Effect of *Bacillus Calmette-Guérin* on Immunosuppression from Cyclophosphamide, Methotrexate, and 5-Fluorouracil

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SUMMARY

Studies were undertaken to determine the effect of *Bacillus Calmette-Guérin* (BCG) on the cellular and humoral response in mice receiving cyclophosphamide, methotrexate, and 5-fluorouracil (CMF). C57BL/6 mice were given i.p. injections of sheep red blood cells and were grafted with BALB/c skin on Day 0. Treatment consisted of CMF, BCG, or both CMF and BCG. The number of plaque-forming spleen cells producing antibody against sheep red blood cells was measured on Days 5, 10, and 14. Skin grafts were evaluated frequently until complete rejection had occurred.

The primary humoral response as measured by number of plaque-forming cells per $10^6$ nucleated spleen cells in mice treated with CMF or CMF and BCG was significantly reduced ($p < 0.05$) as compared to controls on Days 5, 10, and 14. The primary humoral response in mice treated with CMF and BCG was significantly reduced ($p < 0.05$) as compared to mice treated with CMF alone on Day 10. The secondary humoral response in mice treated with CMF or CMF and BCG was significantly reduced ($p < 0.05$) as compared to controls on Day 10. Cellular response as measured by skin graft survival was not altered by CMF or by CMF and BCG, but it was increased significantly ($p < 0.01$) by BCG.

INTRODUCTION

CMF alone (5) or with immunotherapy (33) has been shown to delay early recurrence in women with breast cancer with a high risk of recurrence because of metastases to the axillary nodes. Long-term chemotherapy can be immunosuppressive (9, 14, 16, 19, 23). BCG has been reported to antagonize humoral immunosuppression of methylicholanthrene (34) and cellular immunosuppression of cyclophosphamide (17, 18). Chemoimmunotherapy with BCG and chemotherapeutic drug(s) has been reported to increase the response rate or prolong the remission period in patients with breast cancer (12), malignant lymphoma (28), acute myelogenous leukemia (25), acute lymphoblastic leukemia (21), chronic myelocytic leukemia (27), and melanoma (13). The studies reported herein were undertaken to determine the effect of BCG on the cellular and humoral responses of mice receiving CMF.

MATERIALS AND METHODS

Animals. Female C57BL/6 and BALB/c mice were obtained from the Charles River Breeding Laboratories, Wilmington, Mass. The mice were 8 to 12 weeks old when used and were housed in plastic cages and fed Purina laboratory mouse chow with water ad libitum.

Humoral Response to SRBC. A primary response was elicited in 1 group of mice with i.p. injections of 0.2 ml of a 10% solution of washed SRBC on Day 0. A secondary response was elicited in another group of mice by giving them 2 such injections 42 days apart. The day of the 2nd injection was arbitrarily assigned as Day 0.

Drugs. Tice strain BCG (Research Foundation, University of Illinois, Chicago, Ill.) was administered s.c. at a dose of 5 x $10^6$ cells in 0.1 ml nonbacteriostatic water on Days 0, 5, and 10. In our laboratory, this dose of BCG has caused the regression of established tumors (31) and has prevented death from growth of spontaneous metastases (32).

CMF was given i.p. in the following dosage schedule: cyclophosphamide, 20 mg/kg on Days 3 and 10; methotrexate, 0.5 mg/kg on Days 3 through 10; and 5-fluorouracil, 60 mg/kg on Days 3, 6, and 10. In preliminary studies, cyclophosphamide was given on Days 3 and 10 in doses of 200, 100, 50, 40, and 20 mg/kg while 5-fluorouracil and methotrexate were kept constant as described above. These higher dosage schedules of CMF resulted in increased graft survival but also in a mortality rate of greater than 50%. The dose schedule used was the highest that could be administered without resultant mortality.

WBC and Spleen Weight. A 0.1-ml sample of peripheral blood was removed from the retroorbital plexus before sacrifice, and a WBC was performed using a Coulter counter (Coulter Electronics, Hialeah, Fla.). Mice were sacrificed by cervical dislocation, and the spleens were removed and weighed immediately on a Mettler balance that was accurate to 1 mg.

Assay of Humoral Response to SRBC. The Cunningham modification (8) of the Jerne plaque technique was per-
formed on Days 5, 10, and 14. Spleens were minced in Eagle's medium, passed through a No. 80 grid screen, and centrifuged at 1000 rpm at 5° for 5 min, and the supernatant was removed. The RBC were lysed by resuspending the pellet in 10 ml of 0.85% NH₄Cl for 5 min. Thirty ml of medium were added to the suspension, which was then recentrifuged. After removal of the supernatant, the pellet was resuspended in 1 ml of medium, and a 20-µl aliquot of cells was counted with a Coulter counter. The remaining cells were adjusted to a concentration of 1 x 10⁷ cells/ml. One-tenth ml (10⁶ cells) was mixed with 25 µl of a 10% solution of SRBC, 25 µl of a 1:1 dilution of guinea pig complement, and 50 µl of medium. The resulting 0.2 ml was pipetted into a previously prepared 2-chambered slide, which was then incubated at 37° for 1.5 hr. The number of plaques was then counted in each of the chambers and was added to give the PFC. A plaque was considered to be a zone cleared of SRBC around an antibody-producing spleen cell. The data from each treatment group were averaged and compared to control by Student's t test.

**Skin Graft Technique.** Skin allografts were performed on 128 mice on Day 0 according to the principles of Billingham and Medawar (4). A 1-sq cm piece of BALB/c skin from which the panniculus cannius had been dissected was placed on the lateral chest wall of each C57BL/6 mouse from which the recipient's skin had been removed leaving the s.c. tissue intact. The donor skin was positioned and held in place by Vaseline petroleum jelly-impregnated gauze. A 2.5 x 10-cm plaster of Paris strip was placed over the area and around the animal. Casts were split on Day 5 to observe the grafts. Casts were removed on Day 10. Graft rejection was assessed by observing the degree of alopecia, hemorrhage, scaling, and scarring. All grafts were examined, and the percentage of graft surface showing evidence of graft rejection was expressed to the nearest 25%. The median rejection percentage versus time data were then transformed to yield a linear relationship. The probability that the relationship was linear was significant at p > 0.995 (1). The slopes of the regression lines thus obtained were compared to determine what effect, if any, treatment had on the rate of graft rejection (10).

**RESULTS**

The peripheral WBC was not altered by BCG (Table 1). CMF reduced the peripheral WBC significantly (p < 0.01) only on Day 5. CMF and BCG reduced the peripheral WBC significantly (p < 0.01) on Days 5 and 14. The spleen weight was not altered by any of the treatments except BCG. On Day 10, the spleen weight in BCG-treated mice was significantly greater (p < 0.05) than in controls.

**Effect of Treatment Regimens on Skin Graft Survival.** BCG accelerated (p < 0.01) skin graft rejection (Table 2). CMF or CMF and BCG had no significant effect on skin graft rejection.

**Effect of Treatment on the Primary Humoral Response.** On Day 0, the spleens of 5 mice that had not received any SRBC were assayed. PFC in these mice were less than 1. The primary response to SRBC alone was transient and was greatest on Day 10 (Table 3). PFC rose to 136 on Day 5 and 172 on Day 10 and then declined to 19 on Day 14. BCG had no significant effect on PFC. Both CMF and the combination of CMF and BCG reduced PFC significantly (p < 0.05) relative to controls on Days 5, 10, and 14 (Table 3; Chart 1). PFC in mice treated with CMF and BCG was lower than in mice treated with CMF alone throughout the observation period.

### Table 1

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>No. of animals</th>
<th>WBC ± SE</th>
<th>p</th>
<th>No. of animals</th>
<th>Spleen wt (mg) ± SE</th>
<th>p</th>
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<tbody>
<tr>
<td>0</td>
<td>Control</td>
<td>10</td>
<td>7,580 ± 950³</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>11</td>
<td>10,638 ± 1,189</td>
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<td>61.75 ± 17.52³</td>
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<td></td>
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<tr>
<td></td>
<td>BCG</td>
<td>10</td>
<td>12,780 ± 1,404</td>
<td>4</td>
<td>57.25 ± 7.79</td>
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<tr>
<td></td>
<td>CMF</td>
<td>18</td>
<td>6,410 ± 687</td>
<td>&lt;0.01³</td>
<td>6</td>
<td>42.00 ± 3.71</td>
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<td>BCG-CMF</td>
<td>23</td>
<td>5,393 ± 675</td>
<td>&lt;0.001³</td>
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<td>38.00 ± 2.82</td>
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<tr>
<td>10</td>
<td>Control</td>
<td>6</td>
<td>5,950 ± 1,263</td>
<td>4</td>
<td>43.00 ± 10.92</td>
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<td></td>
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<tr>
<td></td>
<td>BCG</td>
<td>3</td>
<td>7,967 ± 921</td>
<td>3</td>
<td>86.00 ± 12.16</td>
<td>&lt;0.05³</td>
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<tr>
<td></td>
<td>CMF</td>
<td>4</td>
<td>4,780 ± 1,670</td>
<td>6</td>
<td>35.20 ± 4.60</td>
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<tr>
<td></td>
<td>BCG-CMF</td>
<td>11</td>
<td>7,891 ± 1,553</td>
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<td>29.60 ± 1.81</td>
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<tr>
<td>14</td>
<td>Control</td>
<td>10</td>
<td>9,426 ± 1,157</td>
<td>13</td>
<td>63.62 ± 5.62</td>
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<tr>
<td></td>
<td>BCG</td>
<td>3</td>
<td>16,300 ± 2,914</td>
<td>3</td>
<td>75.67 ± 17.79</td>
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<td></td>
<td>CMF</td>
<td>14</td>
<td>10,529 ± 1,137</td>
<td>10</td>
<td>78.40 ± 13.09</td>
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<tr>
<td></td>
<td>BCG-CMF</td>
<td>9</td>
<td>4,000 ± 880</td>
<td>&lt;0.01³</td>
<td>8</td>
<td>59.63 ± 10.21</td>
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</tr>
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</table>

* Mean ± S.E.

* Relative to control.

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Table 3

The effect of treatment on PFC: primary response

<table>
<thead>
<tr>
<th>Day of assay</th>
<th>Treatment</th>
<th>No. of animals</th>
<th>PFC</th>
<th>Mean PFC as% of control</th>
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</thead>
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<tr>
<td></td>
<td>Control 4</td>
<td>0.75 ± 0.48</td>
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<td>100</td>
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<td>5</td>
<td>Control 6</td>
<td>136.00 ± 45.15</td>
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<td>100</td>
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<tr>
<td></td>
<td>BCG</td>
<td>112.33 ± 39.59</td>
<td></td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>CMF</td>
<td>4.67 ± 1.85</td>
<td>&lt;0.01</td>
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<tr>
<td></td>
<td>BCG-CMF</td>
<td>1.92 ± 0.58</td>
<td>&lt;0.01</td>
<td>1</td>
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<tr>
<td>10</td>
<td>Control 21</td>
<td>172.50 ± 35.36</td>
<td></td>
<td>100</td>
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<td></td>
<td>BCG</td>
<td>157.70 ± 28.95</td>
<td></td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>CMF</td>
<td>16.08 ± 2.08</td>
<td>&lt;0.01</td>
<td>9</td>
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<tr>
<td></td>
<td>BCG-CMF</td>
<td>7.36 ± 1.99</td>
<td>&lt;0.01</td>
<td>4</td>
</tr>
<tr>
<td>14</td>
<td>Control 34</td>
<td>19.12 ± 2.44</td>
<td></td>
<td>100</td>
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<tr>
<td></td>
<td>BCG</td>
<td>23.85 ± 3.58</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>CMF</td>
<td>12.88 ± 1.80</td>
<td>&lt;0.05</td>
<td>67</td>
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<tr>
<td></td>
<td>BCG-CMF</td>
<td>10.85 ± 1.76</td>
<td>&lt;0.001</td>
<td>56</td>
</tr>
</tbody>
</table>

a Mean ± S.E.

b p < 0.05 relative to BCG.

c Relative to control.

d p < 0.05 relative to CMF.

Effect of Treatment on the Secondary Humoral Response. The secondary response to SRBC alone was also transient (Table 4; Chart 2). PFC rose to 330 on Day 5 and then declined to 20 on Day 10 and to 8 on Day 14. PFC in mice treated with BCG were significantly greater (p < 0.01) than in controls on Day 14. PFC in mice treated with CMF or CMF and BCG were lower than in controls on Days 10 and 14, although the difference was significant (p < 0.05) only on Day 10. There was no significant difference between PFC in mice treated with CMF and in those treated with CMF and BCG.

DISCUSSION

Cellular immunity as measured by skin allograft survival was accelerated by both BCG and the combination of CMF and BCG in these experiments. Balner et al. (2) reported that BCG accelerated rejection of male skin isografts in female C57BL/6 mice. We have confirmed these observations and have shown that the effect is dose dependent (35).

Because cellular immunity as measured by skin allograft survival was not affected by CMF alone, we were unable to demonstrate that BCG could reverse CMF-induced cellular immunosuppression. In our preliminary studies with higher doses of CMF, skin allograft survival was prolonged, but more than one-half of the mice died from CMF toxicity. Our demonstration that the combination of CMF and BCG increased cellular immunity has application in treating cancer patients.

Humoral immunity, as measured by the Cunningham modification of the Jerne plaque technique, was decreased by CMF. Similar results have been reported using 1-β-D-arabinofuranosylcytosine (11, 15) and the combination of methotrexate and 5-fluorouracil (3, 9). BCG has been reported to increase the antibody response to SRBC (20, 22, 34) and to antagonize humoral immunosuppression of methylcholanthrene (34).

Pretreatment with BCG has not reversed cyclophosphamide-induced immunosuppression of the primary humoral response to SRBC (18). It seemed possible that BCG given concomitantly with CMF might ameliorate the CMF-induced immunosuppression of the antibody response.

Instead, we observed that the combination of BCG and CMF increased immunosuppression of the primary humoral response with reduced PFC on Days 5, 10, and 14. BCG is known to nonspecifically stimulate the reticuloendothelial system (24). We postulate that responding cells would have greater metabolic activity and therefore would be more sensitive to chemotherapeutic agents (2, 6, 26). Consequently, those cells that would most readily respond to the SRBC and BCG antigens would be the most susceptible to CMF-mediated cytotoxicity. This would produce a decrease in the number of antibody-producing cells and would account for the increased suppression of humoral immunity that we observed.
we would expect that potentiation of immunosuppression secondary response (7). In these experiments, the addition of BCG to CMF did not increase or reverse CMF-induced any response. BCG is thought to exert its main effect on the host's response against the cancer would thus be a second and chemotherapeutic agents, have been used to treat human after the diagnosis of cancer. At that time, the tumor might also occur against tumor-associated antigens in hu mans. BCG and CMF, as well as other immunopotentiating and chemotherapeutic agents, have been used to treat humans after the diagnosis of cancer. At that time, the tumor would have been present for a considerable time, and the host's response against the cancer would thus be a secondary response. BCG is thought to exert its main effect on the secondary response (7). In these experiments, the addition of BCG to CMF did not increase or reverse CMF-induced immunosuppression of the secondary humoral response.

Further studies are indicated to gain insight into the effects of chemoimmunotherapy on the cellular and humoral response to antigens. Such studies should help to avoid unpleasant side effects of immunotherapy (29, 31) and to design more effective dose schedules of chemoimmunotherapy for patients with cancer.

The importance of these findings is that the addition of immunotherapy to chemotherapy may actually potentiate chemotherapy-induced immunosuppression. Although SRBC and tumor cells have little in common antigenically, we would expect that potentiation of immunosuppression might also occur against tumor-associated antigens in humans. BCG and CMF, as well as other immunopotentiating and chemotherapeutic agents, have been used to treat humans after the diagnosis of cancer. At that time, the tumor would have been present for a considerable time, and the host's response against the cancer would thus be a secondary response. BCG is thought to exert its main effect on the secondary response (7). In these experiments, the addition of BCG to CMF did not increase or reverse CMF-induced immunosuppression of the secondary humoral response.

Further studies are indicated to gain insight into the effects of chemoimmunotherapy on the cellular and humoral response to antigens. Such studies should help to avoid unpleasant side effects of immunotherapy (29, 31) and to design more effective dose schedules of chemoimmunotherapy for patients with cancer.

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