EFFECT OF ANTI-THYMOCYTE SERUM, ANTI-MACROPHAGE SERUM, AND LATEX PARTICLES ON THE THERAPEUTIC EFFICACY OF BCG OR CORYNEBACTERIUM LIQUEFACIENS (PROPIONIBACTERIUM ACNES C7) IN SYNGENEIC MICE*1

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In a syngeneic mouse-tumor system, anti-thymocyte serum, anti-macrophage serum, and latex particles were used for a comparative study of the immunotherapeutic efficacy of intradermal inoculation of tumor cell-BCG and tumor cell-Corynebacterium liquefaciens (=Propionibacterium acnes C7) vaccines. Anti-thymocyte serum treatment nullified suppression of tumor mediated by BCG (living and nonliving) and living C. liquefaciens. However, the effect of nonliving C. liquefaciens was not affected so much by treatment with anti-thymocyte serum. Treatment with anti-macrophage serum or latex particles also impaired the therapeutic efficacy of living BCG, but had no influence on the efficacy of living and nonliving C. liquefaciens.

In 1970, Zbar et al.38,39) developed an animal model for immunotherapy of cancer, in which intradermal growth of syngeneic guinea pig hepatoma was suppressed when the tumor cells were inoculated with living BCG. Following their work, we have been examining the therapeutic efficacy of tumor cell-BCG vaccine in syngeneic mice.21,29−31,34) This paper deals with Corynebacterium liquefaciens (=Propionibacterium acnes C7), an anaerobic bacteria found in human bone marrow. It was isolated at the National Cancer Center Hospital, Tokyo, in 1965,8,14) and identified, according to Prévôt's classification, as belonging to a group related to C. parvum.24) In the Eighth Edition of Bergey's Manual,41 however, classification of the anaerobic Corynebacterium species was revised as Propionibacterium by their physiological and biochemical properties. Therefore, the new designation Propionibacterium acnes C7 will be used hereafter. The antitumor activity of P. acnes C7 has also been studied in our mouse system, and we established the therapeutic efficacy of tumor cell-P. acnes C7 vaccine; the effective dose of nonliving P. acnes C7, a comparative study of the effect of living and nonliving P. acnes C7, and the effect of combination of tumor cell-BCG-P. acnes C7 vaccine on tumor immunity.32) Recently, the chemical and immunological properties of nonliving P. acnes C7 were investigated by Azuma et al.,3) and by Saino et al.25)

Successful immunotherapy with BCG required participation of cell population in the development of a cell-mediated immune response to mycobacterial antigens, i.e., agents that inhibit the delayed hypersensitivity reactions to BCG antigens abrogate BCG-mediated tumor killing.6,12,40) Notwithstanding the evidence that lymphocytes are required for BCG-mediated tumor suppression, there is

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*1 Research supported in part by Grants-in-Aid for Cancer Research and for Special Cancer Chemotherapy Program from the Ministry of Health and Welfare.

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no convincing evidence that macrophages are necessary for BCG-mediated tumor suppression. In the case of P. acnes C7, its antitumor effect is said to be in some way nonspecific and macrophage-mediated as in C. parvum.28, 36)

In general, the cells of anaerobic coryneform bacteria, such as C. parvum, C. granulosum, and P. acnes C7, have various biological activities, i.e., stimulation of reticuloendothelial systems and resistance to infection,11,19,20,25) adjuvant activity in immune response,3,17~20, 25,38) and antitumor activity.3,15,21,22,25~28,32, 35)

The present work was undertaken to elucidate the mechanism of tumor suppression mediated by BCG and P. acnes C7 by the use of anti-thymocyte serum, anti-macrophage serum, or latex particles which are taken up by and damage macrophages.1,41) Application of these agents may help to clarify the relative role of different host components concerned with the antitumor activity of BCG and P. acnes C7 in our immunotherapy system.

**Materials and Methods**

**Animals** Eight- to 15-week-old Swiss origin inbred male mice (SWM/Ms) were used throughout. **Tumors** The tumors consisted of 3 different lines of sarcoma induced by 3-methylcholanthrene which were produced by a subcutaneous injection of 0.5 mg of 3-methylcholanthrene in olive oil. They were 7th transplant generation of MCA-120574, 8th transplant generation of MCA-072275, and 4th transplant generation of MCA-043076. They are maintained by subcutaneous passage in our laboratory and histological examination showed all the tumors to be a solid spindle cell or fibrosarcoma. These tumors were treated with enzymes, as described previously,29) to make a single cell suspension for intradermal inoculation.

**BCG** Lyophilized *Mycobacterium bovis* of the Japan strain (Japan BCG Laboratory, Tokyo) was suspended in saline solution just before use. For fresh living BCG,29) the Japan strain was cultured in Middlebrook's 7H9 medium (Difco Laboratories, Detroit, U.S.A.) for 10 to 14 days in our laboratory. The organisms were collected by centrifugation, washed with a fresh 7H9 medium, and resuspended. Viable counts were made by a serial dilution

**P. acnes C7** As the living P. acnes C7, freshly cultured organisms were prepared from 5- to 7-day cultures grown in a medium containing 15 g of peptone, 3 g of Phytone, 5 g of glucose, 2.5 g of NaCl, 0.5 g of L-cystine, and 0.5 g of sodium thioglycolate in 1 liter of distilled water. This was centrifuged at 3,000 rpm for 15 min and the sediment was washed with physiological saline. These packed organisms were resuspended in saline and used in a living form. For determination of viable bacteria, 0.1 ml of the culture was plated on GAM agar (Nissui Seiyaku Co., Tokyo). As nonliving P. acnes C7, the phenol-treated preparation41) was kindly provided by Kowa Co., Tokyo. The preparation (20 mg/ml/ampule) was washed with Hanks' balanced salt solution (BSS) before use.

**Preparation of BCG Cell-wall Skeleton in Oil and P. acnes C7 Cell-wall Skeleton Suspension** Cell-wall skeleton (CWS) fractions of BCG and P. acnes C7 were kindly donated by Dr. Azuma, Osaka University Medical School. Oil-attached BCG-CWS (2 mg/ml) was prepared as described elsewhere.2) Since P. acnes C7-CWS fraction does not attach to oil droplets and its antitumor activity was not influenced by the absence of oil droplets (our observation), the fraction was homogenized in Hanks' BSS (2 mg/ml) and used for admixture with tumor cells.

**Anti-thymocyte Serum, Anti-macrophage Serum, and Normal Serum** Rabbit anti-thymocyte serum and normal serum (Lot No. 86412 and Lot No. 15056, respectively, Microbiological Associates, Inc., Bethesda, U.S.A.) were injected, in doses of 0.25 ml into the peritoneal cavity of mice, 4 or 5 times before and after inoculation of tumor cells or of vaccine. Anti-thymocyte serum and normal serum injection extended over a period of 7 to 16 days. Both sera were absorbed with 20% of finely minced mouse liver for 1 hr at room temperature and overnight in the cold. For antimacrophage serum, peritoneal cells of adult ICR mice were obtained by lavage with Hanks' BSS on 4th day after intraperitoneal injection of 1.5 ml of 0.1% glycogen. The cell suspensions were incubated for 20 min in sterile plastic petri dishes at 37°. Adherent cells were removed by a rubber policeman and resuspended in Hanks' BSS to a concentration of 3 × 10⁶ cells/ml for the first injection and 6 × 10⁴ cells for the second injection. Rabbits weighing 3.5 kg received two intravenous injections (ear vein) of mixtures containing 1 ml each of adherent mouse macrophage suspension and Freund's incomplete adjuvant. These injections were separated by 2 weeks. Seven days after the last injection, the rabbits were bled by cardiac puncture. The serum was separated, heat-inactivated at 56° for 30 min, and absorbed with mouse
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erthrocytes (20%) at room temperature for 1 hr and then overnight in the cold (5°C). The potency of these absorbed rabbit anti-thymocyte and anti-macrophage sera was titrated by direct cytolysis test against mouse thymocytes and macrophages by serial dilutions of sera and complement (guinea pig serum), respectively.10) Anti-thymocyte serum showed 50% cytolysis of thymocytes at about 1:340 dilution, and anti-macrophage serum showed 50% cytolysis of macrophages (adherent cells from peritoneal exudate cells) at 1:40 dilution.

Latex Particles Uniform polystyrene particles (diameter 1.099 μm) were obtained from the Dow Chemical Co., U.S.A. They were suspended in Hanks’ BSS in a concentration of 50 mg/ml just before use and were injected intraperitoneally in doses of 30 mg/mouse 2 hr before tumor inoculation.

Measurement of Tumors Intradermal tumors in mice were measured weekly as two diameters of the tumor nodule taken at right angles. The square measures were used as the tumor size. Growth of the individual tumor and the date of death of mice were recorded.

Test for Immunity Animals still alive without tumor for more than 35 to 48 days after the tumor inoculation were challenged intradermally with tumor cells of the appropriate line at the site contralateral to the first tumor inoculation.

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

RESULTS AND DISCUSSION

Experiments 1, 2, and 3 were carried out to compare the antitumor mechanisms of BCG and P. acnes C7 by treatment of mice with anti-thymocyte serum, anti-macrophage serum, or latex particles with a view to clarifying the relative role of different cell populations associated with the antitumor action to BCG or P. acnes C7.

Experiment 1 (No. 061175) Twenty-nine mice were divided into two groups; 17 mice received intraperitoneal injection of 0.25 ml of anti-thymocyte serum on days −2, 0, +2, and +4. On day 0, tumor cells alone, tumor cell-BCG vaccine, or tumor cell-P. acnes C7 vaccine was inoculated intradermally. The adjuvants used were killed P. acnes C7, and BCG-CWS-in-oil preparation, and each of them was mixed with tumor cells (1.4 × 10⁶) from 7th transplant generation of MCA-120574. The effect of anti-thymocyte serum treatment on BCG and P. acnes C7 therapy is shown in Fig. 1. Tumor cells injected without BCG or P. acnes C7 grew progressively in both groups of mice treated with anti-thymocyte serum and in nontreated mice, and all the mice died of tumor within 41 days. The effect of anti-thymocyte serum administration on the antitumor activity of P. acnes C7 and BCG was significantly different, as shown in Fig. 1; tumor growth in mice inoculated the tumor cell-P. acnes C7 vaccine was not influenced by treatment with anti-thymocyte serum, 2 out of 6 mice experienced complete tumor suppression, but not the same with the group of mice inoculated the tumor cell-BCG vaccine. There is a convincing evidence that lymphocytes are required for BCG-mediated tumor regression6, 12) but, in the case of P. acnes C7 therapy, there is evidence that anti-thymocyte serum treatment does not abrogate the efficacy of P. acnes C7-mediated tumor killing in allogeneic mouse tumor system.15)

Recently, following the work of Mackaness et al.,23) Yamada and Ohno37) demonstrated cellular responses in popliteal nodes of mice after inoculation of antitumor agents in the footpads by measuring 3H-TdR incorporation into DNA, and by determining histological features in responding nodes. The animals they used were germ-free mice and athymic mice inoculated with antitumor agents such as BCG-CWS-in-oil vaccine, killed P. acnes C7, or some other agents. The responses elicited by these agents were compared. In normal mice, BCG-CWS-in-oil vaccine produced an obvious antibody response but did not interfere with induction of cell-mediated immunity. BCG-CWS-in-oil vaccine produced no such response in nodes of athymic mice. In mice inoculated with P. acnes C7 (250 and 125 μg), on the contrary, a higher level of response was seen both in normal and in athymic mice, and it was
Fig. 1. Effect of anti-thymocyte serum on the antitumor activity of tumor cell-BCG and tumor cell-P. acnes C7 vaccines (Expt. 1)

Animals: SWM/Ms male mice, 8- to 15-week-old.
Tumor: 7th transplant generation of MCA-120574 line.
Anti-thymocyte serum (ATS) or normal serum (NS) was injected (0.25 ml) intraperitoneally on days -2, 0, +2, and +4. Number in parentheses on the graph indicates number of survivors.

accompanied by a marked and wide granulomatous reaction with histiocytes in responding nodes. These data indicate that the cells responding to BCG are mainly T cells, and those responding to P. acnes C7 (killed) are probably B cells. With a hope of clarifying more exactly the difference between BCG and P. acnes C7 activity, we subsequently conducted the next experiment with anti-macrophage serum treatment.

**Experiment 2 (No. 122775)** Forty-eight mice were divided into 4 groups, and each group of 12 was subdivided into 3 groups for treatment with intraperitoneal injection with 0.25 ml of anti-thymocyte serum, anti-macrophage serum, or normal serum, given on days -5, -3, -1, +1, and +10. On day 0, each group of mice were inoculated intradermally either with tumor cells (4.9 × 10⁵ from 8th transplant generation of MCA-072275) or with the vaccine and, 48 days after inoculation, tumor-free mice were challenged with tumor cells (10⁶) of the same line on the site contralateral to the first inoculation. Adjuvant used was lyophilized BCG, BCG-CWS-in-oil, or P. acnes C7-CWS preparation. As shown in Table I, (1) the results were contrary to the results of Experiment 1 and mice given anti-thymocyte serum died with tumor, even in the group of mice inoculated with tumor cell-P. acnes C7-CWS vaccine, but the growth rate of tumor in the P. acnes C7 group was less affected by anti-thymocyte serum treatment than in other groups. Mean survival time of each of the groups of mice (Table I) treated with anti-thymocyte serum was 35, 30, 25,
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Table I. Effects of Anti-thymocyte Serum (ATS) and Anti-macrophage Serum (AMS) on the Antitumor Activity of Tumor Cell-P. acnes C7 and Tumor Cell-BCG Vaccines (Expt. 2)

<table>
<thead>
<tr>
<th>Treatment (intradermal) with tumor cells (No.)</th>
<th>No. of tumor-free mice/ No. of mice tested on day 48*</th>
<th>No. of tumor-free mice after challenge/ No. of mice challenged on day 65</th>
</tr>
</thead>
<tbody>
<tr>
<td>(4.9 × 10⁶) + P. acnes-CWS (100 µg)</td>
<td>0/4</td>
<td>3/3</td>
</tr>
<tr>
<td>(4.9 × 10⁶) + BCG (7 × 10⁴)</td>
<td>0/4</td>
<td>1/4</td>
</tr>
<tr>
<td>(4.9 × 10⁶) + BCG-CWS (100 µg)</td>
<td>0/4</td>
<td>2/4</td>
</tr>
<tr>
<td>(4.9 × 10⁶) in-oil</td>
<td>0/4</td>
<td>2/4</td>
</tr>
<tr>
<td>(1 × 10⁶)</td>
<td>0/4</td>
<td>0/4</td>
</tr>
</tbody>
</table>

Animal: SWM/Ms male mice, 4- to 8-week-old.
Tumor: 8th transplant generation of MCA-072275 line.
Anti-thymocyte serum, anti-macrophage serum, and normal serum (0.25 ml each) were injected intraperitoneally on days -5, -3, -1, +1, and +10.

1) Tumor-free mice received tumor challenge on day 48 (10⁶ cells, 9th transplant generation of the same tumor).

and 25 days, respectively. (2) When mice were treated with anti-macrophage serum, therapeutic efficacy of tumor cell-P. acnes C7 vaccine and of tumor cell-BCG vaccine was slightly impaired, in contrast to groups of mice treated with normal serum (Table I, AMS vs. normal serum).

The mechanism of living BCG-mediated tumor regression and the induction of systemic tumor immunity was postulated on evidence presented by Zbar and Rapp. The lymphocytes eliminated by anti-thymocyte serum appear to be the mediators of delayed sensitivity and resistance to intracellular infection, and the same cell population is essential for the BCG-mediated tumor regression since anti-thymocyte serum treatment in mice nullifies BCG-mediated tumor killing. In the series of BCG-mediated reaction, macrophages are immobilized and activated by mediators released from specifically sensitized lymphocytes. In vitro observations indicate that these activated macrophages in mice kill tumor cells, and histopathological studies show that histiocytes are in close contact with dying tumor cell. However, there is no distinct in vivo evidence that macrophages are necessary for tumor suppression in BCG-therapy or P. acnes C7-therapy.

Experiment 3 (No. 060776) The last experiment was carried out to confirm the role of macrophages on the immunological efficacy of BCG-mediated or P. acnes C7-mediated function by intraperitoneal administration of uniform latex particles, which are taken up by macrophages and produce selective impairment of macrophage functions, and the influence of this treatment on the therapeutic effect of each vaccine (BCG and P. acnes C7) was examined.

Forty-eight age-matched mice were divided into 16 groups according to a protocol shown in Table II. Anti-thymocyte serum or anti-macrophage serum was injected intraperitoneally on days -2, 0, +5, and +7. Tumor cells (10⁶) from 4th transplant generation of MCA-043076 were mixed with BCG or P. acnes C7, either in living form or nonliving preparation, and inoculated intradermally. The over-all assessment of the effect of BCG or P. acnes C7 on tumor growth was based on the rate of tumor-free animals at the time of tumor challenge (day 35). Adjuvant used was living BCG, BCG-CWS-in-oil preparation, living P. acnes C7, or P. acnes C7-
Fig. 2. Effects of anti-thymocyte serum, anti-macrophage serum, and latex particles on the antitumor activity of tumor cell-BCG and tumor cell-P. acnes C7 vaccines (Expt. 3)

Animals: SWM/Ms male mice, 8- to 10-week-old.
Tumor: 4th transplant generation of MCA-011276 line.
Antisera (0.25 ml) were injected intraperitoneally on days -2, 0, +5, and +7. Latex particles (30 mg) were given intraperitoneally 2 hr before the tumor or vaccine inoculation.

Table II. Effects of Anti-thymocyte Serum (ATS), Anti-macrophage Serum (AMS), and Latex Particles on the Antitumor Activity of Tumor Cell-P. acnes C7 and Tumor Cell-BCG Vaccine (Expt. 3)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment (intradermal) with tumor cells (10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+live BCG (1.4 × 10⁷)</td>
</tr>
<tr>
<td></td>
<td>+BCG-CWS-in-oil (100 µg)</td>
</tr>
<tr>
<td></td>
<td>+live P. acnes (5 × 10⁷)</td>
</tr>
<tr>
<td></td>
<td>+P. acnes-CWS (100 µg)</td>
</tr>
<tr>
<td></td>
<td>+None</td>
</tr>
</tbody>
</table>

Table III. Effects of Anti-thymocyte Serum (ATS), Anti-macrophage Serum (AMS), and Latex Particles on the Antitumor Activity of Tumor Cell-P. acnes C7 and Tumor Cell-BCG Vaccine (Expt. 3)

<table>
<thead>
<tr>
<th>Treatment (intradermal) with tumor cells (10⁶)</th>
<th>No. of tumor-free mice/No. of mice tested on day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>+live BCG (1.4 × 10⁷)</td>
<td>ATS₂</td>
</tr>
<tr>
<td>+BCG-CWS-in-oil (100 µg)</td>
<td>0/3</td>
</tr>
<tr>
<td>+live P. acnes (5 × 10⁷)</td>
<td>0/3</td>
</tr>
<tr>
<td>+P. acnes-CWS (100 µg)</td>
<td>1/3</td>
</tr>
<tr>
<td>+None</td>
<td>0/3</td>
</tr>
</tbody>
</table>

Animals: SWM/Ms male mice, 8- to 10-week-old.
Tumor: 4th transplant generation of MCA-011276 line.

a) Antisera (0.25 ml) were injected intraperitoneally on days -2, 0, +5, and +7.
b) Latex particles (30 mg) were given intraperitoneally 2 hr before the tumor or vaccine inoculation.
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CWS preparation. The results presented in Fig. 2 and Table II can be summarized as follows: (1) Effect of anti-thymocyte serum on the antitumor activity of living P. acnes C7 clearly contrasted to that of non-living P. acnes C7; when mice were treated with anti-thymocyte serum, their response to BCG (both living and nonliving) and to live P. acnes C7 was completely inhibited but, as in Experiments 1 and 2, response to P. acnes C7-CWS was not so affected by anti-thymocyte serum. (2) The effect of anti-macrophage serum on the antitumor activity of living BCG and living P. acnes C7 was significantly different, as will be seen in Fig. 2 and Table II. When mice were treated with anti-macrophage serum the effect of tumor cell-BCG vaccine against tumor growth was completely suppressed in contrast with that of the tumor cell-P. acnes C7 vaccine. Similar results were obtained when mice were injected intraperitoneally with latex beads for blocking macrophage functions. In addition to these experiments (Expts. 1~3), we made a preliminary experiment on latex beads to see whether this treatment abrogates the efficacy of tumor cell-nonliving P. acnes C7 vaccine or not, and confirmed that immune response of mice treated with latex beads was not depressed against the tumor cell-nonliving P. acnes C7 vaccine; 3 out of 4 mice being tumor free at day 47 in contrast to all four mice being tumor free in normal control mice. Tumors grew progressively in both groups of mice nontreated and treated with latex beads, and they all died within 36 days and 27 days, respectively. (3) All the normal mice given tumor cell-BCG (live or CWS-in-oil) vaccine or tumor cell-P. acnes C7 (live or CWS) vaccine rejected the tumor at the site of the inoculation (Fig. 2 and Table II).

There is evidence that intraperitoneal injection of killed C. parvum, as in the case of P. acnes C7, inhibited syngeneic tumor growth in T-cell deprived mice and it was concluded that the antitumor effect of C. parvum depends on macrophage stimulation. On the other hand, tumor growth was not inhibited by BCG therapy in T-cell deprived mice. Our present results were similar to these known evidences, and some additional new findings were presented as follows: (1) Anti-thymocyte serum treatment completely nullified tumor killing mediated by live P. acnes C7 but not in the case of P. acnes C7-CWS preparation. Similarly to tumor cell-live BCG vaccine, the effect of tumor cell-BCG CWS-in-oil vaccine was also inhibited by anti-thymocyte serum. It is apparent that a sequence of reactions of cell-mediated immunity to living bacteria is quite different from the reactions produced by the inoculation of nonliving bacteria, not only at the reaction site but systemically. Recently, the chemical and immunological properties of P. acnes C7 cell wall were reported. The different antigenicities between BCG and P. acnes C7 might produce different responses in mice treated with anti-thymocyte serum. (2) When mice were treated with anti-macrophage serum or latex particles, the effect of live BCG-mediated tumor killing was completely impaired, in contrast with the effect of tumor suppression mediated by live or nonliving P. acnes C7. These results indicate that anti-thymocyte serum or anti-macrophage serum we used reacted with each thymocyte or macrophage specifically and, therefore, they are reflecting the relative roles of different host components which are immunologically committed in the reactions evoked by BCG or P. acnes C7. (3) In the group of normal mice that received tumor cell-BCG or tumor cell-P. acnes C7 vaccines, tumor growth was completely suppressed and, in control mice inoculated with tumor cells, the tumor grew progressively (Fig. 2).

It should be noted here that the anti-macrophage serum we prepared was not clearly specific since we have not done absorption studies with lymphocytes, and in the same way with anti-thymocyte serum whose cross reactivity with macrophages was not
checked. Further studies on the antitumor mechanisms of BCG and P. acnes C7 are being undertaken by the use of highly specific antisera.

The nonspecific effect of nonliving P. acnes C7 has been attributed primarily to stimulation of the reticuloendothelial systems, as in the case of C. parvum (nonliving). The present paper is the first report on a comparative study of macrophage-dependent and lymphocyte-dependent responses in host animals against BCG and P. acnes C7, in both living and nonliving preparations. We believe that elucidation of differences between the antitumor mechanisms of P. acnes C7 and BCG would be an important factor for further development of immunotherapy.

(Received August 13, 1976)

References