EFFECT OF IMMUNOTHERAPY, CHEMOTHERAPY, AND SURGERY ON TUMOR IMMUNITY IN MICE

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The effect of several therapeutic procedures on tumor immunity was compared on three lines of syngeneic tumor grafts of primary or early transplant generation in a group of mice previously sensitized with BCG or xenogeneic cells and in that of nonsensitized mice. For immunotherapy, a mixture of syngeneic tumor cells with BCG, tuberculin purified protein derivative (PPD), xenogeneic cells, or allogeneic cells was inoculated intradermally. For chemotherapy, cyclophosphamide or Mitomycin-C was given peritoneally twice a week for 8 weeks after the intradermal tumor inoculation. When intradermal tumor size became 5~13 mm in diameter the established tumors were surgically excised.

1) Immunotherapy using intradermal inoculation of the tumor cell-BCG mixture resulted in significant suppression of tumor growth (49/60) and tumor immunity (24/49) over controls and groups of other treatment.

2) When a mixture of tumor cell-incompatible cells was injected into normal mice, tumor growth was inhibited at the site of inoculation in 32 of 40 mice, but induced tumor immunity in only one of these 32 mice. On the other hand, inoculation of this mixture into mice presensitized with the same incompatible cells failed to inhibit tumor growth at the site of inoculation.

3) Chemotherapy with cyclophosphamide or Mitomycin-C and excision of established tumor did not affect tumor immunity in spite of suppressing tumor growth (cyclophosphamide) or prolonging the survival time (cyclophosphamide or surgery).

In previous papers, we reported that the intradermal growth of a transplantable, syngeneic guinea pig hepatoma is suppressed in normal guinea pigs if tumor cells are inoculated together with live BCG, and guinea pigs so treated subsequently developed systemic tumor immunity. We also demonstrated a similar effect of BCG in syngeneic mouse system as that in guinea pigs.

The present work was undertaken to see the efficacy of BCG treatment on tumor immunity as immunotherapy, and its result was compared with conventional therapy such as chemotherapy and surgery on the intradermal syngeneic tumor grafts. In order to avoid the immunological artificialities inherent even in the transplantation of syngeneic tissue, all the tumors employed were of primary or fairly recent transplant generation. For immunotherapy, in addition to the BCG treatment, we conducted a new trial using purified protein derivative (PPD) of tuberculin or incompatible cells (xenogeneic or allogeneic), mixed with syngeneic tumor cells in place of BCG. This idea was in our mind for years since knowing the “innocent bystander” effect in immune reaction involving an artificial mixture of target and bystander cells including xenogeneic cells. Actually, studies on the non-specific components of cellular immunity and participation in the rejection of tumors led to the development of a new model for cancer immunotherapy with BCG.

MATERIALS AND METHODS

Animal The animals used throughout these studies were 9- to 16-week-old Swiss mice, SWM/Ms. This inbred subline was separated in 1966 from a colony maintained at the National Institute of Genetics (Mishima). Skin graft among members of the strain was not rejected. Mice were divided into two groups, one of which was presensitized with BCG or xenogeneic cells intradermally and the other was not sensitized.

Tumors Three different syngeneic tumor lines consisted of (1) second transplant generation of 3-methylcholanthrene-induced tumor No. 042573, (2) primary tumor of 3-methylcholanthrene-induced tumor

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No. 111273, and (3) spontaneous mammary tumor No. 111973. These tumors were treated with enzymes as described previously\(^\text{16,19}\) to make a single cell suspension just before the tumor inoculation. More than \(10^6\) cells of viable tumor cells were inoculated intradermally in hind quarters of mice. When mice were presensitized with BCG or xenogeneic cells, tumor cells were inoculated on the site opposite the site of sensitization.

**BCG** Lyophilized *Mycobacterium bovis* of Japan strain (Japan BCG Laboratory, Tokyo) was suspended in saline just before use.

**Tuberculin Purified Protein Derivative (PPD)** Lyophilized PPD was kindly donated by Dr. T. Sawada, Director of Japan BCG Laboratory, Tokyo, and its solution in saline was used.

**Presensitization with BCG** Mice were sensitized with a single intradermal injection of BCG (\(10^7\)) about 4 to 12 weeks before the tumor inoculation.

**Presensitization with Xenogeneic Tumor Cells** Ascites hepatoma AH-62F which had been induced by 4-(dimethylamino)azobenzene in a rat, and maintained by intraperitoneal injection in Donryu rats (random bred) at our Pathology Section, was used as xenogeneic cells. In Experiments 2 and 3, some groups of mice were sensitized with a single intradermal injection of AH-62F cells (\(2.8 \times 10^6\)/mouse) 19 days and 26 days before the tumor inoculation, respectively.

**Preparation of Mixtures for Immunotherapy**

**Tumor Cell-BCG Mixture:** Tumor cells obtained by enzymic dispersion\(^\text{16,19}\) were washed twice in medium 199 and mixed with BCG in 1:10 ratio at room temperature.

**Tumor Cell-Xenogeneic Cell Mixture:** Syngeneic mouse tumor cells were mixed with washed AH-62F cells in a ratio of 1:0.7, 1:3, or 1:3.5.

**Tumor Cell-Allogeneic Cell Mixture:** In Experiment 1, one group was treated with tumor cell-allogeneic cell mixture. Mouse ascites hepatoma cells, MH-134, of C3H/He origin, maintained in our Virus Section, were used as allogeneic cells. Mixing ratio with tumor cells was 1:3.5.

**Tumor Cell-PPD Mixture:** The tumor cells were mixed with PPD and the concentration adjusted to \(10^6\) tumor cells and 20 \(\mu\)g of PPD in 0.1 ml. Mice were inoculated with these mixtures intradermally.

**Chemotherapy** Cyclophosphamide was administered intraperitoneally in 50 mg/kg dose, twice a week for 8 weeks, starting 24 hr after the tumor inoculation. Aqueous solution of cyclophosphamide was prepared freshly just before the injection. Mitomycin-C was given intraperitoneally in 1 mg/kg dose, twice a week for 8 weeks. These dosages were determined according to the data of Hoshi and others\(^\text{11}\).

**Surgery** When the size of intradermal tumor nodules grew to 5~13 mm in diameter, the mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (0.7 mg/10 g of body weight) and their tumors were excised with ophthalmic scissors. The incision was closed with autoclips (Clay-Adams, Parsippany, N.J.).

**Measurement** Intradermal tumors were measured weekly by two diameters of the tumor nodule perpendicular to each other. The date of death and tumor size of each animal were recorded.

**Test for Immunity** Mice still alive without tumor more than 30 to 105 days after the tumor inoculation were challenged intradermally with tumor cells of each line at the site opposite the first tumor inoculation.

**Evaluation** The overall assessment of therapy was based on the rate of tumor-free animals at the time of tumor challenge, and induction of tumor immunity after the tumor challenge.

In Experiment 1, specificity of the tumor immunity was determined by inoculation with 4th transplant generation of 3-methylcholanthrene-induced tumor No. 111273, which was used in Experiment 2, into tumor-free survivors from the challenge inoculation.

**RESULTS**

**Experiment 1** Tumor cells from 3-methylcholanthrene-induced tumor (2nd transplant generation, No. 082173) were inoculated intradermally in groups of mice presensitized with BCG 37 days before the tumor inoculation, and in nonsensitized mice. In some groups (Fig. 1, Groups 2~5, 9, and 10), a mixture of tumor cells with BCG, PPD, xenogeneic cells, or allogeneic cells was inoculated. After 105 days from the tumor inoculation, mice that rejected the tumor were challenged with tumor cells of the same line.

The results presented in Fig. 1 can be summarized as follows: (1) Mice treated with a mixture of tumor cells and BCG (Groups 2 and 9) rejected the tumor growth (9/10 and 8/10) and developed tumor immunity in a high rate (6/9 and 5/8). Immunotherapy using tumor cells mixed with PPD (Groups 3 and 10) did not improve the results compared to the control groups. (2) All normal mice treated with a mixture of syngeneic tumor cells and xenogeneic (Group 4) or allogeneic cells (Group 5) rejected the tumor growth at the site of response to the incompatible cells (10/10 and 10/10), but none of them rejected tumor challenge. (3)
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Cyclophosphamide showed tolerable effect on the tumor growth (Groups 6 and 11), and 2 out of 3 mice alive at the time of tumor challenge in Group 11 acquired tumor immunity. Mitomycin-C did not show any therapeutic effect even with the dosage which was rather toxic. In this experiment, presensitization with BCG did significantly affect therapeutic response; 5 out of 10 control mice sensitized to BCG (Group 8) were free of tumor at the time of tumor challenge in contrast to nonsensitized control mice (Group 1).

Experiment 2 Tumor cells from 3-methylcholanthrene-induced primary tumor (No. 111273) were used. As shown in Fig. 2, mice in Groups 6 and 7 were presensitized with BCG 86 days before, and those in Groups 8 and 9 were presensitized with AH-62F cells 19 days before the tumor inoculation. On 43 days after the tumor inoculation, mice free of tumor were challenged with tumor cells of the same line. The results were as follows.

Fig. 1. Cumulative mortality of different groups of mice according to their therapy, and their effect on tumor immunity (Expt. 1)

Animal: Nine-week-old SWM/Ms males were used, and mice shown in (B) were presensitized with BCG (10⁷) intradermally 37 days before the tumor inoculation.

Tumor: Second transplant generation, 3-methylcholanthrene-induced tumor No. 082173.

No. Treatment
1, 8 Tumor cells (10⁶).
2, 9 Tumor cells (10⁶) + BCG (1:10).
3, 10 Tumor cells (10⁶) + PPD (20 μg).
4 Tumor cells (10⁶) + AH-62F cells (1:3.5).
5 Tumor cells (10⁶) + MH-134 cells (1:3.5).
6, 11 Cyclophosphamide (CPM), 50 mg/kg twice a week for 8 weeks.
7, 12 Mitomycin-C (MMC), 1 mg/kg twice a week for 8 weeks.
13 Tumor cells (7 × 10⁶ cells, 6th transplant generation of the same tumor).
† Tumor-free mice (No. is indicated underneath) received tumor challenge (7 × 10⁶ cells, 6th transplant generation of the same tumor) on day 105.

Cyclophosphamide showed tolerable effect on the tumor growth (Groups 6 and 11), and 2 out of 3 mice alive at the time of tumor challenge in Group 11 acquired tumor immunity. Mitomycin-C did not show any therapeutic effect even with the dosage which was rather toxic. In this experiment, presensitization with BCG did significantly affect therapeutic response; 5 out of 10 control mice sensitized to BCG (Group 8) were free of tumor at the time of tumor challenge in contrast to nonsensitized control mice (Group 1).
Fig. 2. Cumulative mortality of the different groups of mice according to their treatment, and their effect on tumor immunity (Expt. 2)

Animal: Nine- to 14-week-old SWM/Ms males were used, and mice shown in (B) were presensitized with BCG (10^7) or AH-62F (2.8x10^6) intradermally 86 days or 19 days before the tumor inoculation, respectively.

Tumor: 3-Methylcholanthrene-induced primary tumor No. 111273.

No. Treatment
1, 6, 8 Tumor cells (2.8x10^6).
2, 7 Tumor cells (2.8x10^6) + BCG (1:10).
3, 9 Tumor cells (2.8x10^6) + AH-62F (1:3).
4 Cyclophosphamide (CPM), 50 mg/kg twice a week for 7 weeks.
5 Established tumor nodules (av. 8 mm in diameter) were excised 10 days after the tumor inoculation.
10 Tumor cells (5x10^6 cells, 2nd transplant generation of the same tumor).
↓ Chemo.
↓↓ Surgical excision of tumor.
↓↓↓ Tumor-free mice (No. is indicated underneath) received tumor challenge (5x10^6 cells, 2nd transplant generation of the same tumor) on day 43.

(1) Immunotherapy: Mice treated with a mixture of tumor cells and BCG (Groups 2 and 7) rejected the tumor growth in both groups of mice, nonsensitized and presensitized with BCG (5/10 and 8/10), and they developed tumor immunity in a high rate (4/5 and 7/8).

(2) Contrary to the results of Experiment 1, the tumor growth was suppressed in only 2 out of 10 mice treated with a mixture of tumor cell–AH-62F cell (Group 3), and not in mice presensitized with AH-62F cells (Group 9).

(3) Chemotherapy: Cyclophosphamide did not induce complete suppression of tumor growth but had some effect on survival time (Group 4). Average survival time of mice treated with cyclophosphamide was 60 days, while that of the control mice was 38 days (Group 1).

(4) Surgery of the established intradermal tumors did not improve the effect on tumor immunity (Group 5), but it was apparent that it had an effect on survival time for the inoculation of tumor challenge (Group 5 vs. Group 10).
Experiment 3 The procedure was almost the same as in Experiment 2, using spontaneous mammary tumor (No. 111973). Four to 8 weeks before the tumor inoculation some groups of mice (Groups 6 and 7) received BCG sensitization. Thirty days after the tumor inoculation, tumor-free survivors were challenged with tumor cells of the same line. As shown in Fig. 3, all the normal mice treated with a mixture of syngeneic tumor cells and BCG or xenogeneic cells rejected the tumor growth at the site of response (Groups 2 and 3). Mice presensitized with the same xenogeneic cells and which received the tumor cells mixed with xenogeneic cells (Group 9) could not reject the tumor growth at the site of inoculation in contrast to that of Group 3. In spite of marked effect of the tumor cell–BCG mixture on tumor growth, this therapy had little effect on tumor immunity (Groups 2 and 7).

**DISCUSSION**

The work presented here compares the effect on tumor immunity of several therapeutic procedures on three different lines of syngeneic tumor grafts of primary or early transplant
generation in mice. All experimental results are summarized in Table I. These results indicate that immunotherapy by a mixture of tumor cells and BCG or incompatible cells is effective in suppressing tumor growth at the site of delayed cutaneous hypersensitivity reaction to them, and only BCG therapy leads to the development of specific, systemic tumor immunity.

Besides BCG, which is capable of inducing vigorous inflammatory reactions, we attempted the use of PPD or incompatible cells (xenogeneic or allogeneic cells) in place of BCG as an agent for inflammatory reaction at the site of tumor inoculation. It should be noted that incompatible cells are completely rejected in normal mice and this apparently inflicted a damage on the compatible cells at the site of response, i.e., syngeneic tumor growth was completely suppressed in all normal mice (Experiments 1 and 3) at the site of reaction to incompatible cells, but contrary to the effect of BCG therapy, tumor-specific immunity did not develop except in one of 10 mice in Group 3 (Experiment 3), and our attempts to produce tumor immunity by a mixture of tumor cells and incompatible cells failed. Therefore, the effect of these mixtures was local, not systemic. However, tumor suppressing effect diminished when the mixture was inoculated into animals presensitized against incompatible cells, and this may be due to the fact that this sensitization may stimulate more humoral immunity, hence blocking antibodies, than cell-mediated immunity. On the other hand, immunotherapy by a mixture of tumor cells and BCG induced a high incidence of tumor immunity as well as tumor suppression in both groups of mice, presensitized or nonsensitized with BCG. A sequence of reactions of cell-mediated bacterial immunity to BCG at the site of reactions5,8,9,12–14) is an important

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Presensitized with BCG</th>
<th>Treatment</th>
<th>No. tumor-free animals/No. animals tested</th>
<th>No. tumor-free animals after challenge/No. animals challenged</th>
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<tbody>
<tr>
<td>Immunotherapy</td>
<td>1−3</td>
<td>–</td>
<td>Tumor cells mixed with BCG (1:10), id</td>
<td>24/30</td>
</tr>
<tr>
<td></td>
<td>1−3</td>
<td>+</td>
<td>Tumor cells mixed with BCG (1:10), id</td>
<td>25/30</td>
</tr>
<tr>
<td></td>
<td>1, 2, 3</td>
<td>–</td>
<td>Tumor cells mixed with incompatible cells (1:0.7−3.5), id</td>
<td>32/40</td>
</tr>
<tr>
<td></td>
<td>2, 3</td>
<td>–</td>
<td>Cyclophosphamide, ip (50 mg/kg, twice a week for 7 or 8 weeks)</td>
<td>0/20</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>–</td>
<td>Mitomycin-C, ip (1 mg/kg twice a week for 8 weeks)</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>+</td>
<td>Excision of established tumors</td>
<td>(19/19)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>1−3</td>
<td>–</td>
<td>Cyclophosphamide, ip (50 mg/kg, twice a week for 7 or 8 weeks)</td>
<td>0/25</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>+</td>
<td>Mitomycin-C, ip (1 mg/kg twice a week for 8 weeks)</td>
<td>3/5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>+</td>
<td>Excision of established tumors</td>
<td>0/6</td>
</tr>
<tr>
<td>Surgery</td>
<td>2, 3</td>
<td>–</td>
<td>Excision of established tumors</td>
<td>(19/19)</td>
</tr>
<tr>
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<td>–</td>
<td>0/30</td>
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<tr>
<td></td>
<td>1−3</td>
<td>+</td>
<td>–</td>
<td>5/30</td>
</tr>
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</table>
key for the effect of BCG on tumor immunity against the reactions produced by the inoculation of incompatible cells. From our observation, appearance at the reaction site in both groups treated with BCG or incompatible cells was grossly similar. For instance, in Experiment 2, injection of the mixture into the skin of mice evoked a local inflammatory nodule that reached a maximum size at day 2 or 3, and the nodule regressed by day 19. The tumor employed in Experiment 2 (3-methylcholanthrene-induced primary tumor) might have rather a heterogeneous antigenicity, but the tumor cells were highly suppressed at the site of BCG infection in mice presensitized with BCG, which had acquired tumor immunity (Fig. 2, Group 7). It is interesting that one of the mice which received the mixture of mammary tumor cells and AH-62F cells (Expt. 3, Group 3) rejected tumor challenge since, in this experiment, incidence of survivors with tumor immunity in the groups given tumor cell–BCG mixture was equally low (Fig. 3, Groups 2 and 7). Injection of a mixture of tumor cells and PPD (20 μg) enhanced or had no effect on tumor growth and tumor immunity in both groups of mice sensitized with BCG and nonsensitized (Fig. 1, Groups 5 and 10).

Chemotherapy with cyclophosphamide or Mitomycin-C at the dosage indicated did not affect tumor immunity, with one exception. In Experiment 1, Group 11, mice presensitized with BCG were treated with cyclophosphamide. Two of these 5 mice died before the tumor challenge and 2 of the remaining 3 mice acquired tumor immunity. In Group 6, tumor growth was suppressed in mice not sensitized with BCG, by the administration of cyclophosphamide (5/6), but they did not acquire tumor immunity. Before these experiments, the optimal dosage of these drugs to this mouse system should have been checked but this was not done. Therefore, the optimal dose for continuous administration over a long period is still left for future examination. Altogether, in Experiments 1 to 3, cyclophosphamide generally suppressed tumor growth and prolonged the survival time of tumor-bearing animals. Therefore, data shown by Bekesi and Holland4) that administration of a mild chemotherapeutic enhanced the effect of active immunotherapy, and by Thompson et al.20) that chemotherapeutic treatment after surgery is able to protect some animals from tumor death, indicate that further studies on such a combined therapeutic regimens should be conducted. Another immunogenic effect of combination therapy was recently studied by Bartlett et al.,2) in which presurgical infiltration of tumors with BCG improved the surgical results and produced strong tumor immunity in mice.

The present data confirm our previous work that tumor cell–BCG mixtures are highly efficient in producing tumor immunity in mice.17,21) Effect of the BCG therapy on mammary tumors is not so significant compared with results on 3-methylcholanthrene-induced tumors and suggests a possibility of different antigenicities of tumors, also probably due to tolerance state to mammary tumor viruses in this tumor system.

For induction of tumor immunity, intradermal inoculation of live tumor cells was made with syngeneic mouse tumor system since 1937,1,7) and also studied in guinea pigs recently.6,10) Instead of running the risk of inoculation with live tumor cells alone as the source of antigen, intradermal inoculation of autologous living tumor cells mixed with BCG would be a more useful approach for the induction or augmentation of systemic tumor immunity. Further experimental studies are being made on BCG therapy to determine optimal condition for a model of clinical therapeutic efficacy.

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REFERENCES

11) Hoshi, A., Kanzawa, F., Kuretani, K., Gann, 63, 353 (1972).
18) Tanaka, T., unpublished data.