Generation of Anti-MOPC-315 Cytotoxicity in Uneducated or in Vitro Educated Spleen Cells from Normal or MOPC-315 Tumor-bearing Mice Pretreated in Vivo with Bacillus Calmette-Guérin

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ABSTRACT

Cultured spleen cells from normal or MOPC-315 tumor-bearing BALB/c mice that were pretreated in vivo with Bacillus Calmette-Guérin (BCG) exhibited in vitro cytotoxicity against MOPC-315 plasmacytoma. In vitro education of BALB/c spleen cells from normal or tumor-bearing mice by cocultivation with mitomycin C-treated MOPC-315 stimulator cells also resulted in antitumor cytotoxicity. The combination of BCG pretreatment of donor mice with the in vitro education of their spleen cells resulted in a level of anti-MOPC-315 cytotoxicity that was greater than the sum of the levels of cytotoxicity exhibited by spleen cells subjected to either process alone. The levels of cytotoxicity exhibited by educated or uneducated spleen cells from BCG-pretreated mice were dependent on the dose of BCG used and on the time interval between in vivo pretreatment and the initiation of in vitro culture. Thus, our findings suggest that educated spleen cells from tumor-bearing hosts that were pretreated with BCG might be useful in immunotherapeutic regimens requiring histocompatible cells with augmented antitumor cytotoxicity.

INTRODUCTION

The ability of BCG3 to augment antitumor activity has received wide attention in experimental animal models and in clinical trials (1, 2, 5, 19). The mechanism responsible for the heightened antitumor effect induced by BCG has been attributed to either a nonspecific (11, 25) or a "specific" stimulation (12, 13) of the immune system. Most studies with BCG have dealt with its capacity to augment the in vivo generation of antitumor cytotoxicity (15, 16, 21). Since the levels of cell-mediated cytotoxicity produced by in vitro "education" have been shown to be significantly higher than those produced by in vivo sensitization (3, 4, 14), the effects of BCG on the in vitro generation of antitumor cytotoxicity become of interest.

We have previously reported that noncytotoxic spleen cells from MOPC-315 plasmacytoma-bearing mice can become cytotoxic upon cocultivation with mitomycin C-treated MOPC-315 tumor cells (23). Here, we have used BCG pretreatment of donor BALB/c mice in conjunction with the in vitro education of their spleen cells in an attempt to augment further the antitumor cytotoxic activity obtained with "educated" normal or educated MOPC-315 tumor bearer spleen cells.

MATERIALS AND METHODS

Spleen Cell Suspensions. Spleen cell suspensions were prepared from spleens of normal female BALB/c mice (8 to 12 weeks old; Charles River Breeding Laboratory, Wilmington, Mass.), tumor-bearing BALB/c mice, or BALB/c mice pretreated with various doses of BCG bacilli. Single-cell suspensions were prepared by mechanical disruption between sterile glass slides (23). The viability as determined by trypan blue dye (0.4%) exclusion always exceeded 95%.

Tumor Cells Propagated in Vivo. The MOPC-315 plasmacytoma tumor, developed originally by Dr. Michael Potter, was obtained from Dr. Paul Heller, West Side Veterans Administration Hospital, Chicago, Ill., and maintained by serial s.c. inoculation into syngeneic BALB/c mice. Single-cell suspensions prepared as previously described (23) were used as stimulator cells for the in vitro education of spleen cells and as target cells for the 51Cr release assay. The ascites form of leukemia EL4 was obtained through the courtesy of Dr. Frank Fitch, University of Chicago, Chicago, Ill., and was passed weekly in C57BL/6 mice (8 to 12 weeks old; Charles River Breeding Laboratory). These cells were used as target cells in the 51Cr release assay.

Tumor Cells Propagated in Vitro. Tumor cells used as target cells for specificity controls in the 51Cr release assay were derived from continuous culture lines. A syngeneic plasmacytoma of BALB/c origin, S13, an allogeneic plasmacytoma of C3H origin, C1.18.4 (Salk Institute for Biological Studies, San Diego, Calif.), and an allogeneic leukemia, EL4, were maintained in culture by Dr. David Usher of this institution. The tumor cells were grown in Roswell Park Memorial Institute Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 16% fetal calf serum, L-glutamine (300 μg/ml), penicillin (50 units/ml), streptomycin (50 μg/ml), and Fungizone (0.5 μg/ml) (Grand Island Biological). The tumor cells used in the 51Cr release assay were harvested in the logarithmic phase of growth.

BCG. BCG derived from the Trudeau Mycobacterium Culture Collection was obtained from NIH (Bethesda, Md.) and stored at −70° prior to use. Freshly thawed vials of BCG containing 1.5 × 10⁶ bacilli/vial in 1-ml volumes were diluted in Roswell Park Memorial Institute Medium 1640. Various numbers of bacilli ranging from 4 × 10⁴ to 5 × 10⁶ were injected i.p. into normal or MOPC-315-bearing BALB/c mice.

1 Supported in part by NIH Grant USPHS CA-18241.
2 To whom requests for reprints should be addressed, at Department of Microbiology, University of Illinois at the Medical Center, Chicago, 835 South Wolcott Street, P. O. Box 6998, Chicago, Ill. 60680.
3 The abbreviations used are: BCG, Bacillus Calmette-Guérin; MER, methanol extraction residue fraction.

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In Vitro Generation of Cytotoxic Spleen Cells. The in vitro method used to generate cytotoxic spleen cells was described previously (10, 24). Briefly, responder spleen cells (75 x 10⁶) were cultured without stimulator cells or with 2 x 10⁶ mitomycin C-treated MOPC-315 tumor cells (responder:stimulator cell ratio of 37.5:1) for 3 to 7 days at 37° in a humidified atmosphere of 5% CO₂.

In Vitro Cytotoxic Assay. The cell-mediated lysis elicited with "uneducated" or educated spleen cells was determined in the extinction assay (8). Briefly, 5 x 10⁶ tumor cells/ml were labeled with 100 μCi Na¹⁰⁹CrO₄ (Amersham/Searle Corp., Arlington Heights, Ill.) at 37° for 30 min. Mixtures of 5 x 10⁶ (0.4 ml) labeled target cells and various numbers of spleen cells (5 x 10⁶, 1.25 x 10⁷, or 2.5 x 10⁷) (0.4 ml) were incubated in 15- x 75-mm plastic tubes (Falcon Plastics, Oxnard, Calif.) for 3.5 hr at 37°. At the end of the incubation period, cells were pelleted by centrifugation at 250 x g for 10 min, and the supernatants were transferred to identical tubes. Both supernatants (Sup) and pellets (P) were counted in an Auto-Gamma scintillation counter (Packard Instrument Co., Downers Grove, Ill.).

The percentage of specific release of each sample was calculated as follows:

\[
\% \text{ of } ^{109}\text{Cr release} = \frac{cpm \text{ in the Sup}}{cpm \text{ in the Sup} + cpm \text{ in the } P} \times 100
\]

The percentage of specific release was assessed by the following formula:

\[
\% \text{ of specific } ^{109}\text{Cr release} = \frac{T - C}{M - C} \times 100
\]

where T is the percentage of lysis with test spleen cells, C is the percentage of lysis with control spleen cells without stimulator cells (this ranged between 13 and 17%), and M is the percentage of maximal ^{109}Cr release obtained from cells by 3 cycles of freezing and thawing (this ranged between 78 and 81%). The data shown are representative of the data obtained in 3 to 6 individual experiments, and the value for the percentage of ^{109}Cr release is reported in the text as a numerical percentage representing the mean of triplicate samples.

RESULTS

Augmentation of Anti-MOPC-315 Tumor Cytotoxicity in Uneducated or Educated Spleen Cells from BALB/c Mice Pretreated in Vivo with BCG. Normal mice were given injections of 2 x 10⁷ BCG i.p. Spleen cells were prepared 7 days following BCG injection and cocultivated in vitro with mitomycin C-treated MOPC-315 cells at a spleen:tumor cell ratio of 37.5:1. The cells were harvested 3 to 7 days later and tested for in vitro anti-MOPC-315 cytotoxic activity in the extinction assay at an effector:target cell ratio of 100:1 (Table 1). No significant cytotoxicity was observed with normal spleen cells cultured without stimulator cells for 3 to 7 days; cytotoxicity was <5%, compared to that with tumor cells alone (data not shown). Cytotoxicity was observed, however, with normal spleen cells cocultivated with tumor cells for 4 to 7 days with maximal cytotoxicity exhibited on Days 5 and 6 of the education (67 and 64%, respectively). In addition, substantial cytotoxicity against MOPC-315 was also observed when spleen cells from BCG-pretreated mice were cultured in the absence of stimulator cells for 5, 6, or 7 days (39, 60, or 49%, respectively). Educated spleen cells from BCG-pretreated mice exhibited higher levels of cytotoxicity on Day 4, 5, 6, or 7 than were exhibited by educated spleen cells from normal mice or uneducated spleen cells from BCG-pretreated mice. The maximum cytotoxicity (100%) was obtained on the fifth day of the culture, and high levels of cytotoxicity (85%) were maintained through the seventh day of the culture. Thus, BCG treatment in conjunction with the in vitro education method is a means for generating in vitro antitumor cytotoxicity in normal BALB/c spleen cells than superior to either BCG pretreatment or in vitro education alone.

The Effect of Pretreating BALB/c Mice with Different Doses of BCG on the Generation of in Vitro Anti-MOPC-315 Tumor Cytotoxicity in Uneducated or Educated Spleen Cells. Spleen cells from mice given injections 7 days previously with various doses of BCG were cultivated for 5 and 6 days in vitro in the presence or absence of MOPC-315 stimulator cells and tested subsequently for cytotoxicity against MOPC-315 target cells (Table 2). On the fifth day of culture at an effector:target cell ratio of 100:1, a high level of cytotoxicity was obtained with uneducated spleen cells from mice that had received 5 x 10⁷ BCG (71%). Decreasing the BCG pretreatment dosage led to decreasing levels of anti-MOPC-315 cytotoxicity in uneducated spleen cells so that pretreatment with 4 x 10⁶ BCG did not lead to antitumor cytotoxicity (~3%). The correlation between the dose of BCG used for pretreatment and the resultant in vitro antitumor cytotoxicity was also evident at lower effector:target cell ratios (25:1 and 5:1) as well as on the sixth day of the culture. The levels of antitumor cytotoxicity exhibited by educated spleen cells from BCG-pretreated mice were also BCG dose dependent on Days 5 and 6 of the culture and at all effector:target cell ratios tested, so that

<table>
<thead>
<tr>
<th>Source of responder spleen cells</th>
<th>Stimulator tumor cells</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>MOPC-315</td>
<td>-0.2 ± 0.5</td>
<td>28.1 ± 1.0</td>
<td>67.4 ± 1.2</td>
<td>64.1 ± 3.9</td>
<td>27.2 ± 0.5</td>
</tr>
<tr>
<td>2 x 10⁷ BCG</td>
<td>None</td>
<td>0.6 ± 0.6</td>
<td>1.5 ± 0.2</td>
<td>36.7 ± 0.8</td>
<td>60.4 ± 0.7</td>
<td>48.9 ± 3.9</td>
</tr>
<tr>
<td>2 x 10⁷ BCG</td>
<td>MOPC-315</td>
<td>3.0 ± 0.5</td>
<td>49.7 ± 1.0</td>
<td>100 ± 2.3</td>
<td>85.3 ± 1.1</td>
<td>84.8 ± 2.1</td>
</tr>
</tbody>
</table>

a Extinction assay was performed at an effector:target cell ratio of 100:1.  
b Mean ± S.E.  
c Injected i.p. into BALB/c mice 7 days prior to the in vitro culture of their spleen cells.

Table 1

Effect of in vivo pretreatment of BALB/c mice with BCG on the generation of in vitro anti-MOPC-315 tumor cytotoxicity in uneducated or MOPC-315-educated spleen cells cultured for 3 to 7 days

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increasing the BCG pretreatment dosage led to increasing levels of cytotoxicity.

The augmented levels of cytotoxicity obtained with educated spleen cells from BCG-pretreated mice, compared to either uneducated spleen cells from BCG-pretreated mice or educated spleen cells from normal mice, were evaluated further (Table 2). Pretreatment of mice with 1 x 10^7 or 5 x 10^7 BCG led to a substantial augmentation of in vitro cytotoxicity. A smaller pretreatment dose of BCG, 2 x 10^6 bacilli, was able to augment the in vitro cytotoxic activity of educated spleen cells only on the sixth day of culture. On the other hand a BCG pretreatment dose of 4 x 10^7 bacilli depressed the cytotoxic activity of educated spleen cells relative to the activity of educated spleen cells from untreated mice.

On the fifth day of culture, similar levels of cytotoxicity were obtained with uneducated spleen cells from mice pretreated with 5 x 10^7 BCG and with educated spleen cells from normal, untreated mice at all effector:target cell ratios tested, i.e., 71 versus 71%, 63 versus 56%, and 29 versus 21%. Combining a BCG pretreatment dose of 1 x 10^7 or 5 x 10^7 bacilli with in vitro education led to higher levels of cytotoxicity than either process alone. Augmentation in cytotoxicity was evident at all effector to target cell ratios tested, both on the fifth and sixth days of culture. This augmentation was most evident at an effector:target cell ratio of 5:1 and led to cytotoxic activity (63 and 76% with 1 x 10^7 and 5 x 10^7 BCG, respectively) in the range of cytotoxic activity exhibited by educated spleen cells from untreated mice tested at an effector:target cell ratio of 100:1 (71%) (Table 2, Day 5). Furthermore, at an effector:target cell ratio of 5:1, the level of cytotoxic activity exhibited by educated spleen cells from BCG-pretreated mice was greater than the sum of the levels of cytotoxicity exhibited by spleen cells subjected to either process alone, e.g., at a dose of 5 x 10^7 BCG, 76 versus 21 plus 29%, and at a dose of 1 x 10^7 BCG, 63 versus 21 plus 11%.

Cytotoxicity of Uneducated or MOPC-315-educated Spleen Cells from BCG-pretreated Mice against Syngeneic or Allogeneic Tumors. Uneducated or educated spleen cells from BCG-pretreated mice were tested for in vitro antitumor cytotoxicity against MOPC-315, against another BALB/c plasmacytoma, S13, and against an allogeneic plasmacytoma of C3H origin, C1.18.4 (Table 3). Uneducated spleen cells from mice pretreated with 1 x 10^7 or 5 x 10^7 BCG exhibited cytotoxicity against all of the plasmacytomas tested. Pretreating donor mice with 5 x 10^7 BCG led to the highest levels of cytotoxicity against MOPC-315 target cells (51%), with appreciable cytotoxicity against S13 (36%) and C1.18.4 (34%). The similar levels of cytotoxicity seen against the syngeneic plasmacytoma, S13, and the allogeneic plasmacytoma, C1.18.4, suggest that BCG pretreatment did not elicit sensitization against BALB/c histocompatibility antigens. Combined BCG pretreatment with in vitro education with MOPC-315 stimulator cells led to an augmentation of cytotoxicity against all of the plasmacytomas tested.

Since cytotoxicity against syngeneic and allogeneic plasmacytommas was elicited by BCG pretreatment of donor mice, experiments were performed to determine whether some of the observed cytotoxicity was directed against a nonplasmacytoma tumor. Uneducated or MOPC-315-educated spleen cells from mice pretreated with 1 x 10^7 BCG were tested against in vitro- or in vivo-propagated EL4 leukemia cells of C57BL/6 origin. Uneducated spleen cells exhibited poor cytotoxicity (3%) against in vitro-propagated EL4 cells, whereas they exhibited some cytotoxicity against in vivo-propagated EL4 cells (7%) (Table 4). Educated spleen cells from BCG-pretreated mice exhibited heightened anti-EL4 cytotoxicity against in vivo-propagated target cells (30%), with less cytotoxicity against in vitro-propagated target cells (5%) (Table 4). In all experiments the level of antitumor cytotoxicity achieved with uneducated or educated spleen cells from BCG-pretreated mice was greater against plasmacytoma target cells than against EL4 cells. These data suggest that the augmented capacity of uneducated or educated spleen cells from BCG-pretreated mice to mediate in vitro antiplasma cytotoxicity is due to an increase in both specific and nonspecific components of cytotoxicity.

### Table 2

**Effect of pretreating BALB/c mice with different doses of BCG on the generation of in vitro anti-MOPC-315 tumor cytotoxicity in uneducated or MOPC-315-educated spleen cells**

<table>
<thead>
<tr>
<th>Responder spleen cells from mice pretreated with BCG</th>
<th>Stimulator tumor cells</th>
<th>% of specific ^51Cr release</th>
<th>Day 5</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>MOPC-315</td>
<td>70.6 ± 1.8</td>
<td>56.4 ± 1.0</td>
<td>21.4 ± 0.5</td>
</tr>
<tr>
<td>4 x 10^6</td>
<td>None</td>
<td>-3.3 ± 0.7</td>
<td>-2.5 ± 0.4</td>
<td>0.7 ± 0.8</td>
</tr>
<tr>
<td>2 x 10^6</td>
<td>None</td>
<td>12.8 ± 1.3</td>
<td>9.3 ± 0.7</td>
<td>2.6 ± 0.8</td>
</tr>
<tr>
<td>1 x 10^7</td>
<td>None</td>
<td>40.0 ± 1.0</td>
<td>25.5 ± 0.8</td>
<td>10.6 ± 0.3</td>
</tr>
<tr>
<td>5 x 10^7</td>
<td>None</td>
<td>70.7 ± 1.9</td>
<td>63.4 ± 0.6</td>
<td>29.2 ± 0.7</td>
</tr>
<tr>
<td>4 x 10^6</td>
<td>MOPC-315</td>
<td>31.7 ± 1.3</td>
<td>18.1 ± 1.7</td>
<td>6.1 ± 0.8</td>
</tr>
<tr>
<td>2 x 10^6</td>
<td>MOPC-315</td>
<td>77.4 ± 1.2</td>
<td>60.6 ± 1.1</td>
<td>23.1 ± 1.0</td>
</tr>
<tr>
<td>1 x 10^7</td>
<td>MOPC-315</td>
<td>90.9 ± 1.0</td>
<td>89.8 ± 4.4</td>
<td>62.8 ± 1.4</td>
</tr>
<tr>
<td>5 x 10^7</td>
<td>MOPC-315</td>
<td>94.4 ± 0.6</td>
<td>96.9 ± 2.0</td>
<td>75.9 ± 2.5</td>
</tr>
</tbody>
</table>

* a Injected i.p. into BALB/c mice 7 days prior to the in vitro culture of their spleen cells.
* b Effector:target cell ratio.
* c Mean ± S.E.
* d ND, not done.
The Effect of Pretreating BALB/c Mice with BCG at Various Time Intervals Prior to the Education of Their Spleen Cells on the Generation of in Vitro Anti-MOPC-315 Tumor Cytotoxicity. Experiments were performed to determine the minimum time interval between the in vivo administration of BCG to mice and the in vitro education of their spleen cells, which still leads to augmented antitumor cytotoxicity. BALB/c mice were pretreated with 1 × 10⁷ BCG 11, 7, 5, or 2 days prior to the education of their spleen cells (Tables 5 and 6). The cytotoxic activity of spleen cells from mice pretreated with BCG 11 or 7 days prior to education of their spleen cells was augmented, compared to the cytotoxicity exhibited by educated spleen cells from normal, untreated mice. Augmentation was evident on Days 5 and 7 of the in vitro culture and at effector:target cell ratios of 100:1 and 25:1. The levels of cytotoxicity exhibited by educated spleen cells from mice pretreated with BCG 11 or 7 days prior to education were only slightly higher than those exhibited by spleen cells from mice pretreated with BCG 2 days prior to the education. The results of these experiments demonstrate that the heightened levels of cytotoxicity obtained with educated spleen cells from BCG-pretreated mice are dependent on the interval between BCG pretreatment and the initiation of the culture (Table 6).

Augmentation of Anti-MOPC-315 Cytotoxicity in Educated or Uneducated Spleen Cells from MOPC-315 Tumor-bearing Mice Pretreated In Vivo with BCG. BALB/c mice were inoculated with 1 × 10⁶ viable MOPC-315 tumor cells s.c. (a dose that leads to progressively growing tumors that kill the mice in 17 ± 2 days); 3 days later, one-half of the mice with BCG 5 days prior to the education of their spleen cells led to augmented cytotoxicity (compared to the cytotoxicity exhibited by educated spleen cells from untreated mice) when the education was performed at a responder:stimulator cell ratio of 100:1, 33:1, and 10:1. In contrast, pretreatment of mice with BCG 2 days prior to the education of their spleen cells led to augmented cytotoxic activity only when the education was performed at a responder:stimulator cell ratio of 100:1. Even when the spleen cell education was performed at a responder:stimulator cell ratio of 100:1, the level of cytotoxicity exhibited by educated spleen cells from mice pretreated with BCG 5 days prior to the education was higher than the level of cytotoxicity exhibited by educated spleen cells from mice pretreated with BCG 2 days prior to the education. The results of these experiments demonstrate that the heightened levels of cytotoxicity obtained with educated spleen cells from BCG-pretreated mice are dependent on the interval between BCG pretreatment and the initiation of the culture (Table 6).

### Table 3

<table>
<thead>
<tr>
<th>Responder spleen cells from mice pretreated with BCG (× 10⁷)</th>
<th>Stimulator tumor cells</th>
<th>% of specific ¹⁵⁷Cr release</th>
<th>Days following in vitro-propagation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOPC-315</td>
<td>28.4 ± 1.4^c</td>
<td>13.3 ± 1.0</td>
<td>Day 5: 67.2 ± 1.9</td>
</tr>
<tr>
<td>MOPC-315</td>
<td>51.2 ± 2.7</td>
<td>36.1 ± 2.8</td>
<td>Day 7: 71.0 ± 3.1</td>
</tr>
<tr>
<td>MOPC-315</td>
<td>77.1 ± 2.1</td>
<td>29.8 ± 0.9</td>
<td>100:1: 29.1 ± 0.8</td>
</tr>
<tr>
<td>MOPC-315</td>
<td>89.0 ± 2.1</td>
<td>51.2 ± 3.1</td>
<td>25:1: 29.1 ± 0.8</td>
</tr>
</tbody>
</table>

a Injected i.p. into BALB/c mice 7 days prior to the in vitro culture of their spleen cells.
b ¹⁵⁷Cr release assay was performed at an effector:target cell ratio of 25:1.
c Mean ± S.E.

d ¹⁵⁷Cr release assay was performed at an effector:target cell ratio of 100:1, the level of cytotoxicity exhibited by educated spleen cells from mice pretreated with BCG 5 days prior to the education was higher than the level of cytotoxicity exhibited by educated spleen cells from mice pretreated with BCG 2 days prior to the education. The results of these experiments demonstrate that the heightened levels of cytotoxicity obtained with educated spleen cells from BCG-pretreated mice are dependent on the interval between BCG pretreatment and the initiation of the culture (Table 6).

### Table 5

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</tr>
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<td>MOPC-315</td>
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<td>25:1: 29.1 ± 0.8</td>
</tr>
</tbody>
</table>

a Injected i.p. into BALB/c mice 7 days prior to the in vitro culture of their spleen cells.
b ¹⁵⁷Cr release assay was performed at an effector:target cell ratio of 25:1.
c Mean ± S.E.

d ¹⁵⁷Cr release assay was performed at an effector:target cell ratio of 100:1, the level of cytotoxicity exhibited by educated spleen cells from mice pretreated with BCG 5 days prior to the education was higher than the level of cytotoxicity exhibited by educated spleen cells from mice pretreated with BCG 2 days prior to the education. The results of these experiments demonstrate that the heightened levels of cytotoxicity obtained with educated spleen cells from BCG-pretreated mice are dependent on the interval between BCG pretreatment and the initiation of the culture (Table 6).

### Table 4

<table>
<thead>
<tr>
<th>Responder spleen cells from mice pretreated with BCG (× 10⁷)</th>
<th>Stimulator tumor cells</th>
<th>% of specific ¹⁵⁷Cr release</th>
<th>Days following in vitro-propagation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOPC-315</td>
<td>33.9 ± 2.6^c</td>
<td>14.7 ± 1.2</td>
<td>Day 5: 67.2 ± 1.9</td>
</tr>
<tr>
<td>MOPC-315</td>
<td>73.8 ± 3.1</td>
<td>45.1 ± 1.2</td>
<td>Day 7: 71.0 ± 3.1</td>
</tr>
</tbody>
</table>

a Injected i.p. into BALB/c mice 7 days prior to the in vitro culture of their spleen cells.
b ¹⁵⁷Cr release assay was performed at an effector:target cell ratio of 25:1.
c Mean ± S.E.
tumor-bearing animals received $1 \times 10^7$ BCG i.p. Fourteen days following tumor inoculation, BCG-pretreated or unpretreated mice were sacrificed, and their spleen cells were cultured in vitro in the presence or absence of MOPC-315 stimulator cells. The spleen cells were harvested on the fifth or sixth day of the culture and tested for in vitro anti-MOPC-315 cytotoxicity (Table 7). Educated spleen cells from tumor-bearing mice exhibited anti-MOPC-315 cytotoxicity on the fifth day of the culture (21%), which decreased by the sixth day of the culture (13%). Similar levels of cytotoxicity were seen on the fifth day of the culture with uneducated spleen cells from BCG-pretreated, tumor-bearing mice (21%), which was maintained through the sixth day of the culture (21%). Combined in vivo BCG pretreatment of tumor-bearing mice with the in vitro education of their spleen cells led to a substantial augmentation of anti-MOPC-315 cytotoxicity on the fifth day of the culture (61%), which persisted through the sixth day of the culture (65%) and was also evident at an effector to target cell ratio of 25:1 (38%). The level of cytotoxicity obtained with educated spleen cells from BCG-pretreated, tumor-bearing mice was greater than the sum of the levels of cytotoxicity obtained with tumor bearer spleen cells subjected to either process alone.

**DISCUSSION**

The present study demonstrates that in vivo pretreatment of normal or MOPC-315 tumor-bearing BALB/c mice with BCG prior to the culture of their spleen cells results in the generation of in vitro anti-MOPC-315 cytotoxicity. Cultured spleen cells from BCG-pretreated mice exhibited some cytotoxicity against a nonplasmacytomatous, allogeneic tumor (EL4) and stronger cytotoxicity against syngeneic and allogeneic plasmacytomas. When spleen cells from BCG-pretreated mice were educated in vitro with MOPC-315 stimulator cells, they exhibited higher levels of anti-MOPC-315 cytotoxicity than the sum of the levels of cytotoxicity exhibited by spleen cells subjected to either process alone. The levels of anti-MOPC-315 cytotoxicity obtained with uneducated or educated spleen cells from BCG-pretreated mice were dependent on the BCG dose used for the pretreatment and on the interval between the BCG inoculation and the initiation of the in vitro culture.

Antitumor immunity elicited by BCG has been attributed to either a nonspecific stimulation (17, 25) or a specific stimulation of the immune system (12, 13). Nonspecific antitumor immunity has been widely studied and has been ascribed to the actions of macrophages (11, 17), lymphocytes (28), or nonadherent, nonphagocytic, natural killer cells (27). Specific antitumor immunity has only recently been reported and has been ascribed to shared antigenic determinants between some tumors and mycobacteria (12, 13). Cross-reactivity between BCG and a guinea pig hepato- toma, line 10, was indicated by the observation that antibodies elicited with either BCG or line 10 cells bound both the bacilli and the tumor cells (6, 9, 20). Shared antigenicity between melanoma cells and BCG has also been implicated. In a human melanoma system, Bucana and Hanna (9) used an immunoelectron-microscopy technique to demonstrate the binding of anti-BCG antibodies to the surface of melanoma cells. In a murine system Feraci et al. (12, 13) reported that spleen cells from BCG hyperimmune BALB/c mice exhibited some in vitro cytotoxicity against the S91 melanoma (an allogeneic tumor of CBA origin, which grows progressively in BALB/c mice) but that they exhibited no cytotoxicity against a syngeneic mammary carcinoma or a syngeneic methylcholanthrene-induced sarcoma. Our data, demonstrating that uneducated spleen cells from BCG-pretreated mice exhibit cytotoxicity against syngeneic and allogeneic plasmacytomas as well as against an allogeneic EL4 leukemia, suggest that BCG stimulates nonspecific components of the immune system. The levels of cytotoxicity, however, were higher against all 3 plasmacytomas tested than against EL4, suggesting the existence of shared antigenicity between BCG and plasmacytoma tumors, although other explanations relating to the biological differences between plasmacytomas and EL4 cannot be ruled out. Nevertheless, testing of human tumors for antigenic cross-reactivity with microorganisms might lead to a more rational selection of microbial immunostimulants (such as

### Table 6

Effect of pretreating BALB/c mice with $1 \times 10^7$ BCG 5 or 2 days prior to the education of their spleen cells with various numbers of MOPC-315 tumor cells on the generation of anti-MOPC-315 cytotoxicity

<table>
<thead>
<tr>
<th>Responder spleen cells from mice pretreated with BCG on the following days</th>
<th>Stimulator tumor cells</th>
<th>Responder: stimulator ratio</th>
<th>% of specific $^{a}$Cr release$^{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>MOPC-315</td>
<td>10:1</td>
<td>$55.3 \pm 0.5^{c}$</td>
</tr>
<tr>
<td>None</td>
<td>MOPC-315</td>
<td>33:1</td>
<td>$55.0 \pm 3.2$</td>
</tr>
<tr>
<td>None</td>
<td>MOPC-315</td>
<td>100:1</td>
<td>$27.4 \pm 1.7$</td>
</tr>
<tr>
<td>-2</td>
<td>None</td>
<td>10:1</td>
<td>$-0.6 \pm 1.6$</td>
</tr>
<tr>
<td>-2</td>
<td>MOPC-315</td>
<td>33:1</td>
<td>$56.9 \pm 2.8$</td>
</tr>
<tr>
<td>-2</td>
<td>MOPC-315</td>
<td>100:1</td>
<td>$55.0 \pm 4.9$</td>
</tr>
<tr>
<td>-5</td>
<td>None</td>
<td>100:1</td>
<td>$41.2 \pm 2.3$</td>
</tr>
<tr>
<td>-5</td>
<td>MOPC-315</td>
<td>100:1</td>
<td>$11.3 \pm 3.8$</td>
</tr>
<tr>
<td>-5</td>
<td>MOPC-315</td>
<td>33:1</td>
<td>$79.0 \pm 3.3$</td>
</tr>
<tr>
<td>-5</td>
<td>MOPC-315</td>
<td>100:1</td>
<td>$78.3 \pm 1.5$</td>
</tr>
<tr>
<td>-5</td>
<td>MOPC-315</td>
<td>1000:1</td>
<td>$66.9 \pm 1.3$</td>
</tr>
</tbody>
</table>

$^{a}$ Mice were pretreated with $1 \times 10^7$ BCG 5 or 2 days prior to the initiation of the in vitro culture of their spleen cells.  
$^{b}$ $^{a}$Cr release assay was performed on the fifth day of the culture at an effector:target cell ratio of 25:1.  
$^{c}$ Mean ± S.E.

### Table 7

Generation of in vitro cytotoxicity in uneducated or MOPC-315-educated spleen cells from MOPC-315 tumor-bearing mice pretreated in vivo with BCG

<table>
<thead>
<tr>
<th>Responder spleen cells from mice pretreated with BCG</th>
<th>Stimulator tumor cells</th>
<th>% of specific $^{a}$Cr release</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>MOPC-315</td>
<td>20.8 ± 0.4$^{c}$</td>
</tr>
<tr>
<td>$1 \times 10^7$</td>
<td>MOPC-315</td>
<td>21.1 ± 2.2</td>
</tr>
<tr>
<td>$1 \times 10^8$</td>
<td>MOPC-315</td>
<td>61.2 ± 1.1</td>
</tr>
</tbody>
</table>

$^{a}$ BCG was injected i.p. into Day 3 MOPC-315 tumor-bearing mice 11 days prior to the in vitro culture of their cells.  
$^{b}$ Effector:target cell ratio.  
$^{c}$ Mean ± S.E.
Augmenting Antitumor Activity of Autochthonous Cells

BCG, Corynebacterium parvum, Bordetella pertussis, Listeria monocytogenes, etc.) in immunotherapy.

BCG has received wide attention as an immunostimulator of antitumor immunity. In most cases, however, the effect of BCG has been evaluated on the in vivo generation of antitumor immunity (15, 16, 21). Since it was shown that the levels of cell-mediated cytotoxicity produced by in vitro education are significantly higher than those produced by in vivo sensitization (3, 4, 14), it is important to evaluate the effects of BCG on the in vitro generation of cytotoxic cells. Weiss et al. (26) have shown in preliminary experiments that pretreatment of BALB/c mice with the MER of tubercle bacilli prior to the in vitro education of their spleen cells with an allogeneic tumor, EL4, led to greater anti-EL4 cytotoxicity than was exhibited by educated spleen cells from normal mice. This augmenting effect was restricted to a narrow range of MER doses and to a pretreatment interval so that pretreatment of donor mice with 100 but not 500 μg MER 2 but not 8 or 15 days prior to in vitro education led to augmented anti-EL4 cytotoxicity. In our study, pretreatment of BALB/c mice with BCG prior to the in vitro education of their spleen cells with a syngeneic plasmacytoma tumor, MOPC-315, led to greater anti-MOPC-315 cytotoxicity than was exhibited by educated spleen cells from normal mice. This augmentation was evident with BCG pretreatment doses ranging from 2 x 10^6 to 5 x 10^7 bacilli and when mice were pretreated with BCG 2 to 11 days prior to the education of their spleen cells. Moreover, pretreatment 2 days prior to in vitro education was less effective in augmenting antitumor cytotoxicity than was pretreatment 5, 7, or 11 days prior to in vitro education.

The levels of cytotoxicity obtained with educated spleen cells from BCG-pretreated mice were dependent on the dose of BCG used for the pretreatment. BCG doses of 1 x 10^7 or 5 x 10^7 bacilli led to augmented cytotoxicity in educated spleen cells on the fifth and sixth days of the culture, compared to the cytotoxicity obtained with educated spleen cells from normal, untreated mice. These levels of cytotoxicity were higher than the levels of cytotoxicity obtained with spleen cells subjected to either process alone. Moreover, these levels of cytotoxicity obtained with the combined processes at an effectortarget cell ratio of 5:1 were similar to the level of cytotoxicity obtained with educated spleen cells from normal mice at an effectortarget cell ratio of 100:1. Educated spleen cells from mice pretreated with the smallest amount of BCG used in this study (4 x 10^6 bacilli) exhibited a lower level of cytotoxicity than that of educated spleen cells from untreated mice. Since pretreatment with an intermediate dose of BCG (2 x 10^6 bacilli) was able to augment the cytotoxicity of educated spleen cells only on the sixth day but not on the fifth day of the culture, lower pretreatment doses of BCG may require longer incubation periods to exhibit augmentation.

BCG has been reported to exert immunostimulatory effects on lymphoid cells in vitro (22, 24). Thus, the addition of BCG to education mixtures might lead to augmented cytotoxicity of educated lymphoid cells obtained from untreated or BCG-pretreated mice. In preliminary experiments Weiss et al. (26) reported that the addition of 0.8 to 66 μg MER to a syngeneic in vitro education mixture of C57BL/6 spleen cells and EL4 tumor cells augmented cytotoxicity of the educated spleen cells, whereas the addition of 200 μg MER inhibited their cytotoxicity. In a preliminary experiment conducted in our laboratory, we added 4 x 10^5 to 5 x 10^7 BCG to the education mixture but did not observe augmented cytotoxicity; however, the effect of adding lower numbers of BCG to the education mixture should be evaluated.

BCG has been shown to activate both macrophages and thymus-derived lymphocytes (T-cells) in vitro (22, 24) and in vivo (18, 21). However, the activation of T-cells was obtained only in the presence of macrophages (24). In addition, macrophages produced higher levels of lymphocyte-activating factor in the presence of BCG-stimulated T-cells, which in turn led to further activation of T-cells. Mokyr and Mitchell (24) have suggested that the consequence of these events is the development of highly stimulated populations of lymphocytes and macrophages in which response to new antigenic stimuli results in earlier development and higher levels of immunity than is seen with normal cells exposed to the same antigen. Our data are compatible with this mechanism since we have found that the cytotoxicity exhibited by educated spleen cells from BCG-pretreated mice developed earlier, reached higher levels, and persisted longer than did the cytotoxicity exhibited by educated spleen cells from untreated, normal mice.

The therapeutic efficacy of in vitro educated lymphoid cells from BCG-pretreated hosts is particularly feasible when histocompatibility between donor and recipient is assured. Such cells can be provided by autochthonous lymphoid cells from tumor-bearing hosts. Previous work from this laboratory demonstrated that unresponsive lymphoid cells from MOPC-315 tumor-bearing mice can be converted to a state of immunological responsiveness by immune RNA (7). The in vitro education method was also used in our laboratory, as another means to evoke antitumor activity in noncytotoxic spleen cells from mice bearing plasmacytoma tumors (23). In the present study the in vitro cytotoxic activity of educated spleen cells from MOPC-315 tumor-bearing mice was further augmented when tumor-bearing donors were pretreated with BCG prior to the education of their spleen cells. This approach might offer some therapeutic advantages since parenterally administered BCG can exert antitumor effects in tumor-bearing hosts and at the same time lead to augmented cytotoxic activity upon in vitro education of their lymphoid cells. Since we have previously demonstrated that educated spleen cells from tumor-bearing mice exhibited greater in vivo antitumor cytotoxicity than did educated cells from syngeneic normal donors (23), the in vivo antitumor activity of educated spleen cells from BCG-pretreated, MOPC-315 tumor-bearing donors is presently being evaluated.

REFERENCES


Generation of Anti-MOPC-315 Cytotoxicity in Uneducated or *in Vitro* Educated Spleen Cells from Normal or MOPC-315 Tumor-bearing Mice Pretreated *in Vivo* with *Bacillus Calmette-Guérin*

Donald P. Braun, Margalit B. Mokyr and Sheldon Dray


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