger, C. Chemical oncogenesis in culture. *Adv. Cancer Res.*, **18**, 317–366 (1973).
2) Fujimoto, S., Green, M. I. and Sehon, A. H. Regulation of the immune response to tumor antigens. I. Immunosuppressor cells in tumor-bearing hosts. *J. Immunol.*, **116**, 791–799 (1976).
3) Old, L. C. Cancer immunology. *Sci. Am.*, **237**, 62–79 (1977).
4) Dye, E. S. and North, R. J. T cell-mediated immunosuppression as an obstacle to adoptive immunotherapy of the P815 mastocytoma and its metastases. *J. Exp. Med.*, **154**, 1033–1041 (1981).
5) Hosakawa, M., Sawamura, Y., Morikage, T., Okada, F., Xu, Z. Y., Morikawa, K., Itoh, J. and Kobayashi, H. Improved therapeutic effects of interleukin 2 after the accumulation of lymphokine-activated killed cells in tumor tissue of mice previously treated with cyclophosphamide. *Cancer Immunol. Immunother.*, **26**, 250–256 (1988).
6) Lotze, M. T., Line, B. R., Mathisen, D. J. and Rosenberg, S. T. The *in vivo* distribution of autologous human and murine lymphoid cells grown in T cell growth factor (TCGF): implications for the adoptive immunotherapy of tumors. *J. Immunol.*, **125**, 1487–1493 (1980).
7) Trial, J. Cooperation between early acting delayed-hypersensitivity T-cell and cultured cells in tumor rejection. *Cancer Res.*, **48**, 5922–5926 (1988).
8) Hamuro, J. and Chihara, G. Lentinan, a T-cell-oriented immunopotentiator: its experimental and clinical applications and possible mechanism of immune modulation. *In* "Immune Modulation Agents and Their Mechanisms," ed. R. L. Feniche, pp. 409–435 (1085). Marcel Dekker Inc., New York.
9) Suga, T., Shiio, T., Maeda, Y. and Chihara, G. Antitumor activity of lentinan in murine syngeneic and autochthonous hosts and its suppressive effects on 3-methylcholanthrene-induced carcinogenesis. *Cancer Res.*, **44**, 5132–5137 (1984).
10) Maeda, Y. and Chihara, G. The effects of neonatal thymectomy on the antitumor activity of lentinan, carboxymethylpachymaran and zymosan, and their effects on various immune responses. *Int. J. Cancer*, **8**, 41–46 (1973).
11) Suzuki, M., Higuchi, S., Taki, Y., Taki, S., Miwa, K. and Hamuro, J. Induction of endogenous lymphokine-activated killer activity by combined administration of lentinan and interleukin 2. *Int. J. Immunopharm.*, **12**, 613–619 (1990).
12) Masuko, Y., Nakajima, H., Tsubouchi, J., Yamazaki, M., Mizuno, D. and Abe, S. Changes of antitumor immunity of hosts with murine mammary tumors regressed by lentinan: potentiation of antitumor delayed hypersensitivity reaction. *Gann*, **73**, 790–797 (1982).
13) Iwashiro, M., Kondo, T., Shimizu, T., Yamagishi, H., Takahashi, K., Matsubayashi, Y., Masuda, T., Otaka, A., Fujii, N., Ishimoto, A., Miyazawa, M., Robertson, M. N., Chesebro, B. and Kuribayashi, K. Multiplicity of virus-encoded helper T-cell epitopes expressed on FBL-3 tumor cells. *J. Virol.*, **67**, 4533–4542 (1993).
14) Sasaki, T. and Takasuka, N. Further study of the structure of lentinan, an antitumor polysaccharide from *Lentinus edodes*. *Carbohydrate Res.*, **47**, 99–104 (1976).
15) Dialynas, D. P., Quan, Z. S., Wall, K. A., Pires, A., Quintous, J., Loken, M. R., Pierres, M. and Fitch, F. W. Characterization of the murine T call surface molecule, designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human Leu3/T4 molecule. *J. Immunol.*, **131**, 2445–2451 (1983).
16) Nakayama, E. and Uenaka, A. Effects of *in vivo* administration of Lyt antibodies. Lyt phenotype of T cells in lymphoid tissues and blocking of tumor rejection. *J. Exp. Med.*, **161**, 345–355 (1985).
17) Koo, G. C., Dumont, F. J., Tutt, M., Hackett, J. and Kumar, V. The NK1.1(−)mouse: a model to study differentiation of murine NK cells. *J. Immunol.*, **137**, 3742–3747 (1986).
18) Greenberg, P. D., Kern, D. E. and Cheever, M. A. Therapy of disseminated murine leukemia with cyclophosphamide and immune Lyt-1$^+$2$^-$ T cells; tumor eradication does not require participation of cytotoxic T cells. *J. Exp. Med.*, **161**, 1122–1134 (1985).
19) Mills, C. D. and North, R. J. Expression of passively transferred immunity against an established tumor depends on generation of cytolytic T cells in recipients: inhibition by suppressor T cells. *J. Exp. Med.*, **157**, 1448–1460 (1983).
20) Shu, S., Chou, T. and Rosenberg, S. A. Generation from tumor-bearing mice of lymphocytes with *in vivo* therapeutic efficacy. *J. Immunol.*, **139**, 295–304 (1987).
21) Fujiwara, H., Hamaoka, T., Shearer, G. M., Yamamoto, H. and Terry, W. D. The augmentation of *in vivo* tumor-specific T cell-mediated immunity by amplifier T lymphocytes. *J. Immunol.*, **124**, 863–869 (1980).
22) Fujiwara, H., Fukuzawa, M., Yoshioka, T., Nakajima, H. and Hamaoka, T. The role of tumor-specific Lyt-1$^+$2$^-$ T cells in eradicating tumor cells *in vivo*. I. Lyt-1$^+$2$^-$ T cells do not necessarily require recruitment of host's cytotoxic T cell precursors for implementation of *in vivo* immunity. *J. Immunol.*, **133**, 1671–1676 (1984).
23) Mowat, A. M., Borland, A. and Parrott, D. M. Augmentation of natural killer cell activity by anti-host delayed-type hypersensitivity during the graft-versus-host reaction in mice. *Scand. J. Immunol.*, **22**, 389–399 (1985).
24) Golding, H. T., Munitz, T. I. and Singer, A. Characterization of antigen-specific, Ia-restricted, L3T4$^+$ cytolytic T lymphocytes and assessment of thymic influence on their self specificity. *J. Exp. Med.*, **162**, 943–961 (1985).
25) Greenberg, P. D. Therapy of murine leukemia with cyclophosphamide and immune Lyt2$^+$ cells: cytolytic T cells can mediate eradication of disseminated leukemia.

J. Immunol., **136**, 1917–1922 (1986).
26) Udono, H., Mieno, M., Shiku, H. and Nakayama, E. The roles of $CD8^+$ and $CD4^+$ cells in tumor rejection. *Jpn. J. Cancer Res.*, **80**, 649–654 (1989).
27) Yoshimura, A., Shiku, H. and Nakayama, E. Rejection of an I-A^+ variant line of FBL-3 leukemia by cytotoxic T lymphocytes with $CD4^+$ and $CD4^-$ $CD8^-$ T cell receptor-$\alpha\beta$ phenotypes generated in CD8-depleted C57BL/6 mice. *J. Immunol.*, **150**, 4900–4910 (1993).
28) Zinkernagel, R. M. H-2 restriction of virus-specific T-cell-mediated effector functions *in vivo*. II Adoptive transfer of delayed-type hypersensitivity to murine lymphocytic choriomeningitis virus is restricted by the K and D region of H-2. *J. Exp. Med.*, **144**, 776–787 (1976).
29) Hamaoka, T. and Fujiwara, H. Phenotypically and functionally distinct T-cell subsets in anti-tumor responses. *Immunol. Today*, **8**, 267–269 (1987).
30) Askenase, P. W., Bursztajn, S., Gershon, M. D. and Gershon, R. K. T cell-dependent mast cell degranulation and release of serotonin in murine delayed-type hypersensitivity. *J. Exp. Med.*, **152**, 1358–1374 (1980).
31) Askenase, P. W., Metzler, C. M. and Gershon, R. K. Localization of leucocytes in site of delayed-type hypersensitivity and in lymph nodes: dependence on vasoactive amines. *Immunology*, **47**, 239–246 (1982).
32) Maeda, Y., Watanabe, T., Chihara, G. and Rokutanda, M. T-cell mediated vascular dilatation and hemorrhage induced by antitumor polysaccharides. *Int. J. Immunopharm.*, **6**, 493–501 (1984).
33) Maeda, Y., Sakaizumi, M., Moriwaki, K. and Yonekawa, H. Genetic control of the expression of two biological activities of an antitumor polysaccharide, Lentinan. *Int. J. Immunopharm.*, **13**, 977–986 (1991).
34) Homma, R. and Kuratsuka, K. The histamine-sensitizing activity of lentinan, an antitumor polysaccharide. *Experientia*, **29**, 290–293 (1973).