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K Takatsu; ... et. al

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AUGMENTED INDUCTION OF TUMOR-SPECIFIC RESISTANCE BY PRIMING WITH *MYCOBACTERIUM TUBERCULOSIS* (TBC) AND SUBSEQUENT IMMUNIZATION WITH PPD-COUPLED SYNGENEIC TUMOR CELLS[1]

KIYOSHI TAKATSU, TOSHIYUKI HAMAOKA, AKIRA TOMINAGA, AND YASUHIRO KANAMASA[2]

From the Department of Oncogenesis, Institute for Cancer Research, Osaka University Medical School, Osaka, 553, Japan

The present study investigates the augmenting effect of tuberculin- (PPD) reactive amplifier T cells on the induction of syngeneic tumor immunity. PPD-reactive helper (amplifier) T cell activity was generated in C3H/HeJ mice by appropriate immunization with heat-killed *Mycobacterium* (Tbc). Immunization of these Tbc-primed mice with PPD-coupled syngeneic X5563 tumor cells led to augmented generation of *in vivo* tumor-neutralizing activity contingent on the presence of PPD-reactive amplifier T cell activity. Splenic T cells from these mice exhibited potent tumor-neutralizing activity using Winn's assay, whereas spleen cells from mice not primed with Tbc before PPD-X5563 immunization failed to neutralize viable X5563 tumor cells. After establishing that the neutralizing activity was tumor specific and mediated by T cells, the applicability of this augmentation of tumor-specific immunity to an immunotherapy model was explored. Immunization with PPD-X5563 in the early stages of the tumor-bearing state induced potent anti-tumor activity sufficient to reject the growing tumor. Pretreatment of mice with cyclophosphamide or light x-irradiation (250 R), procedures that eliminate suppressor cell activity nonspecifically, before priming with Tbc further potentiated the anti-tumor activity under these conditions. Thus, the present study elucidates the augmenting effect of PPD-reactive amplifier T cells in the induction of tumor-specific immunity and provides an effective method of immunotherapy in tumor-bearing animals.

The use of surface modified tumor cells in the induction of specific active immunization has received considerable attention. In the past decade, numerous attempts to increase the immunogenicity of tumor-associated transplantation antigens (TATA)[3] by coupling additional antigenic determinants to the tumor cell surface have been reported (1–6). This approach was based on the theoretical concept of a cellular cooperation in tumor immunity that might augment tumor rejection (7). Although there is some evidence that immunization with surface modified tumor cells augments tumor-specific immunity against relatively weak immunogenic syngeneic tumors, the underlying mechanisms remain obscure and sometimes the immunity thus induced has not been potent enough to reject tumor cells.

It is well established that amplifier T cells collaborate with cytotoxic T cells leading to increased cytotoxicity against alloantigens (8, 9). It is conceivable, therefore, that increasing amplifier T cell activity may result in potentiation of the cytotoxic T cell response against TATA. In our previous report (10), conditions were established under which augmentation of killer T cell activity against TATA was attained by inducing of hapten-reactive helper (amplifier) T lymphocytes, followed by immunization with hapten-modified syngeneic tumor cells. The obvious goal of the application of this theoretical concept of hapten-reactive amplifier T cell activity in the augmentation of tumor-specific immunity is to develop an effective mode of immunotherapy with which to substantially improve host resistance to already growing tumors. For this purpose, it is necessary to devise a potent antigen system applicable to humans that is able to induce amplifier T cells. *Mycobacterium tuberculosis* (Tbc) may be a good candidate as a potent immunogen for the evoking of such T cell activity. Previous work from our laboratory has shown that tuberculin (purified protein derivative, PPD) is a powerful carrier in inducing a primary anti-DNP antibody response when used with DNP-PPD to immunize mice (11). We have also demonstrated that in the secondary anti-DNP antibody response PPD-reactive T cells in Tbc-primed mice exert potent helper activity toward DNP-primed B cells via DNP-PPD (12–14). On the other hand, PPD itself induces neither delayed type hypersensitivity nor antibody response but elicits delayed type reactions in Tbc-presensitized hosts only. This potent immunoreactivity of PPD led us to predict that the weak immunogenicity of TATA can be strengthened by means T-T cell interaction using chemical coupling of tumor with PPD.

The results in this paper demonstrated that 1) mice primed with Tbc and subsequently immunized with PPD-coupled, mitomycin C-treated syngeneic tumor cells generated greater tumor-specific T cell activity than mice that were not previously

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[2] The Department of Microbiology, Okayama University Medical School, Okayama, 730, Japan.
[3] Abbreviations used in this paper: TATA, tumor-associated transplantation antigen; Tbc, heat-killed *Mycobacterium tuberculosis* human type Aoyama B; PPD, purified protein derivative from *Mycobacterium tuberculosis*; PPD-X5563, PPD-coupled mitomycin C-treated X5563 plasmacytoma; BAT, brain-associated T cell antigen; C, selected rabbit complement; MMC, mitomycin C; KLH, Keyhole limpet hemocyanin; ECDI, 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide.

immunized with Tbc, and 2) immunization of Tbc-primed mice with PPD-coupled tumor cells immediately after viable tumor inoculation also generated potent anti-tumor activity sufficient to suppress and reject the growing tumor. Thus, the potential therapeutic usefulness of this approach in the management of tumor-bearing individuals is obvious.

MATERIALS AND METHODS

Mice. C3H/HeJ mice of both sexes, originally obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in our Institute, were used at 7 to 9 wk of age.

Antigens and preparation of hapten-carrier conjugates. Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem, San Diego, Calif.; tuberculin (PPD) was partially purified from *Mycobacterium tuberculosis* (Tbc) strain H37Rv culture supernatant according to the method previously described (12).

The DNP conjugates DNP_7-KLH and DNP_2-PPD were prepared using sodium 2,4-dinitrobenzene sulfonate. Subscripts refer to the average number of DNP groups per molecule of the proteins; these were calculated from the absorption reading at 348 nm. In the above calculations of the number of haptens per molecule of carrier, the m.w. of KLH and PPD were taken to be 100,000 and 25,000, respectively.

Tumor. Spontaneously occurring X5563 plasmacytoma and MM102 mammary tumor (MM) (15), both derived from the C3H/HeJ strain, were used. Both tumors were maintained by serial i.p. passage in syngeneic mice in ascitic form.

Preparation of PPD-coupled tumor cells. PPD-coupled tumor cells were prepared according to the method described previously (16). Briefly, 100×10^6 tumor cells pretreated with 20 ml mitomycin-C (MMC) solution at a concentration of 40 μg/ml for 45 min at 37°C were suspended in 10 ml of phosphate-buffered saline containing 1 mg/ml of PPD. One milliliter of 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide (ECDI) (Sigma Chemicals, St. Louis, MO) solution (5 mg/ml) was added to the mixture, kept standing for 1 hr at room temperature, and shaken gently several times. The mixture was then thoroughly washed with MEM, and viability of the cells was determined by the trypan blue dye exclusion test. Using this procedure, more than 90% of the tumor cells were found to be viable after coupling with PPD.

Induction of tumor-specific immunity. To sensitize the host with PPD, C3H/HeJ mice were given subcutaneous injections of 500 μg of an acetone powder of heat-killed Tbc in 0.2 ml paraffin oil, and booster injections of the same dose as a primary immunization 6 wk after the initial immunization. Three weeks after the booster injection, the mice were inoculated i.p. with 0.5 to 1×10^7 PPD-coupled tumor cells or MMC-attenuated unmodified tumor cells to induce specific immunity against the tumor. To detect the presence of protective immunity against the tumor, spleen cells from the host were submitted to an *in vivo* tumor neutralization test as described below, or the host was challenged intradermally with 0.5 to 1×10^6 viable unmodified tumor cells and tumor growth was measured by caliper.

In vivo tumor neutralization test. The spleens from nonimmunized mice or from mice immunized with X5563 cells were removed aseptically, and single-cell suspensions were prepared in MEM. One million viable tumor cells were mixed with 100×10^6 spleen cells from either nonimmunized or immunized mice and injected i.p. into normal syngeneic mice. Intraperitoneal tumor growth was measured by quantitation of myeloma protein in the sera of tumor-inoculated mice by using a radial immunodiffusion technique utilizing rabbit anti-idiotypic antibody to X5563 myeloma protein, as described previously by Yutoku *et al.* (17)

Fractionation of spleen cells. T cells in the spleen cell suspensions were removed by treatment with rabbit anti-brain-associated Thy 1 (BAT) antiserum and rabbit complement (C). The preparation of anti-BAT antiserum and the method for depleting T cells from spleen cell suspensions were the same as those described previously (11). Purification of splenic T cells was carried out according to the method of Julius *et al.* (18) using a glass wool column and a nylon wool column (nylon wool in LP-1 Leuko-Pak leukocyte filters, Fenwal Laboratories, Morton Grove, IL). Briefly, adherent cells in a spleen cell suspension were removed by passing the cell suspension through a glass wool column. Nonadherent cells were applied to a nylon wool column to obtain the T cell-rich fraction. The proportion of T cells in a given cell preparation was determined by treatment of the cells with anti-BAT antiserum and C. Unless specified otherwise, 80 to 85% of the cells in the T cell-rich fraction were killed by treatment with anti-BAT antiserum and C as judged by the trypan blue dye exclusion test.

Assay system for measuring helper activity of PPD-reactive T lymphocyte populations. Single-cell suspensions were prepared from lymph nodes (axillary, inguinal, and popliteal) and spleens of Tbc-immunized C3H/HeJ mice and used as a source of PPD-reactive T cells. Single-cell suspensions from spleens of syngeneic mice that had been immunized with 100 μg of DNP-KLH in incomplete Freund's adjuvant by i.p. injection 8 to 10 wk earlier were used as responding B cells and transferred i.v. together with Tbc-primed cells into recipients previously x-irradiated with 600 rads. To measure the helper T cell activity, a secondary antigenic challenge was given by i.p. injection of 20 μg DNP-PPD in saline immediately after the cell transfer. The degree of helper T cell activity in the Tbc-primed cell population was detected by comparing the magnitude of the anti-DNP antibody responses in the presence of transferred DNP-specific B cells with those of normal cells. The anti-DNP antibody responses were measured by the number of hemolytic plaques in the recipient's spleen 7 days after adoptive cell transfer and antigenic stimulation. The number of anti-DNP plaque-forming cells were enumerated using TNP-SRBC as indicator cells as described previously (11). Since the number of anti-DNP IgM-PFC was consistently negligible, only anti-DNP IgG-PFC are listed in *Results.*

Statistical analysis. Test data were logarithmically transformed before means and standard errors were calculated. Group comparisons were made employing Student's *t*-test. For mice in whose sera no myeloma protein could be detected, a value of 312 μg/ml was arbitrarily assigned to allow logarithmic transformation of the data. This value corresponds to the minimum concentration of myeloma protein detectable in our assay.

RESULTS

Establishment of conditions for effective induction of PPD-reactive T cell activity in low-responder C3H/HeJ mice by Tbc priming. It has been demonstrated that C3H/HeJ mice constitute a relatively low responder strain to Tbc (14). In order to establish conditions for raising an effective PPD-reactive T cell population in C3H/HeJ mice by Tbc immunization, the immunization regimen was studied monitoring the generation of PPD-reactive helper T cell activity.

Two groups of mice were immunized subcutaneously with

500 μg of an acetone powder of heat-killed Tbc in 0.2 ml paraffin oil. Six weeks thereafter, 1 group received booster injections using the same dose of Tbc. Lymph node and spleen cell suspensions were prepared from either nonprimed, Tbc-primed, or Tbc-primed and boosted mice 9 wk after the priming. To measure the helper activity of the Tbc-primed cells, cells from each group of mice (20×10^6) were mixed with spleen cells (40×10^6) from mice that had been immunized with DNP-KLH 8 wk previously, and were adoptively transferred into x-irradiated (600 R) syngeneic recipient mice. All recipients were secondarily challenged with antigen immediately after the cell transfer. The anti-DNP IgG-PFC in the spleen were enumerated 7 days after the cell transfer. It is evident from Table I that the Tbc-priming and boosting regimen induced potent PPD-reactive helper activity in the C3H/HeJ mice (compare groups 2 and 4), whereas Tbc-priming without a booster injection generated only marginal helper activity (group 3). The dependency of the generated PPD-reactive helper activity on T cells was confirmed by abrogation of the activity after treatment of the cells with anti-BAT antiserum plus C (group 5). This was further confirmed by the potent helper activity observed in the nylon wool-purified T cell-enriched population (group 6). In the generation of potent PPD-reactive T cell activity in C3H/HeJ mice, the above Tbc-immunization regimen was routinely used throughout the subsequent experiments.

TABLE I

Development of PPD-reactive helper T cells by immunization of C3H/HeJ mice with killed M. tuberculosis (Tbc)[a]

Group No.	B Cell Source	Helper Cells Primed with (20×10^6)	Secondary Antigen (20 μg)	Anti-DNP IgG-PFC on Day 7[b]
1		None	DNP-KLH	33,900 (1.19)
2		None	DNP-PPD	3,120 (1.12)
3	DNP-KLH primed cells	Tbc 1° (whole spleen)	DNP-PPD	5,460 (1.46)
4		Tbc 1° & 2° (whole spleen)	DNP-PPD	36,400 (1.18)
5		Tbc 1° & 2° (anti-BAT + C)	DNP-PPD	4,028 (1.08)
6		Tbc 1° & 2° (T cell-enriched)[c]	DNP-PPD	61,500 (1.28)

[a] Spleen cells (40×10^6) from DNP-KLH-primed mice were mixed with helper cells (20×10^6) from mice immunized with 500 μg of Tbc in paraffin oil and transferred into x-irradiated (600 R) syngeneic recipient mice. Secondary antigenic stimulation was performed i.p. without adjuvant immediately after the cell transfer.

[b] The numbers denote geometric means from 5 mice, and the values in parentheses represent the SE.

[c] Nylon wool nonadherent splenic cells.

Augmented induction of anti-tumor activity in mice presensitized with Tbc followed by immunization with PPD-coupled tumor. To explore the feasibility of utilizing this PPD-reactive T cell system to facilitate the development of tumor-specific immunity, PPD-conjugated MMC attenuated X5563 (PPD-X5563) cells were utilized as a tumor vaccine and tested in syngeneic C3H/HeJ recipient mice for their capacity to develop tumor resistance depending on whether such mice had been preimmunized with Tbc.

Two groups of 6 mice each were immunized with Tbc and received booster injections with Tbc 3 wk before experimentation. They were further immunized i.p. with either MMC-treated X5563 myeloma cells without PPD coupling (5×10^6 cells) or the same number of MMC-treated PPD-X5563 cells. Each group of mice received the same immunization regimen at 4-day intervals for a total of 3 times (groups 1 and 2). As controls, 2 other groups of Tbc nonprimed normal mice were also immunized either with X5563 cells or PPD-X5563 cells under the same conditions as above (groups 3 and 4). Four days after the final immunization, all mice were challenged with 5×10^5 viable X5563 cells intradermally. Nontreated normal mice were also challenged with X5563 cells (group 5). Tumor growth and mean survival times of the mice are summarized in Table II. It is evident that tumor growth as determined by tumor diameter and the serum level of the IgG2a produced by the X5563 cells was considerably suppressed in Tbc-presensitized and PPD-X5563-immunized mice, all the mice, in fact, eventually surviving without tumor growth, whereas tumor growth in mice that had been immunized with PPD-X5563 cells alone without prior Tbc-priming was only slightly slowed or remained similar to that in nonimmunized mice (group 3 *vs* group 5). This indicates that tumor resistance was effectively induced by immunization with PPD-coated tumor cells in the presence of PPD-reactive T cells.

Direct coupling of PPD to tumor cells as a requirement for augmented induction of anti-tumor activity. In order to test the role of PPD coupled to the tumor cell surface in the augmented induction of anti-tumor activity, Tbc-primed mice were immunized with either PPD-coupled X5563 cells (1×10^7) or a mixture of MMC-attenuated X5563 cells (1×10^7) plus 25 μg of PPD, which was calculated to be the amount of PPD attached to the 1×10^7 PPD-X5563 cells as determined by ^{125}I-

TABLE II

Antitumor activity against X5563 in Tbc-presensitized PPD-X5563-immunized mice

Group No.	Presensitized with[a]	Immunized with[b]	Tumor Growth[c]		Survivors on Day 40/Total[d]	Mean Survival Time
			Tumor diameter	Myeloma protein in serum		
			mm	*μg/ml*		*days*
1	Tbc	PPD-X5563	3.70 (1.16)	<312	6/6	—
2		X5563	11.3 (1.06)	4318 (1.08)	0/6	16.4 (1.08)
3	None	PPD-X5563	12.3 (1.08)	4264 (1.10)	0/6	16.2 (1.07)
4		X5563	13.2 (1.09)	5247 (1.19)	0/6	15.5 (1.08)
5		None	13.6 (1.08)	4650 (1.09)	0/6	13.5 (1.07)

[a] Mice were presensitized with 500 μg heat-killed Tbc and boosted with the same dose 6 weeks after presensitization.

[b] Tumor immunization was begun 3 weeks after the booster injection. Five million mitomycin C-treated X5563 cells or PPD-conjugated-mitomycin C-treated X5563 cells were injected ip at 4-day intervals for a total of 3 times.

[c] Viable X5563 cells (5×10^5) were inoculated intradermally 4 days after the final tumor immunization. Tumor diameter and serum level of IgG2a myeloma protein on day 9 are listed.

[d] Survivors were observed on day 40: number of survivors/number of mice inoculated with tumor.

PPD binding. Tumor growth and survival times in the respective groups are summarized in Table III. As expected, significant suppression of tumor growth was observed in mice that had been primed with Tbc followed by immunization with PPD-X5563 cells. By contrast, immunization with X5563 cells mixed with PPD did not induce any significant anti-tumor activity, indicating that for potentiation of anti-tumor activity, PPD must be covalently bound to the tumor cell surface.

To confirm the acquisition of tumor resistance in mice that have once rejected the tumor, a second set of tumor cells (2×10^6 cells) were inoculated. As expected, all the mice resisted this second challenge with tumor (data not listed). These results indicate that anti-tumor activity was effectively potentiated by immunization of mice with PPD-coupled tumor cells in the presence of PPD-reactive T cells.

Specificity of anti-tumor activity induced by immunization with PPD-coupled tumor cells in the presence of PPD-reactive T cells. The specificity of the anti-tumor activity augmented by the above system was next examined using the tumor neutralization test (Winn's assay).

Spleen cells (50 to 100×10^6) from mice presensitized with Tbc, followed by immunization with either PPD-X5563 cells or attenuated X5563 cells without PPD coupling, were mixed with 1×10^6 viable X5563 cells or MM102 cells and injected i.p. into naive syngeneic C3H/HeJ mice. Tumor growth was determined by mean survival time. As shown in Table IV, significant prolongation of mean survival time after challenge with X5563 cells was observed in mice receiving spleen cells from mice primed with Tbc followed by immunization with PPD-X5563 cells (group 5) compared with control groups (groups 1 through 4). This tumor-neutralizing activity of the cells was specific against X5563 cells, since growth of the MM102 cells was not inhibited by cells with potent anti-X5563 tumor-neutralizing activity (group 10). These results indicate that the tumor resistance induced by immunization with PPD-coated tumor cells in the presence of PPD-reactive T cells is specific for the X5563 tumor cells.

Analysis of the effector mechanism in potentiated anti-tumor activity. The effector mechanism underlying the anti-tumor activity potentiated in Tbc-primed mice after immunization with PPD-coupled tumor cells was next analyzed using the tumor neutralization test (Winn's assay). Although the data are not shown here, the tumor-neutralizing activity demonstrated in group 5 in Table IV was not mediated by the serum, since mixing 1×10^6 viable X5563 cells with 0.5 ml of serum from group 5 did not give rise to any significant inhibition of the tumor growth.

TABLE III

Failure of induction of anti-tumor activity in mice presensitized with Tbc followed by immunization with a mixture of X5563 and PPD[a]

Group No.	Presensitized with	Immunized with	Tumor Growth on Day 10		Survivors on Day 40/Total Mice
			Tumor diameter	Myeloma protein in serum	
			mm	*μg/ml*	
1	Tbc	PPD-X5563	4.9 (1.02)	<312	8/10
2		PPD + X5563[b]	13.2 (1.28)	3183 (1.15)	0/10
3		None	11.2 (1.21)	2383 (1.21)	0/7
4	None	PPD-X5563	11.2 (1.16)	2765 (1.09)	0/10
5		PPD + X5563	12.1 (1.09)	3428 (1.28)	0/10
6		X5563	10.8 (1.24)	3229 (1.01)	0/8
7		None	11.8 (1.04)	2992 (1.21)	0/10

[a] Immunization was carried out by i.p. injection of 1×10^7 PPD-X5563 cells at 3-day intervals for a total of 3 times.

[b] PPD (25 μg) was mixed with MMC-attenuated X5563 cells immediately before immunization.

TABLE IV

Specificity of in vivo tumor neutralizing activity of spleen cells from mice presensitized with Tbc followed by immunization with PPD-X5563

Group No.	Presensitized with	Immunized with[a]	Challenged with[b]	Survivors on Day 40/Total Mice	Mean Survival Time
					days
1	None	None	X5563	0/7	15.3 (1.14)
2	None	X5563		0/7	13.8 (1.29)
3		PPD-X5563		0/7	19.0 (1.18)
4	Tbc	X5563		0/7	16.7 (1.15)
5		PPD-X5563		3/6	30.5 (1.18)
6	None	None	MM102	0/7	14.0 (1.19)
7	None	X5563		0/7	13.8 (1.08)
8		PPD-X5563		0/7	14.9 (1.17)
9	Tbc	X5563		0/7	13.7 (1.27)
10		PPD-X5563		0/7	13.1 (1.14)

[a] Immunization was performed by i.p. injection of attenuated tumor cells at 3-day intervals for a total of 3 times. The first injection was carried out 3 weeks after the booter injection.

[b] One million of either X5563 cells or MM102 cells were mixed with spleen cells (100×10^6) (effector to target = 100:1) and implanted i.p. 4 days after the final immunization.

To delineate the cell type responsible for anti-tumor activity, a portion of spleen cells from mice immunized in the same manner as group 5 in Table IV was treated with anti-BAT antiserum and C. Another portion of spleen cells was loaded onto a glass wool column and subsequently passed through a nylon wool column to obtain an enriched T cell population. Nylon wool nonadherent cells were used as the splenic T cell source. This population contained more than 85% T cells as determined by the cytotoxicity test using anti-BAT antiserum plus C. One hundred million cells from each of the cell populations were mixed with 1×10^6 viable X5563 cells (effector: target = 100:1) and implanted i.p. into normal naive mice. A group of normal mice also received a mixture of X5563 cells (1×10^6) and normal spleen cells (100×10^6) as a control. Tumor growth was measured by the serum level of IgG2a protein produced by the X5563 cells as depicted in Figure 1.

It is evident that the tumor-neutralizing activity observed in whole spleen cells (group 2) was also present in the nylon wool-recovered T cell-enriched fraction (group 3), whereas this activity was completely abolished by treatment with anti-BAT antiserum plus C (group 4). These experimental data indicate that the T cells are responsible for this augmented anti-tumor activity.

Suppression of tumor growth in tumor-bearing mice by augmented induction of anti-tumor activity. We next explored the feasibility of applying the aforementioned immunizing regimen to an immunotherapy model. Two groups of Tbc-primed mice, each comprised of 8 animals, were challenged intradermally with 10^6 viable X5563 tumor cells on day 0. One group was placed on an immunizing regimen of PPD-X5563 i.p. at 3-day intervals times 3, begun immediately after inoculation with viable tumor. The other group did not receive PPD-X5563 immunization. Likewise, after viable tumor inoculation, 2 other Tbc-nonprimed groups were also treated with PPD-X5563 or not treated, respectively. Tumor-growth was measured by

means of tumor diameter and the results summarized in Figure 2.

As is evident from this figure, tumor that initially reached more than 5 mm in diameter started to regress around 10 days after tumor inoculation in the group of mice primed with Tbc and receiving the PPD-X5563 immunotherapy regimen, and interestingly, 5 out of the 8 mice ultimately rejected the tumor. This was in sharp contrast to tumor growth in the other experimental groups, which similarily received PPD-X5563 immunotherapy but were not primed with Tbc, or were Tbc-primed but did not receive PPD-X5563 immunotherapy. Thus, commencement of immunization with PPD-X5563 in the early phases of the tumor-bearing state augmented the induction of tumor-specific immunity and rendered the host resistant to the tumor.

In the above immunotherapy protocol, however, 3 out of 8 mice eventually died due to tumor overgrowth at around day 30 (data are not depicted in Fig. 3), even after immunization with PPD-X5563. In order to further potentiate the development of host tumor immunity, Tbc priming was performed after elimination of suppressor activity by treatment with cyclophosphamide or x-irradiation.

Two groups of mice were pretreated subcutaneously with cyclophosphamid (200 mg/kg) 2 days before Tbc priming. Two other groups of mice were irradiated (250 R) immediately before Tbc priming. As a positive control, another group of mice was primed with Tbc without such pretreatment. Six weeks after Tbc priming, all mice received a booster injection of Tbc in the same dose as that used in priming, and 3 wk thereafter were challenged with viable X5563 cells (5×10^5). Immediately after tumor challenge, the 3 groups of mice primed with Tbc after no treatment, cyclophosphamide treatment, or x-irradiation were placed on the immunotherapy regimen using PPD-X5563 cells (1×10^7). Two other groups of mice primed with Tbc after either treatment with cyclophosphamide or x-irradiation did not undergo the tumor immunization procedure. Tumor growth as measured by tumor diameters is displayed in Figure 3, and the percentage of survivors is depicted in Figure 4. It is evident that immunization of Tbc-primed mice with PPD-X5563 cells immediately after tumor challenge again suppressed tumor growth as compared with that in Tbc-primed mice without PPD-X5563 immunization (Fig. 3). Quite striking was the observation that cyclophosphamide treatment or x-ray irradiation before Tbc priming further augmented the induction of anti-tumor activity, and eventually almost all of the mice suppressed and rejected the growing tumor (Fig. 4). Thus, elimination of suppressor cell activity before Tbc priming augmented the induction of tumor-specific immunity sufficiently to enable rejection of an already growing tumor.

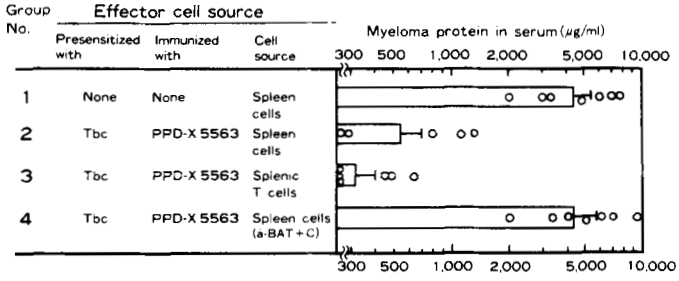

Figure 1. T cell-mediation of augmented anti-tumor activity in C3H/HeJ mice presensitized with Tbc followed by immunization with PPD-X5563 cells. Immunization regimens were substantially the same as shown in Table IV. One hundred million effector cells were mixed with 1×10^6 viable X5563 cells and implanted i.p. into naive normal mice. Tumor growth was determined by the serum level of IgG2a protein produced by the X5563 cells with the use of a radial immunodiffusion technique utilizing rabbit anti-idiotypic antibody to X5563 myeloma protein. Individual values for tumor growth in each group on day 10, and their geometric means and standard errors are illustrated.

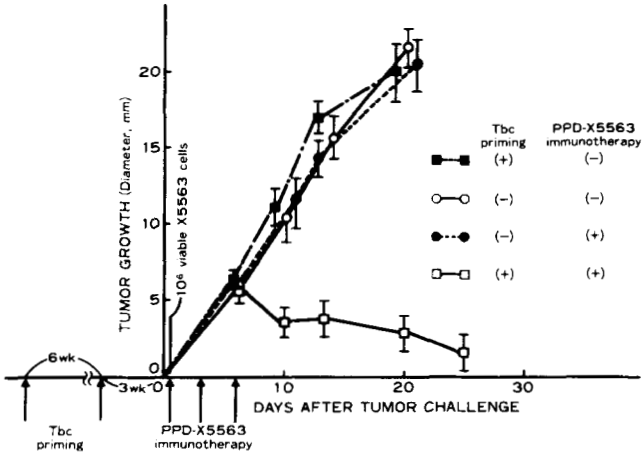

Figure 2. Suppression of tumor growth in tumor-bearing mice obtained by augmented induction of tumor-specific immunity. Two groups of C3H/HeJ mice primed and boosted with Tbc received intradermal implantations of 1×10^6 viable X5563 cells 3 weeks after Tbc boosting. One group of mice was placed on an immunizing regimen of PPD-X5563 cells (1×10^7) i.p. of 3-day intervals times 3 begun immediately after challenge with viable tumor (□——□). The other group did not undergo the PPD-X5563 cell immunization regimen (■-·-·■). Non-Tbc-primed normal mice were also immunized with PPD-X5563 cells after tumor challenge (●-----●), or not immunized after tumor challenge (○——○).

DISCUSSION

It is generally accepted that the immune response to syngeneic tumors is relatively weak and not sufficient to cause regression of the growing tumors. In the past decade, there

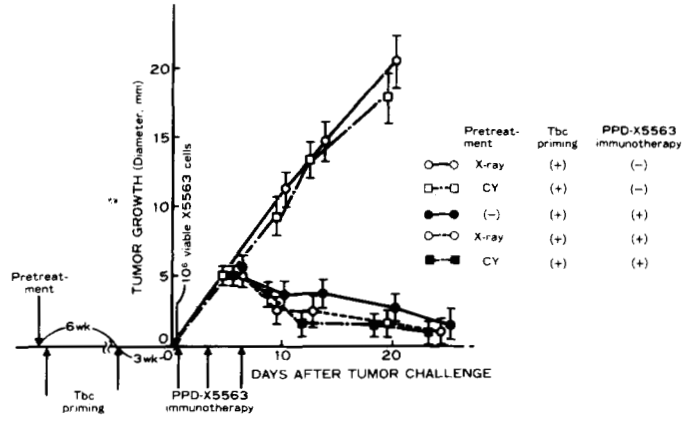

Figure 3. Further potentiation of the induction of host anti-tumor activity by treating with cyclophosphamide (CY) or x-irradiation before Tbc presensitization. Two groups of normal mice were x-irradiated (250 R) immediately before Tbc presensitization. Two other groups of mice received i.p. administration of CY (250 mg/kg) 2 days before Tbc priming. These 4 groups of mice then received booster injections of Tbc 6 weeks after Tbc priming. Another group of mice was primed with Tbc without such pretreatment. Viable X5563 cells (5×10^5) were implanted intradermally into these mice 3 weeks after the booster injection. Immunotherapy regimens with PPD-X5563 cells (1×10^7) were started immediately after tumor challenge according to exactly the same protocol as in Figure 2.

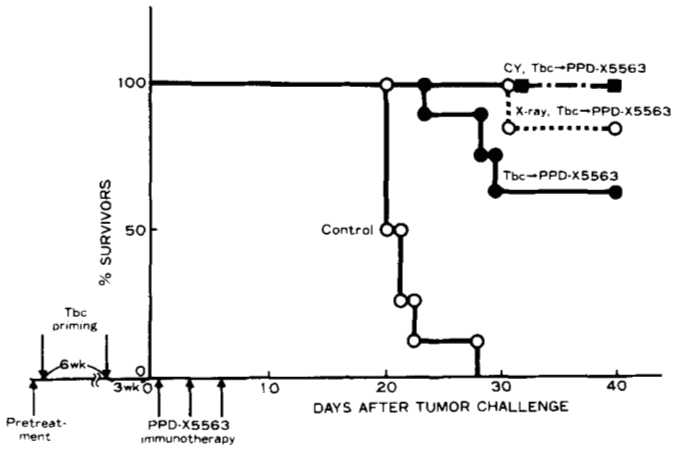

Figure 4. Survival of X5563-bearing mice after immunotherapy with PPD-X5563 cells. Percentage of survivors in the respective groups shown in Figure 3 are displayed. Control denotes the groups that were pretreated with either CY or x-ray, followed by priming with Tbc but not immunized with PPD-X5563.

have been numerous reports of attempts to increase the immunogenicity of TATA by coupling additional antigenic determinants to the tumor cell surface such as haptens, proteins, new transplantation antigens, viral coat proteins, or xenogeneic cell antigens. Some studies (1-6) have suggested that such treatment of tumor cells results in increased TATA immunogenicity. However, the augmented activity is not consistently high enough to reject the tumor, and the underlying mechanisms remain obscure.

In our previous study (10, 19), a trinitrophenyl (TNP) residue was introduced to the surface of tumor cells as an additional determinant, and syngeneic mice were repeatedly immunized with those tumor cells after TNP-reactive amplifier T cell activity had been generated by an appropriate immunization regimen using TNP-mouse γ-globulin (MGG). The results revealed that those mice developed higher cytotoxic effector T cell responses and *in vivo* anti-tumor activity than control mice not primed with TNP-MGG.

In the present study, we extended this idea of T-T cell interactions into the area of augmented induction of tumor-specific immunity in tumor-bearing animals, and tested the feasibility of utilizing a PPD-reactive T cell system for tumor immunotherapy. The relatively immunogenically weak syngeneic plasmacytoma X5563 was used in C3H/HeJ mice. The reasons why we chose PPD-reactive T cells as the amplifier T cell system in the induction of tumor-specific immunity were 2-fold: a) it was established in our previous study (11-14) that PPD-reactive T cells served as a potent helper T cell system in the augmentation of the anti-hapten antibody response to hapten-derivatized PPD, and b) vaccination of human beings with BCG is ethically feasible, and this can induce potent PPD-reactive T cell activity. In the course of testing the applicability of such a PPD-reactive T cell system to tumor immunotherapy, we first established experimental systems for the induction of potent anti-tumor activity by immunizing Tbc-primed mice with PPD-coupled tumor cells. As shown in Table II, animals appropriately presensitized with Tbc and subsequently immunized repeatedly with PPD-X5563 cells developed potent anti-tumor activity that inhibited the growth of the challenging tumor. Under these experimental conditions, however, mice not presensitized with Tbc died from overgrowth of the challenging tumor, even after being immunized with PPD-X5563 cells. The immunoprophylactic effect induced by immunization with PPD-coupled tumor cells in the presence of PPD-reactive T cells is T cell mediated and tumor specific (Fig. 1 and Table IV).

The feasibility of utilizing PPD-reactive T cells for augmenting tumor-specific immune responses was also demonstrated in recent studies reported by Lachman and Sikora (20). In their studies, mice previously sensitized with BCG followed by immunization with PPD-coujugated Con A-derivatized tumor cells developed a higher degree of anti-tumor activity than that developed by control mice not sensitized with BCG. Our present results are consistent with some aspects of their studies, although certain differences were also found. One of the prominent differences was the method used for the coupling of PPD to tumor cell surface. We used ECDI as the coupling agent, whereas they coupled PPD to Con A using glutaraldehyde and used the lectin to bind the PPD to the tumor cell membrane. As discussed previously (16), direct coupling of PPD to an attenuated tumor cell surface using ECDI is a rather easy, mild, and reproducible procedure. Above 5 to 8% of the added PPD bound to the tumor cells, and the viability of the tumor cells was not changed by this coupling procedure. As in Lachman's experiment, covalent coupling of the PPD molecule to the tumor cells was essential for augmented induction of tumor-specific resistance. Immunization of mice with a mixture of PPD and tumor cells was ineffective even in the presence of PPD-reactive T cells (Table III), indicating that T-T cell interactions between PPD-reactive T cells and TATA-reactive T cells through PPD-coupled tumor cells are required to induce potent anti-tumor activity *in vivo*.

Another prominent feature of the present study is that we extended this experimental model into a tumor immunotherapy system by applying it to tumor-bearing animals. It is noteworthy that commencement of the immunizing regimen with PPD-coupled tumor cells after inoculation of viable tumor in the host, which had been appropriately primed with Tbc, was effective in augmenting the induction of tumor resistance and that the tumor-bearing host was able to suppress and ultimately reject a tumor that had grown to palpable proportions (Figs. 2 and 3). This clearly indicates the feasibility of application of the present system in tumor-bearing animals for the purpose of augmenting tumor-specific immunity. If one wishes to induce effective host resistance against an already growing tumor, one approach would be to attempt to resect the tumor first, then to preimmunize or boost the host with Tbc, followed by immunization of the individual with PPD-coupled attenuated tumor cells. This might suppress the growth of the remaining tumor by augmenting the tumor-specific immunity. We are now further testing the feasibility of application of the present system to an autochthonous tumor system induced by a chemical carcinogen.

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