Antitumor Activity of Cyclophosphamide and Lipopolysaccharide in Tumor-Bearing Mice Pretreated with BCG

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Antitumor effects of cyclophosphamide (CY) and lipopolysaccharide (LPS) were investigated in BCG-treated mice. C3H/He mice and CDF1 mice were injected with BCG and were inoculated subcutaneously with syngeneic mouse hepatoma and mastocytoma P815 respectively. A subsequent injection of LPS caused hemorrhagic necrosis and retarded growth of tumor. When mice were treated with LPS plus suboptimal dose of CY, tumor growth was retarded and survival time was prolonged. The antitumor effects were more remarkable when mice were treated with CY prior to the injection of LPS. Without BCG pretreatment, LPS showed no antitumor activity in mice. Sera from mice treated with BCG plus PLS was cytotoxic for cultured tumor cells. However treatment of mice with CY did not increase the in vitro cytotoxicity. In this experimental condition, CY had no effect on delayed type hypersensitivity when evaluated by the footpad reaction to purified protein derivative (PPD). These results seem to indicate that the antitumor effects of the treatment with CY and LPS in BCG-treated mice are mediated by the reduction of tumor burden by CY and a serum factor induced by LPS. ——— Antitumor activity; cyclophosphamide; LPS; BCG; serum factor

We have observed that an injection of bacterial lipopolysaccharide retarded the growth of tumors inoculated subcutaneously in mice pretreated with BCG (Sato et al. 1986). The antitumor activity of LPS seemed to be mediated by a serum factor. However prolongation of survival time was not significant statistically. Because of toxicity and tolerance (Madonna and Vogel 1985), repeated injections of LPS are not feasible. There have been a number of reports dealing with the antitumor effect of chemotherapeutic agents in BCG-treated animals. However no publication is available concerning the antitumor activity of LPS in combination with anticancer drugs in mice pretreated with BCG. In this study,
the antitumor effects of LPS plus cyclophosphamide (CY), an antineoplastic agent, were investigated in mice pretreated with BCG.

**MATERIALS AND METHODS**

**Materials**

**Mice.** Male C3H/He mice and hybrid CDF₁ (BALB/C × DBA/2) mice were purchased from Shizuoka Experimental Animal Farm (Shizuoka). Mice were 6 to 8 weeks old at the start of experiments.

**Tumor cells.** Mouse hepatoma MH134, originally induced with carbon tetrachloride in C3H mice and converted into the ascites form, has been maintained in C3H/He mice by intraperitoneal inoculations. Mastocytoma P815, induced in DBA/2 mice, has been maintained in ascites by intraperitoneal transplantations in histocompatible CDF₁ mice. Tumor cells were harvested from the abdominal cavity and washed with saline immediately before inoculation.

**Drugs**

Lyophilized *Mycobacterium bovis*, strain BCG (Japanese strain Tokyo No. 172 supplied by the National Institute of Health, Tokyo) was used throughout this series of experiments. Lipopolysaccharide (LPS) derived from *Escherichia coli* was purchased from Difco (Detroit, MI, USA). Cyclophosphamide monohydrate (Shionogi Pharmaceutical Co., Tokyo) was dissolved in saline and a final volume of 0.25 ml was injected intraperitoneally. Purified protein derivative (PPD) from *Mycobacterium tuberculosis* strain Aoyama B from Nihon BCG Co. (Tokyo) was dissolved in saline immediately before use.

**Animal experiments**

Male C3H/He mice and CDF₁ mice were injected intravenously with 1 mg (5×10⁶) of viable BCG organisms. After 7 days they were inoculated subcutaneously with 1.0×10⁶ cells of MH134 and 1.0×10⁶ cells of P815 respectively. After 8 days 15 μg of LPS was injected intravenously. Cyclophosphamide (Hereafter CY, 40 mg/kg) was injected intraperitoneally 4 days after injection of LPS. In experiments which were carried out in parallel, 40 mg/kg of CY was injected 8 days after inoculation of tumor cells and 15 μg of LPS was given 4 days thereafter. Tumor size was expressed in terms of the mean of two perpendicular diameters. In another experiment, CDF₁ mice without previous BCG injection were treated in the similar manner as described above.

**Preparation of sera for cytotoxicity test**

Mice were injected intravenously with 1 mg (5×10⁶) of viable BCG organisms. After 10 days, 40 mg/kg of CY was injected intraperitoneally. LPS (15 μg) was injected intravenously 4 days after injection of CY and blood was taken 2 hr after injection of LPS. Blood from the BCG-treated mice was taken 14 days after injection of BCG. Blood from the LPS-treated mice was taken 2 hr after injection of LPS.

**Cytotoxicity test**

P815 cells were cultured in RPMI 1640 medium (Nissui Co., Tokyo) supplemented with 10% heat-inactivated fetal calf serum. Sera to be tested from CDF₁ mice, heated at 56°C for 30 min, were added and the cell suspension was incubated in 5% CO₂ in air at 37°C for 48 hr. Viable cells were counted using the trypan blue dye exclusion method. Cytotoxicity was expressed by the formula:

\[
\text{Cytotoxicity} \, (\%) = (1 - a/b)\times100
\]

where “a” is the number of viable cells in culture with test serum and “b” is the number of viable cells in culture without test serum.
Footpad reaction

Male CDF1 mice received an intravenous injection of 1 mg (5 x 10^6) of viable BCG organisms. After 7 days, they were inoculated subcutaneously with 1.0 x 10^6 cells of P815. The mice thus treated were divided into two groups. One group was injected intraperitoneally with 40 mg/kg of CY and the other group with saline alone 8 days after inoculation of tumor cells. After 4 days, both groups were given an intradermal injection of 5 µg of PPD in 0.04 ml of saline into the right hind footpad. The thickness of the right footpad was compared with that of the control (left) footpad after 24 hr.

Statistical analysis

Student's t-test was used in all statistical evaluations. Any p value less than 0.05 was considered significant.

RESULTS

Effects of LPS and/or CY on tumor size in, and survival time of, BCG-pretreated mice bearing tumor

Male CDF1 mice and C3H/He mice, injected intravenously with 1 mg of BCG, were inoculated subcutaneously with syngeneic mastocytoma P815 and syngeneic ascites hepatoma MH134 respectively. Antitumor effects of CY and LPS on tumor size and survival time were investigated in these animals. Figs. 1 and 2 show the results of experiments in which LPS was injected before CY. When CDF1 mice were inoculated with P815 cells (Fig. 1), an injection of LPS
caused hemorrhagic necrosis (groups 1 and 3). Regrowth of tumor was retarded in these two groups as compared with that in control group (group 4). However there was no significant difference in survival time between LPS-injected group (group 3) and the control group. The tumor size of the CY-injected groups (groups 1 and 2) remained smaller than that of control group. The survival time of groups 1 and 2 was significantly prolonged as compared with that of control group. However the mean survival time of the group treated with LPS plus CY (group 1) did not differ from that of CY-injected group (group 2) statistically.

In C3H/He mice previously inoculated with MH134 cells (Fig. 2) hemorrhagic necrosis of tumor was observed after injection of LPS (groups 1 and 3). However in CY-injected mice (group 2), although regrowth of tumor was retarded, the effect of CY was not so remarkable as in CDF1 mice. The mean survival time of the LPS-injected mice (group 3) and that of the CY-injected mice (group 2) was not prolonged significantly as compared with that of the control group. In the group treated with LPS plus CY (group 1), tumor growth was retarded. The prolongation of survival time in this group was statistically significant as compared with that of the control group.

Figs. 3 and 4 show the results of experiments in which CY was injected before LPS.

As shown in Fig. 3, when CDF1 mice had been inoculated with P815 cells the tumor size was unchanged for 4 days after injection of CY (groups 1 and 2).
Fig. 3. Antitumor activity on P815
Male CDF1 mice, 8 animals per group injected i.v. with 1 mg of BCG 7 days previously, were inoculated with $1.0 \times 10^6$ cells of P815 (day 0). Groups 1 and 2 received an i.p. injection of 40 mg/kg of CY on day 8. Groups 1 and 3 were injected i.v. with 15 μg of LPS on day 12. Group 4 served as a control group. Mean tumor diameter of each group was compared.

Mean survival days (± s.d.): group 1, 28.5±1.6; group 2, 24.8±1.0; group 3, 20.4±2.5; group 4, 20.1±1.8.

Fig. 4. Antitumor activity on MH134
Male C3H/He mice, 8 animals per group injected i.v. with 1 mg of BCG 7 days previously, were inoculated with $1.0 \times 10^6$ cells of MH134 (day 0). Groups 1 and 2 received an i.p. injection of 40 mg/kg of CY on day 8. Groups 1 and 3 were injected i.v. with 15 μg of LPS on day 12. Group 4 served as a control group. Mean tumor diameter of each group was compared.

Mean survival days (± s.d.): group 1, 38.2±2.1; group 2, 34.0±1.8; group 3, 33.6±2.0; group 4, 30.5±1.3.
Tumor grew anew thereafter in the CY-injected mice (group 2). On the other hand, the regrowth of tumor was retarded in mice treated with CY plus LPS (group 1). The mean survival time of the CY-injected group (group 2) was prolonged significantly as compared with that of the control group. There was also significant difference in survival time between the group treated with CY plus LPS (group 1) and the CY-injected group (group 2).

In C3H/He mice which received an inoculation of MH134 cells (Fig. 4), the effect of CY was not so remarkable as in CDF1 mice. Tumor growth was retarded after injection of LPS (groups 1 and 3). While the rate of regrowth of the tumor in CY-injected mice (group 2) was similar to that in LPS-injected mice (group 3), the mean survival time was significantly prolonged in the CY-injected group as compared with that in the control group. In this case also the survival time in the group treated with CY plus LPS (group 1) was prolonged significantly as compared with that in the CY-injected group (group 2).

In order to eliminate the effect of BCG on antitumor activity of CY and LPS, an experiment was performed in which P815-bearing CDF1 mice were treated with CY and/or LPS without BCG pretreatment. As shown in Fig. 5, the rate of regrowth of tumor of the group treated with CY at 8th day and then with LPS at 12th day (group 2) was similar to that of the group treated with CY 8 days after inoculation of tumor cells (group 4). Moreover there was no difference in mean survival time between groups 2 and 4. The mean survival time of the group

Fig. 5. Antitumor activity on P815

Five groups of 6 male CDF1 mice were inoculated with $1.0 \times 10^6$ cells of P815 (day 0). Group 1 received an i.v. injection of 15 μg of LPS on day 8 and an i.p. injection of 40 mg/kg of CY on day 12. Group 2 was injected i.p. with 40 mg/kg of CY on day 8 and i.v. with 15 μg of LPS on day 12. Groups 3 and 4 received an i.v. injection of 15 μg of LPS and an i.p. injection of 40 mg/kg of CY respectively on day 8. Group 5 served as a control group. Mean tumor diameter of each group was compared.

- - - group 1; ••••, group 2; △△△, group 3; ▲▲▲, group 4; ×××, group 5.

Mean survival days (± s.d.): group 1, 20.1 ± 1.1; group 2, 24.1 ± 0.8; group 3, 15.0 ± 1.7; group 4, 22.0 ± 2.6; group 5, 15.0 ± 1.8.
treated with LPS 8 days after inoculation of tumor cells (group 3) was not prolonged as compared with that of the control group. In mice treated with LPS at 8th day and then with CY at 12th day (group 1), no effect of CY on tumor size was observed. However the mean survival time of this group was prolonged significantly as compared with that of the control group. Unlike the experiment in which BCG had been injected, no hemorrhagic necrosis of tumor was observed after injection of LPS.

**Effect of sera on cultured tumor cells**

Cytotoxicity of sera from BCG-treated CDF1 mice which had been injected with LPS and/or CY on cultured P815 cells was investigated. Neither the serum from LPS-injected mice, nor that from BCG-treated mice was cytotoxic for cultured P815 cells. On the other hand, the serum from mice treated with BCG plus LPS was highly cytotoxic. However there was no significant difference in cytotoxicity between the serum from mice treated with CY in addition to BCG plus LPS and that from mice treated with BCG plus LPS (Table 1).

**Footpad reaction**

To investigate the effect of CY in relation to cell-mediated immunity in tumor-bearing mice, the results of footpad reaction to PPD in BCG-injected CDF1 mice was evaluated. As indicated in Table 2, a significant increase of the thickness of footpad was observed in BCG-injected mice. When BCG-injected CDF1 mice were inoculated with P815 cells, the rate of the increase of the thickness of footpad was significantly reduced. Treatment with CY did not lessen the rate of reduction, suggesting that CY had no effect on cell-mediated immunity in this

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<th>Table 1. Cytotoxicity of sera on cultured tumor cells</th>
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<td>Group</td>
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<tr>
<td>A</td>
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* p < 0.05 against values of Groups A, B and C.

P815 cells (2×10⁵) were suspended in 0.2 ml of RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum.

Sera to be tested (0.01 ml) from CDF1 mice were added. Cell suspension was incubated in 5% CO₂ in air at 37°C in round-bottomed micro wells.

After 48 hr, the number of viable cells was counted with a hemocytometer by the trypan blue dye exclusion method. Each serum was assayed in triplicate cultures.
DISCUSSION

Previously we reported that the antitumor activity of LPS on tumors inoculated subcutaneously in BCG-treated mice was mediated by a humoral factor (Sato et al. 1986). It is well known that activated macrophages release cytotoxic factors by stimulation with LPS. The tumor necrosis factor (TNF) reported by Carswell et al. (1975) is one of these factors. There are number of reports concerning the antitumor effect of TNF. Regenass et al. (1987) observed the synergistic antitumor effects of the partially purified TNF and chemotherapeutic agents. However it should be taken into account that interferon and interleukin 1 participate in the antitumor activity when mice are treated with LPS (Ruff and Gifford 1980; Lovett et al. 1986). Though, it is unclear whether a serum factor is identical with TNF in the present investigation, the sera from mice treated with BCG plus LPS were cytotoxic for cultured P815 cells. No augmentation of cytotoxicity was observed in sera from BCG-treated mice that were then injected with CY and LPS, suggesting that CY seems to exert no effect on cytotoxicity of the serum factor. Without BCG-pretreatment, no significant difference of antitumor effect was observed between the mice treated with CY and the group treated with CY plus LPS. Therefore the mice had to be treated with BCG prior to the injection of LPS for the production of a serum factor which was cytotoxic for tumor cells in vivo as well as in vitro.

The antitumor activity of BCG in experimental animal models has already been established. However the timing of injection of BCG relative to the inoculation of tumor cells is very important as shown by the fact when BCG was injected after inoculation of tumor cells tumor growth was accelerated. In the

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<th>Mice treated with</th>
<th>Increase of thickness of footpad (10^-1 mm) (means±s.d.)</th>
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<tr>
<td>BCG</td>
<td>14.2±1.1</td>
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<tr>
<td>BCG+P815</td>
<td>9.3±1.2</td>
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<tr>
<td>BCG+P815+CY</td>
<td>8.6±1.0</td>
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<tr>
<td>Saline</td>
<td>1.1±0.2</td>
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Male CDF, mice, 8 animals per group, were injected i.v. with 1 mg of BCG (day 0). On day 7 two groups of mice were inoculated subcutaneously with 1.0x10^6 cells of P815. CY (40 mg/kg) was given i.p. 8 days after inoculation of tumor cells (day 15). All groups received an intradecal injection of 5 μg of PPD in the right hind footpad on day 19. The thickness of footpad was measured after 24 hr.
present study mice were treated with BCG before inoculation of tumor cells.

Cyclophosphamide (CY), a nonspecific alkylating agent, is a cytoreductive drug that is commonly used in the control of a variety of tumors and is also known to modulate immune responses (Chassoux et al. 1978; Hengst et al. 1981; Berd et al. 1984). For example suppressor T-cells are known to be specifically sensitive to CY (Rollinghof et al. 1977; Glaser 1979; North 1982). It has been reported that elimination of suppressor T-cells by an injection of CY prior to sensitization leads to an augmentation of delayed-type hypersensitivity (Askenase et al. 1975; Mitsuoka et al. 1976; Thomson et al. 1983; Bovbjerg et al. 1986). In the present study, purified protein derivative (PPD) was injected into the footpad of BCG-treated mice to demonstrate delayed-type hypersensitivity. As a result an increase of the thickness of the footpad was observed. However when P815 cells were inoculated into BCG-pretreated mice, the increase of footpad thickness became less remarkable. An injection of CY did not lessen the rate of reduction. Apparently CY does not modulate the cell-mediated immunity in this experimental condition.

In the present investigation the antitumor effect in mice was more remarkable when injection of CY preceded that of LPS. Immunotherapy has been reported to be more effective when a tumor burden is small (Bast and Bast 1976; Schlick et al. 1986). Reduction of the tumor burden by CY would be favorable for the serum factor to exert antitumor effects.

Hoon and Rhamshaw (1985) reported that the tumor-bearing animals which had been cured with CY were partially immune against a rechallenge of the same tumor cells and that spleen cells were responsible for this immune protection. In the present study all the mice succumbed to tumors. Hence it was impossible to investigate whether immune mechanism contributes to the improvement of the results of treatment with CY. Further studies would be necessary in order to investigate the action mechanism of CY and LPS. It is very important to find out the optimal treatment schedule by which complete regression of tumors occurs and to identify the cytotoxic serum factor from mice treated with BCG plus LPS. Experiments are now being carried out along this line.

References