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Augmentation of anti-tumor activity by immunization with Mycobacterium tuberculosis (Tbc) and tuberculin-coupled tumor cells.

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Abstract

The anti-tumor effect of immunization with heat-killed Mycobacterium tuberculosis (Tbc) and Tuberculin (PPD)-coupled syngeneic tumor cells was examined in vivo. Three tumor cell lines were employed. Immunization of Tbc-primed BALB/c mice with PPD-coupled syngeneic Meth-A tumor cells displayed a potent anti-tumor effect on viable Meth-A cells inoculated subcutaneously. Neither PPD-coupled LLC (Lewis Lung Carcinoma) cells nor sonicated PPD-coupled Meth-A cells were capable of immunizing these mice. PPD-coupled syngeneic whole tumor cells were indispensable for induction of this tumor-specific resistance. Immunization of Tbc-primed C3H/He mice with PPD-coupled syngeneic MH134 tumor cells did not elicit anti-tumor activity against MH134, but additional pretreatment of mice with cyclophosphamide brought on an anti-tumor effect. Antimetastatic reactivity was investigated in C57BL/6 mice bearing LLC, with a reduction in metastases noted. This antimetastatic effect was observed even when the mice were immunized with PPD-coupled LLC cells three days after removal of the initial tumor. Immunization with Tbc and PPD-coupled Meth-A cells together with intraperitoneal administration of murine or rat interleukin 2 (IL 2) further augmented anti-Meth-A resistance. Murine IL 2 further inhibited tumor growth during the early stage, while rat IL 2 showed an anti-tumor effect throughout the course of tumor growth.

KEYWORDS: mycobacterium, Tuberculin, interleukin 2, coupling

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AUGMENTATION OF ANTI-TUMOR ACTIVITY BY IMMUNIZATION WITH *MYCOBACTERIUM TUBERCULOSIS* (TBC) AND TUBERCULIN-COUPLED TUMOR CELLS

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Abstract. The anti-tumor effect of immunization with heat-killed *Mycobacterium tuberculosis* (Tbc) and Tuberculin (PPD)-coupled syngeneic tumor cells was examined *in vivo.* Three tumor cell lines were employed. Immunization of Tbc-primed BALB/c mice with PPD-coupled syngeneic Meth-A tumor cells displayed a potent anti-tumor effect on viable Meth-A cells inoculated subcutaneously. Neither PPD-coupled LLC (Lewis Lung Carcinoma) cells nor sonicated PPD-coupled Meth-A cells were capable of immunizing these mice. PPD-coupled syngeneic whole tumor cells were indispensable for induction of this tumor-specific resistance. Immunization of Tbc-primed C3H/He mice with PPD-coupled syngeneic MH134 tumor cells did not elicit anti-tumor activity against MH134, but additional pretreatment of mice with cyclophosphamide brought on an anti-tumor effect. Antimetastatic reactivity was investigated in C57BL/6 mice bearing LLC, with a reduction in metastases noted. This antimetastatic effect was observed even when the mice were immunized with PPD-coupled LLC cells three days after removal of the initial tumor. Immunization with Tbc and PPD-coupled Meth-A cells together with intraperitoneal administration of murine or rat interleukin 2 (IL 2) further augmented anti-Meth-A resistance. Murine IL 2 further inhibited tumor growth during the early stage, while rat IL 2 showed an anti-tumor effect throughout the course of tumor growth.

Key words : mycobacterium, Tuberculin, interleukin 2, coupling.

Although there is evidence for the existence of tumor-associated transplantation antigens (TATA), syngeneic hosts are incapable of rejecting tumor cells inoculated into them (1, 2). Attempts to amplyfy TATA immunogenicity by modifying the tumor cell surface have somewhat successfully enhanced the response, but not strongly enough to reject the tumor challenge (3, 4). TNP (trinitrophenyl)-reactive amplifier T cell activity was induced in mice by immunization with TNP-conjugated mouse γ-globulin (MGG) (5, 6). These mice presented anti-tumor activity, both *in vitro* and *in vivo*, after secondary immunization with TNP-conjugated syngeneic tumor cells. Induction of interaction between hapten-reactive helper T-cells and cytotoxic T-cells was applied to the experimental model of immunization with Tbc followed by PPD-coupled syngeneic tumor cells; PPD sensitization generated PPD-

reactive helper T-cell activity (7). C3H/He mice immunized sequentially with Tbc and PPD-coupled syngeneic tumor cells possessed immunity against syngeneic X5563 plasmacytoma cells (8). The growth of transplanted 3-methylcholanthrene-induced tumors was also suppressed in BCG-primed mice after immunization with PPD-coupled syngeneic tumor cells (9). In this report, immunization with Tbc and PPD-coupled tumor cells was followed by challenge with one of three cell lines, Meth-A, MH134 or LLC. Tumor growth as well as metastases were examined. The additional anti-tumor effect of interleukin 2 (IL 2) administration also was investigated.

MATERIALS AND METHODS

Mice. Female C3H/He mice were purchased from the Funabashi Farm (Chiba, Japan). C57BL/6 and BALB/c mice, also female, were purchased from the Shizuoka Animal Laboratory (Hamamatsu, Japan). At the time of testing the mice were 5 to 6 wk of age.

Tumors. Murine hepatoma MH134 (C3H/HeJ origin) was maintained as an ascitic tumor by passage in syngeneic mice (10^6 cells/animal). A 3-methylcholanthrene-induced sarcoma, Meth-A (BALB/c origin), was maintained similarly. A spontaneously occurring Lewis Lung Carcinoma (LLC, C57BL/6 origin) was maintained as a solid tumor in gluteal muscles of syngeneic mice (10^6 cells/animal).

Preparation of PPD-coupled tumor cells. PPD-coupled tumor cells were prepared according to established methods (8). LLC cells were suspended by treating them with 250 mg/dl trypsin for 15 min at 37℃. After treatment with 20 ml of 0.05 mg/ml mitomycin-C (MMC) solution for 45 min at 37℃, 10^8 tumor cells were suspended in 10 ml of phosphate-buffered saline containing 0.1 mg/ml of PPD (Mitsui Pharmaceutical Industry Co.,Tokyo, Japan). One milliliter of a 3-(3-dimethylaminopropyl)-1-ethylcarbodiimid (ECDI) (Peptide Laboratory, Kyoto, Japan) solution (5 mg/ml) was added to the suspension which was shaken gently for 1 h at room temperature. The conjugated cells were washed by pelletting and stored at -80℃ until used. Foot pad tests were performed to ascertain successful PPD-coupling. One foot pad of mice preimmunized with Tbc was injected with the PPD-coupled tumor cells (5×10^5 cells); another pad was injected with MMC-treated tumor cells (5×10^5 cells). Both foot pads were measured for thickness 48 h later, and obvious swelling was observed with the pad inoculated with PPD-coupled tumor cells.

Induction of tumor specific immunity. Mice were inoculated subcutaneously with 500 μg of acetone powder of heat-killed Tbc dissolved in 0.1 ml paraffin oil, twice at a 4-6 wk interval. During the second week after the last Tbc injection, the mice underwent three i.p. injections of 10^7 PPD-coupled tumor cells. At the same time tumor cells were inoculated subcutaneously. Tumor growth was quantified by multiplying the long diameter by the short diameter.

Induction of antimetastatic effect. Viable LLC cells (10^6 cells) were introduced into the foot pads of C57BL/6 mice primed with Tbc. Two weeks later the tumor was resected by amputation. Some mice underwent immunization with PPD-coupled LLC cells simultaneously with the amputation, and others from the third day after tumor resection. All mice were sacrificed on the tenth or fourteenth day after tumor resection, and the lungs were extirpated to measure metastases. Injection of carbon ink in the bronchia of these lungs partially fixed with Feket solution simplified lung metastasis counting.

Preparation of semipurified interleukin 2 (IL 2). Murine IL 2 was purified from tissue culture

medium of C57BL/6 spleen cells containing 10 γ/ml concanavalin A (Con A). Briefly, purification involved ammonium sulfate precipitation (50 % saturated) followed by Sephadex G-75 (Pharmacia Uppsala, Sweden) column chromatography in phosphate-buffered saline. The collected material was concentrated by CX-10 (Millipore, USA). This material was subjected again to column chromatography and concentrated to a final 50 ml. These procedures were performed at 4℃. The titer of the IL 2 material was determined by the co-stimulation assay of murine thymocytes with Con A and the IL 2 material, assessed by tritiated thymidine uptake. The final titer of the semipurified murine IL 2 material was estimated to be 100 μ/ml.

Rat IL 2 was purchased from Collaborative Research, Inc. (Lexington, USA). The titer was 200 μ/ml, and the material contained 0.37 mg/ml bovine serum albumin.

Human IL 2 was presented by Ajinomoto Laboratory (Tokyo, Japan). Human IL 2 material was obtained from the condition medium of mixed human lymphocytes and BALL-1 cells. The human IL 2 material possessed a titer of 500 μ/ml.

Statistical analyses. The statistical significance of differences observed between mean values was calculated by the Student unpaired *t* test.

RESULTS

Effect of PPD-coupled tumor cells on Meth-A sarcoma challenge. Two groups of BALB/c mice were prepared: one group was immunized twice with 500 μg Tbc injected subcutaneously in the hip region; the other group was not treated. Both groups were subdivided into two subgroups, one vaccinated with PPD-coupled Meth-A cells (10^7cells/animal) and the other not vaccinated. Simultaneously, viable Meth-A cells (10^6cells/animal) were inoculated subcutaneously in the back of each BALB/c mouse. PPD-coupled Meth-A cells (10^7cells/animal) were injected intraperitoneally three times within one week into the mice (Fig. 1).

In the group immunized with a combination of Tbc and PPD-coupled tumor

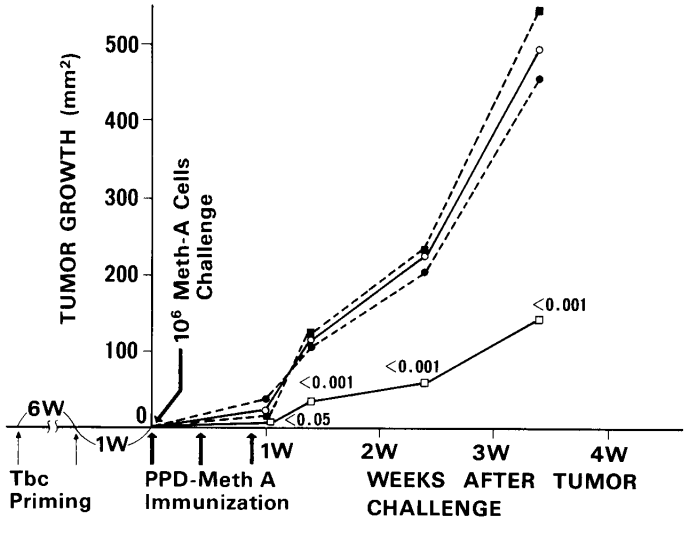

Fig. 1. Suppression of tumor growth; BALB/c mice challenged with Meth-A tumor cells. (●) Tbc (−), PPD-Meth-A (−); (■) Tbc (−), PPD-Meth-A (+); (○) Tbc (+), PPD-Meth-A (−); (□) Tbc (+), PPD-Meth-A (+).

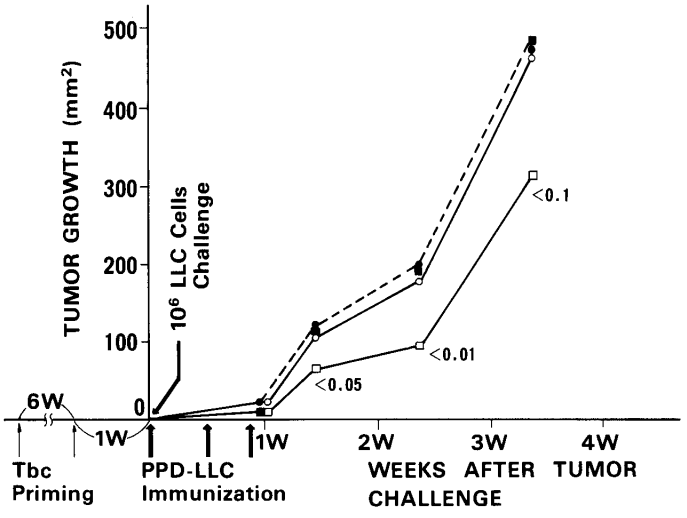

Fig. 2. Suppression of tumor growth; C57BL/6 mice challenged with Lewis Lung Carcinoma. (●) Tbc (−), PPD-LLC (−); (■) Tbc (−), PPD-LLC (+); (○) Tbc (+), PPD-LLC (−); (□) Tbc (+), PPD-LLC (+).

cells, tumor growth was suppressed significantly from the seventh day to more than three weeks after the tumor challenge (Fig. 1). In the single immunization groups, either with Tbc or PPD-coupled tumor cells, no suppression of tumor growth was observed. Mean survival time was apparently prolonged by the combined immunization (36.5 days vs 29.5 days in the controls; $p<0.05$), and one-fifth of these mice satisfactorily rejected the challenged sarcoma. Moreover, these mice which survived the first challenge survived another challenge with the same Meth-A sarcoma (data not shown).

Effect of PPD-coupled LLC cells on LLC challenge. Similarly to the first experimental series, C57BL/6 mice were divided into four groups, and immunized with Tbc and PPD-coupled LLC cells. Viable LLC cells (10^6 cells/animal) were inoculated subcutaneously in the backs of these mice, and PPD-coupled LLC cells (10^7 cells/animal) were injected intraperitoneally three times within a week (Fig. 2). In the combined immunization group, tumor growth was suppressed from the tenth day. Again, tumor growth was similar between the single immunization group and the controls. Mean survival time was elongated by the combined immunization (31.9 days vs 24.1 days; $p<0.01$). However, no mouse was able to reject the tumor challenge.

Effect of PPD-coupled MH134 cells on MH134 challenge. When MH134 tumor cells were inoculated into C3H/He mice, Tbc preimmunization alone and co-treatment with PPD-coupled MH134 cells in combination with Tbc priming resulted in suppressed tumor growth from the tenth day (Fig. 3), but this suppression was not significant.

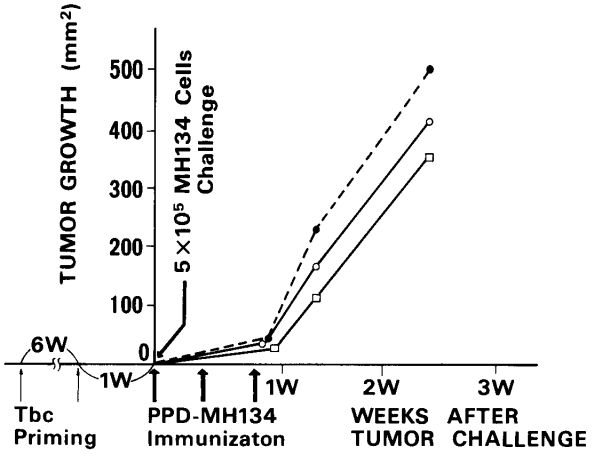

Fig. 3. Suppression of tumor growth; C3H/He mice challenged with MH134 tumor cells. (●) Tbc (−), PPD-MH134 (−); (○) Tbc (+), PPD-MH134 (−); (□) Tbc (+), PPD-MH134 (+).

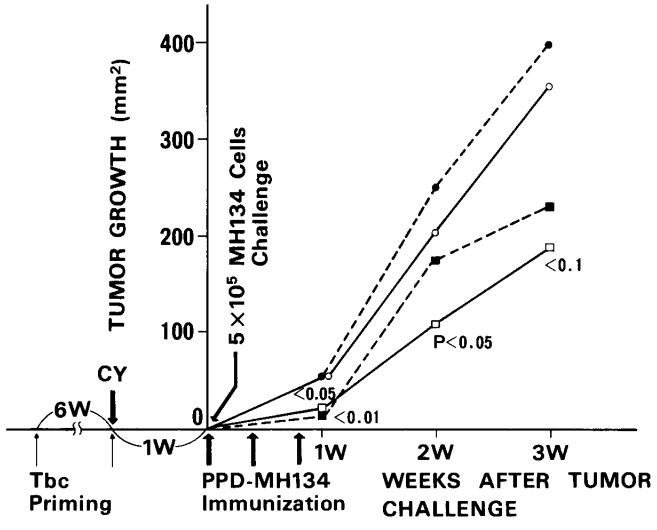

Fig. 4. Suppression of tumor growth; C3H/He mice challenged with MH134 tumor cells. Cyclophosphamide was administered i.p. to every mouse (CY). (●) Tbc (−), PPD-MH134 (−); (■) Tbc (−), PPD-MH134 (+); (○) Tbc (+), PPD-MH134 (−); (□) Tbc (+), PPD-MH134 (+).

Cyclophosphamide, 150 mg/kg, was administered i.p. to mice one week before the tumor challenge to reduce suppressor T-cell activity. From the first week, tumor growth was suppressed more significantly in the combined immunization group (Fig. 4). Mean survival time was prolonged by 8 days in this group as compared to the control group, but no mouse was able to reject the MH134 challenge.

Specificity of anti-tumor activity generated by immunization with PPD-coupled tumor cells following Tbc priming. BALB/c mice were primed with Tbc, followed by immunization with PPD-coupled LLC cells (10^7cells/animal; three times) or PPD-coupled Meth-A cells (10^7cells/animal; three times) destroyed by sonication (150 mA, 7 min). The mice were challenged with viable Meth-A cells (10^6cells/animal). Tumor growth suppression was not significant in these immunization groups as

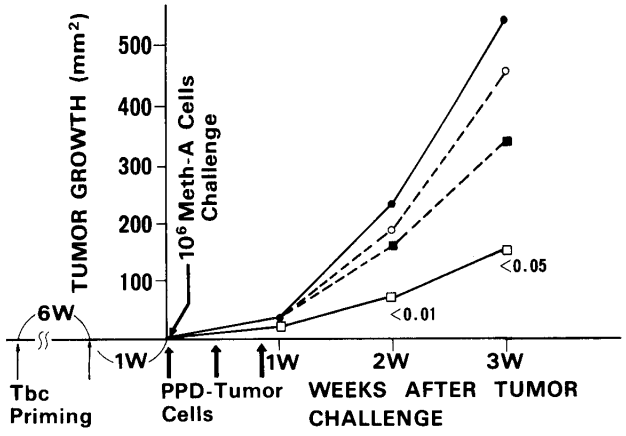

Fig. 5. Suppression of tumor growth; BALB/c mice challenged with Meth-A tumor cells. (■) Tbc (+); (○) Tbc (+), sonicated PPD-Meth-A (+); (●) Tbc (+), PPD-LLC (+); (□) Tbc (+), PPD-Meth-A (+).

compared to the group with only Tbc priming (Fig. 5).

Effect of Tbc and PPD-coupled LLC cell immunization on LLC metastasis. Viable LLC cells (10^6 cells/animal) were injected into the foot pads of C57BL/6 mice primed with Tbc. The tumor was resected by amputation two weeks later. At this time the mice were immunized with PPD-coupled LLC cells. This treatment resulted in the reduction of lung metastases (Fig. 6-a). This antimetastatic effect was noted also in the group treated with PPD-coupled LLC cells alone. If immunization commenced on the third day after tumor resection, significant suppression of lung metastases was accomplished only in the Tbc and PPD-coupled LLC cell group (Fig. 6-b).

Synergy of action of PPD-coupled Meth-A cell immunization and IL 2. BALB/c mice were primed with Tbc (500 μg/animal) once, and subcutaneously inoculated with viable Meth-A cells (10^6 cells/animal) in the back four weeks later. The mice were treated in four groups: a) untreated, b) murine IL 2 (100 unit in 1 ml) intraperitoneally on both the third and fourth days after tumor challenge, c) PPD-Meth-A cells (10^7 cells/animal; once) simultaneously with tumor challenge, and d) both murine IL 2 and PPD-Meth-A cells. On the seventh day after tumor challenge, slight suppression of tumor growth was observed in the single treatment groups, and significant suppression in the combined treatment group (Fig. 7). On the fourteenth day, tumor growth was suppressed significantly only in the group undergoing combined treatment. This anti-tumor effect was not observed in the third week or later.

The efficacy of IL 2 from other species was explored (Fig. 8). Either rat IL 2 (200 U, 1 ml/animal) or human IL 2 (500 U, 1 ml/animal) was administered once intraperitoneally along with PPD-coupled Meth-A cells (10^7 cells/animal; once).

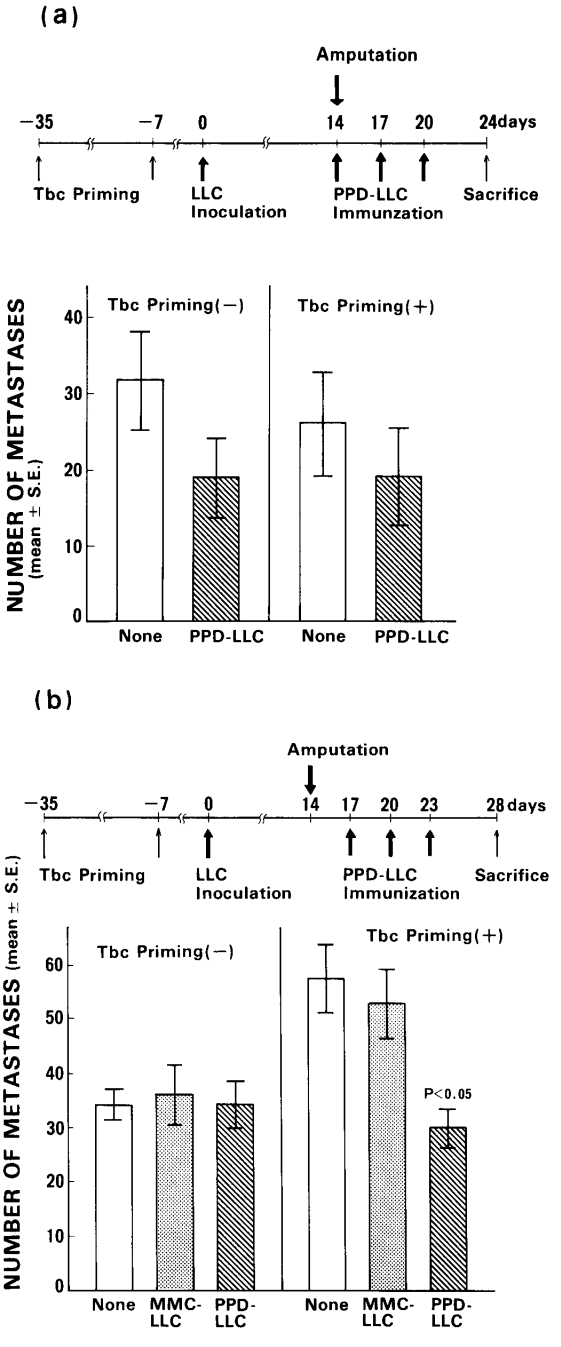

Fig. 6-(a), 6-(b). Inhibitory effect on lung metastasis by immunization with PPD-coupled LLC cells. C57BL/6 mice challenged with Lewis Lung Carcinoma. MMC-LLC; immunization with MMC treated LLC cells. PPD-LLC; immunization with PPD-coupled LLC cells.

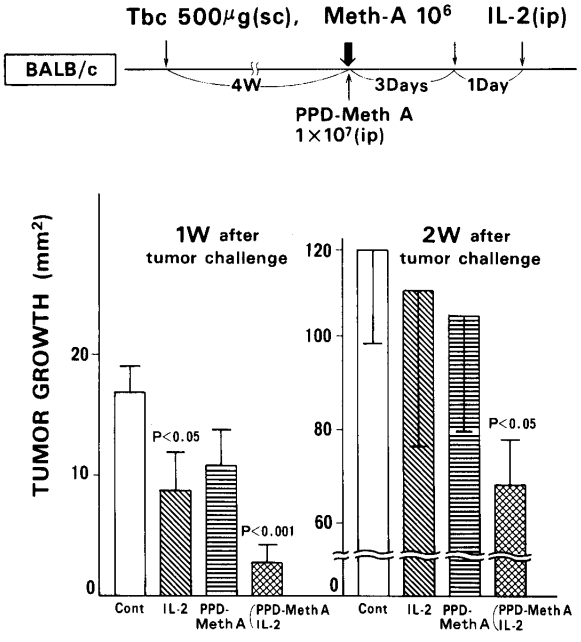

Fig. 7. Suppression of tumor growth; BALB/c mice challenged with Meth-A tumor cells. (□) Tbc (+); (▨) Tbc (+), murine IL 2 (+); (▤) Tbc (+), PPD-Meth-A (+); (▩) Tbc (+), murine IL 2 (+), PPD-Meth-A (+).

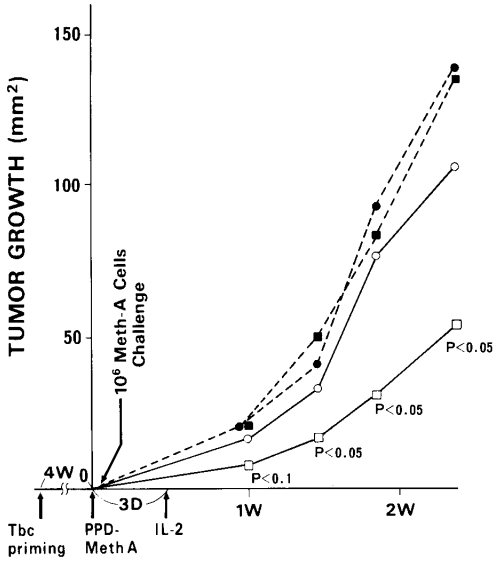

Fig. 8. Suppression of tumor growth; BALB/c mice challenged with Meth-A tumor cells. (●) Tbc (+), PPD-Meth-A (−), IL 2 (−); (■) Tbc (+), PPD-Meth-A (+), IL 2 (−); (○) Tbc (+), PPD-Meth-A (+), human IL 2 (+); (□) Tbc (+), PPD-Meth-A (+), rat IL 2 (+).

In the group receiving rat IL 2, tumor growth was significantly suppressed and mean survival time was prolonged by 14 days (50.1 days vs 36.1 days in controls; $p<0.01$). The group with human IL 2 administration, however, showed no suppression of tumor growth.

DISCUSSION

Previous studies have suggested that some malignant neoplasms have tumor-associated transplantation antigens (TATA) (1, 2). Generally an organism is incapable of rejecting an inoculated syngeneic malignant tumor; this challenge usually results in death. The immune surveillance mechanism of the host individual is considered insufficient to recognize the TATA or mount a vigorous enough response. There have been many attempts to increase the immunogenicity of TATA by modifying the tumor cell membrane, for instance with proteins, haptens or viral proteins, or by cell-fusion. These trials have been partially successful in increasing the immunogenicity, but the evolved response is insufficient to reject a challenged tumor (3, 4, 11). Takatsu *et al.* (10), reported immunization of Tbc-primed mice with syngeneic X5563 tumor cells modified by PPD, and elicited augmentation of tumor neutralizing activity *in vivo*, conditional upon the presence of PPD-reactive helper T cell activity. Thus, Tbc-primed C3H/HeJ mice were challenged with X5563 tumor cells, and immunized with PPD-coupled X5563 tumor cells during early stages of the tumor-bearing state. This regimen induced satisfactory anti-tumor activity, resulting in rejection of the tumor.

In the present study, this immunotherapy regimen was applied to other cell lines. Tbc-primed BALB/c mice were challenged with Meth-A tumor cells, and immunized with PPD-coupled Meth-A tumor cells. According to expectation, growth of the Meth-A tumor was suppressed from the early stages, and one-fifth of the mice rejected the tumor. Tbc-primed C57BL/6 mice were challenged with LLC tumor cells, and immunized with PPD-coupled LLC cells. Indeed, growth of LLC was significantly suppressed, but all mice eventually died. Tbc-primed C3H/He mice were challenged with MH134 tumor cells, and immunized with PPD-coupled MH134 cells. Growth of MH134 was not suppressed by this procedure. Additional intraperitoneal administration of cyclophosphamide led to suppression of tumor growth. In brief, elimination of suppressor cell activity by cyclophosphamide augmented the tumor-specific immunity. This result is supported by a previous work (10).

Destruction of PPD-coupled Meth-A cells by sonication eliminated the anti-tumor effect. Additionally, immunization with PPD-coupled LLC cells did not suppress the growth of inoculated Meth-A cells. Thus, whole, syngeneic PPD-coupled tumor cells were indispensable for this tumor-specific immunotherapy. It is necessary, however, to assure that PPD-coupled tumor cells never cause a tumor *in vivo*. Immunization with both Tbc and PPD-coupled LLC cells elicited an anti-

metastatic effect on an LLC challenge. This effect was observed even when the mice were immunized three days after tumor resection. Thus, it is feasible to apply this immunization program clinically. However, as mentioned above, the anti-tumor effects were not invariable with all three tumor cell lines investigated.

The varying responses to the immunization regimen might be attributed to the TATA of each tumor, or to differences in the effector cells. Tumor associated transplantation antigens have not been accurately characterized, and each may be different. Therefore, different TATA may induce different immunological responses (12). Almost all effector cells that are capable of attacking tumor cells, have been regarded as killer T cells (13, 14). Recently, however, A.K. Bhan *et al.* (15) reported that effector cells responding to a methylcholanthrene-induced sarcoma (S1509a) *in vivo* were Lyt-1 positive and Lyt-2 negative, and that the tumor infiltrating cells appeared to be macrophages. Effector cells may indeed be cytotoxic T lymphocytes, macrophages activated by Lyt-1 cells, NK cells activated by Lyt-1 cells, or Lyt-1 cells themselves (15). Exact analysis of effector mechanisms is required.

Interleukin 2 (IL 2), a helper factor derived from Lyt-1 positive T cells, has been shown to enhance antigen- or mitogen-activated cytotoxic T lymphocyte expansion *in vitro* (16, 17). Systemic *in vivo* application of interleukin 2 plus alloantigen in nude mice was shown to trigger induction of alloreactive cytotoxic T lymphocytes (18). *In vivo* treatment with purified murine interleukin 2 results in augmented cytotoxic T lymphocyte activity against allogeneic tumors (19). Proliferation of helper T cells in mixed-lymphocyte-tumor-culture (MLTC) was promoted by additional IL 2, and the helper T cells seemed to be the main effector cells which attacked a tumor *in vivo* (20). In this experiment, intraperitoneal administration of IL 2 enhanced the anti-tumor effect against Meth-A tumor cells inoculated into BALB/c mice immunized with Tbc and PPD-coupled Meth-A cells. This suggested that IL 2 might augment tumor-specific cytotoxic T lymphocyte activity *in vivo*. On the other hand, IL 2 may lead to helper T cell proliferation of cells activated by the immunization with Tbc and PPD-coupled tumor cells. The precise mechanisms of these anti-tumor activities remain to be clarified.

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