Ability of Cyclophosphamide in the Absence of Cross-Linking Activity to Exert the Immunomodulatory Effect Required for the Cure of Mice Bearing a Large MOPC-315 Tumor

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ABSTRACT

We have previously shown that mice bearing a late-stage, large primary MOPC-315 plasmacytoma and extensive metastases can be cured by a low dose of the bifunctional alkylating drug, cyclophosphamide (BiCY) [J. C. D. Hengst et al., Cancer Res., 40: 2135-2141, 1980]. Here we show that therapy with the monofunctional form of cyclophosphamide (MoCY) can also cure such mice. However, a dose of at least 150 mg of MoCY per kg is required to approximate the curative effectiveness of the lowest curative dose of BiCY, i.e., 15 mg/kg. This need for a 10-fold higher dose of MoCY is due, at least in part, to the 10-fold lower direct tumoricidal and/or tumoristatic activity of MoCY compared to BiCY. Consequently, a 10-fold higher dose of MoCY is required to directly reduce the tumor burden to the level reduced by 15 mg of BiCY per kg. Other than dose, the therapy of the mice with 150 mg of MoCY per kg was similar in its essential features to that shown previously for therapy with 15 mg of BiCY per kg [J. C. D. Hengst et al., Cancer Res., 40: 2135-2141, 1980; J. C. D. Hengst et al., Cancer Res., 41: 2163-2167, 1981; Q-W. Ye et al., Cancer Immunol. Immunother., 16: 162-169, 1984; Q-W. Ye and M. B. Mokyr, Cancer Res., 44: 3873-3879, 1984; M. B. Mokyr and S. Dray, Cancer Res., 43: 3112-3119, 1983], namely: (a) the drug does not directly eradicate all tumor cells; (b) host T-cell-dependent antitumor immunity is also required for the curative effect; (c) the therapy of tumor bearers leads to the rapid appearance of an augmented antitumor immune potential in their hitherto immunosuppressed spleen; and (d) the cured mice are resistant to a subsequent challenge with at least 300-fold the minimal lethal tumor dose. Thus, cross-linking is not an essential property for the immunomodulatory activity of BiCY nor for its direct antitumor effect. However, in the presence of cross-linking activity, a much lower dose of drug is effective.

INTRODUCTION

The immunosuppressive potential of many cancer chemotherapeutic drugs has been known for some time (1). However, it is now recognized that under appropriate conditions, at least some of these anticancer drugs can potentiate the immune response (2, 3). For example, a potent immunosuppressive therapeutic drug, BiCY, enhances antibody production (4, 5) and cell-mediated immunity (6-9) if the drug is administered prior to immunization rather than shortly after immunization. The immunopotentiating activity of BiCY pretreatment was attributed to the selective toxicity of the drug for precursors of suppressor T-cells (6-9).

We have recently shown that BiCY can also enhance the antitumor immune responsiveness of tumor-bearing mice (10-13). In those studies, administration of a low dose of BiCY, which represents less than one-tenth the maximal tolerable dose of drug, was shown to reverse the immunosuppression that operates during late stages of MOPC-315 tumor growth, thereby resulting in the appearance of potent antitumor immunity. The resultant potent antitumor immunity eradicated the large primary and metastatic tumor load that remained after clearance of CY and its active metabolites from the circulation, and consequently brought about the cure of the tumor bearers. In the absence of potent host antitumor immunity, a low dose of BiCY was not curative for mice even at an early stage of tumor growth when they had a nonpalpable tumor (14). Mice cured of a large tumor burden by a low dose of BiCY in cooperation with host antitumor immunity were shown to exhibit a strong, long-lasting antitumor immunity as evident by their ability to reject a subsequent tumor challenge of at least 300 times the minimal lethal tumor dose (11, 15).

The studies reported herein were aimed at determining whether the cross-linking activity of the alkylating agent BiCY is essential for the curative effectiveness of the drug for mice bearing a large s.c. MOPC-315 tumor and extensive metastases. Particular emphasis was placed on determining whether the cross-linking capability of BiCY is required for the immunomodulatory activity of the drug and/or for its direct tumoricidal/tumoristatic effect.

MATERIALS AND METHODS

Animals. Female BALB/c (H-2d) 5 to 7 wk old were purchased from Goodwin Institute for Cancer Research (Plantation, FL).

Tumors. The weakly immunogenic (16) MOPC-315 plasmacytoma was maintained by serial s.c. inoculation in syngeneic BALB/c mice. Single cell suspensions were prepared by mechanical disruption (17), and the viability as determined by trypan blue dye exclusion (0.4%) always exceeded 85%. Routinely, mice were inoculated with 1 x 10^6 viable MOPC-315 cells, a dose which is at least 300 times greater than the minimal lethal tumor dose and kills the mice in 18 ± 1 days.

Chemotherapy. The BiCY (Cytoxan) was purchased from Mead Johnson and Co. (Evansville, IN). The BiCY was dissolved in sterilized water at a concentration of 20 mg/kg and further diluted in distilled water to a concentration of 20 mg/kg. The BiCY was administered as a single i.p. injection of either BiCY or MoCY, was given to mice 10 to 12 days after tumor inoculation when the mice had a 22-mm s.c. tumor. The mice were monitored 3 times weekly for the presence of a primary s.c. tumor, and mice remaining tumor free 60 days after chemotherapy were considered to be cured.

Rabbit Anti-Mouse Thymocyte Serum. Rabbit ATS (Microbiological
The in vitro generation of antitumor cytotoxicity (20). To use. The cultures were incubated for 5 days, the time required for
volume of 20 ml Roswell Park Memorial Institute Tissue Culture
of the incubation period, each well was pulsed with 1 ¿iCi of [3H-
| ìl| Ihy niidiiu- Incorporation. We determined the percentage of re
\[\text{% of specific \(^{51}\text{Cr} \text{ release} = \frac{(T- C)}{(M - C)} \times 100}\]

where \(T\) is the percentage of release with test spleen cells, \(C\) is the percentage of spontaneous release by the target cells alone (which
ranged between 17 and 25%), and \(M\) is the percentage of maximal \(^{51}\text{Cr} \text{ release obtained by 3 cycles of freezing and thawing (which ranged}
\text{between 82 and 91%). Each experiment was performed 2 to 4 times.}

The level of antitumor cytotoxicity is presented as the mean \(^{51}\text{Cr} \text{ release of triplicate samples. Variations in the \(^{51}\text{Cr} \text{ release between individual}
\text{samples rarely exceeded 5% of the mean. All points that differ by 10% or}

more of \(^{51}\text{Cr} \text{ release were found to be significantly different by Student's t test (P < 0.05).}

Isolation of Sephadex G-10-adherent Spleen Cells. Spleen cell sus-
pensions depleted of RBC by NH4Cl were subjected to fractionation on

Sephadex G-10 columns by the method of Ly and Mishell (22) with
some modifications as we have described previously (12). Briefly, 2 ml
of spleen cell suspensions (1 to 1.5 \times 10^8 cells/ml) in RPMI-1640
medium supplemented by 10% FCS were applied to Sephadex G-10
columns previously equilibrated with the same medium. The columns
were washed extensively with RPMI-1640 medium supplemented by
10% FCS until the effluent was essentially free of cells (<5 \times 10^6 cells/
ml). Subsequently, the Sephadex G-10-adherent cells were collected
according to the method of Chang et al. (23). Briefly, the columns
were incubated with 30 mM Lidocaine (Sigma Chemical Co., St. Louis, MO)
solution in RPMI-1640 medium containing 20% FCS at 37°C, in a 5% 
CO\(_2\) atmosphere for 20 min. Following incubation, the adherent cells
were harvested by rapidly eluting the column with RPMI-1640 medium
supplemented with 10% FCS. Of the original number of spleen cells
applied to the column, approximately 10% was recovered as adherent
cells from the Sephadex G-10 column. The viability of these adherent
cells as determined by trypan blue dye exclusion always exceeded 85%. The Sephadex G-10-adherent spleen cell population contained primarily
macrophages (~70%); however, cells bearing Thy 1.2 antigens and
cells bearing surface immunoglobulin were also present (~15 and 25%,
respectively).

Depletion of T-Cells by Anti-Thy 1.2 Antibody and Complement. One
ml of Sephadex G-10-adherent cells (2 \times 10^7/ml) was mixed with an
appropriate volume of monoclonal anti-Thy 1.2 antibody (30-H12; see
Ref. 24) to give a final dilution of 1/10. The cells were incubated at
4°C for 45 min and then mixed with an appropriate volume of low
toxicity rabbit complement (Pel Freez, Roger, AR) to give a final
dilution of 1/20. After a further incubation at 37°C for 30 min, the
cells were washed 3 times and resuspended in RPMI-1640 supple-
mented with 5% FCS before addition to the immunization culture of
normal spleen cells. This treatment reduced the percentage of Thy 1.2-
positive cells in the Sephadex G-10-adherent spleen cell fraction from
approximately 15% to less than 0.3%. The effectiveness of the protocol
in depleting T-cells was also illustrated by the ability of the monoclonal
anti-Thy 1.2 antibody and complement to abolish the ability of spleen
cells to generate T-cell-dependent antitumor immunity and to prolif-
erate in response to a T-cell mitogen, phytohemagglutinin.

Depletion of Phagocytic Cells by Carbonyl Iron and Magnet. About 2
ml of a Sephadex G-10-adherent cell suspension (2 \times 10^7/ml) in RPMI-1640
medium containing 10% FCS were added to an equal volume of
lymphocyte-separating reagent (Technicon Instruments Corp., Tarry-
town, NY) containing carbonyl iron particles. The mixture was
 incubated with constant rocking at 37°C for 30 min. The cells that
ingested carbonyl iron were removed by the use of a magnet according
to the method described by Kirchner et al. (25). The effectiveness of
the procedure in depleting macrophages was illustrated by reduction in
the percentage of esterase-positive cells from ~70% to less than 5%.

Isolation of Nucleus-Wool-nonadherent Spleen Cells. To enrich for T-
cells, the Sephadex G-10-adherent spleen cell population was subjected
to nylon-wool fractionation according to the method of Julius et al. (26)
with slight modifications (12). Briefly, 600 mg of sterile nylon
wool were packed in a 10-ml sterile syringe down to the 6-ml mark,
and the columns were equilibrated at 37°C with RPMI-1640 supple-
mented with 5% FCS. Subsequently, 3 \times 10^7 Sephadex G-10-adherent
splenic cells in 1 to 2 ml of warm medium were applied to each column,
and the columns were incubated at 37°C for 45 min with occasional
rotation. The nonadherent cells were collected by washing with 10 ml
of medium.

\[\text{Mo CY} \quad \text{Bi CY} \quad \text{MONOFUNCTIONAL CYCLOPHOSPHAMIDE} \]

\[\text{Cl-CH-CH}_2 \quad \text{Cl-CH-CH}_2 \quad \text{N-CH}_2 \]

\[\text{CH}_2, \quad \text{H} \quad \text{H} \quad \text{N-CH}_2 \]

\[\text{H} \quad \text{H} \quad \text{MONOFUNCTIONAL CYCLOPHOSPHAMIDE} \]

\[\text{Cl-CH-CH}_2 \quad \text{Cl-CH-CH}_2 \quad \text{N-CH}_2 \]

\[\text{CH}_2, \quad \text{H} \quad \text{H} \quad \text{N-CH}_2 \]

\[\text{H} \quad \text{H} \quad \text{MONOFUNCTIONAL CYCLOPHOSPHAMIDE} \]
Determination of the Minimum Dose of MoCY Required for the Cure of Mice Bearing a Large s.c. MOPC-315 Tumor and Extensive Metastases. Mice bearing a 22-mm tumor were given a single i.p. injection of MoCY in doses ranging from 15 to 1000 mg/kg. Subsequently, for a period of 60 days, the mice were monitored for the presence of a primary s.c. tumor (Table 1). As a reference point, we provide information regarding the curative effectiveness of 15 mg of BiCY per kg (Table 1). As expected (10), BiCY (15 mg/kg) cured most treated mice (72%). On the other hand, MoCY (15 mg/kg) was not curative for any of the tumor bearers, and a 150-mg/kg dose of the MoCY was required to cure most tumor bearers. Most tumor bearers were also cured with 450 mg of MoCY per kg. However, when 1000 mg of MoCY per kg were used, although a marked tumor regression was observed, the mice died within 4 days postchemotherapy of general toxicity of the drug. Complete regression of the primary tumor nodule was observed within 7 days postchemotherapy with 15 mg of BiCY per kg or within 9 days postchemotherapy with 150 mg of MoCY per kg. Thus, although the MoCY is effective in the therapy of MOPC-315 tumor bearers, the dose of MoCY required is at least 10-fold higher than that of the BiCY.

Comparison of MoCY and BiCY Therapy of Tumor Bearers for Their Ability to Inhibit the Proliferative Capacity of Tumor Cells from the Primary Tumor Nodule. The direct tumoricidal/tumorstatic effect of graded doses of MoCY and BiCY was assessed by the use of [3H]thymidine incorporation assay as an indirect estimate of tumor cell proliferation. Two h postchemotherapy of mice bearing a 22-mm s.c. tumor, the primary tumor was excised, single cell suspension was prepared, and the cells were then cultured in vitro for 72 h, the last 16 of which were in the presence of [3H]thymidine (Table 2). With both MoCY or BiCY therapy, an increase in the dose of drug used for the chemotherapy of the tumor bearers resulted in a decrease in the ability of the tumor cells to incorporate [3H]thymidine. However, the MoCY was much less effective than the BiCY, since the dose of MoCY required to approach the inhibitory activity of the BiCY was at least 10-fold higher (and probably more) than the dose of BiCY used; i.e., 150 mg of MoCY per kg were required to approximate the inhibitory activity of 15 mg of BiCY per kg. It should be noted, however, that although treatment of tumor bearers with MoCY (150 mg/kg) drastically reduced the ability of the tumor cells to incorporate [3H]thymidine, approximately 2% of the proliferative capacity was retained. When tumor cells from mice treated with various doses of BiCY or MoCY were incubated in vitro for 4 days (instead of just 3 days) with [3H]thymidine present during the last 16 h of culture, the magnitude of inhibition of [3H]thymidine incorporation observed following chemotherapy was essentially the same as that provided in Table 2 for tumor cells cultured in vitro for 3 days (data not shown).

Effect of ATS on the Curative Effectiveness of the Lowest Curative Dose of MoCY for Mice Bearing a Large Tumor. In light of our previous observations that the curative effectiveness of the lowest curative dose of BiCY for mice bearing a large MOPC-315 tumor burden requires the participation of T-cell-dependent host antitumor immunity (11), we have evaluated the role of T-cell-dependent antitumor immunity in the cure of tumor bearers with the lowest curative dose of MoCY. Mice bearing a 22-mm s.c. MOPC-315 tumor were treated with MoCY (150 mg/kg), and subsequently some mice were given injections of ATS on Days 0, 2, and 4 postchemotherapy (Table 3). Treatment of tumor bearers with the MoCY alone cured a substantial number of the tumor bearers (16 of 33); however, when MoCY therapy was followed by treatment with ATS, tumor regression was abrogated and all mice died of tumors. Thus, the eradication of a large MOPC-315 tumor burden induced by MoCY (150 mg/kg) requires the participation of host T-cell-dependent antitumor immunity.

Effect of Chemotherapy with MoCY on the Antitumor Immune Potential of Hitherto Immunosuppressed Tumor Bearer Spleen Cells. Mice bearing a 22-mm MOPC-315 tumor received a single i.p. injection of the lowest curative dose of either MoCY (150 mg/kg) or BiCY (15 mg/kg). On Day 3 postchemotherapy, spleen cells obtained from untreated or CY-treated tumor bearers were evaluated for their antitumor immune potential by determining their ability to mount an antitumor cytotoxic response following in vitro immunization with mitomycin C-treated stimulator tumor cells. As a reference point, we provide the level of antitumor cytotoxicity exhibited by in vitro immunized spleen cells from normal mice. Since only 50% of the tumor bearers were cured by 150 mg of MoCY per kg in

### Table 1 Effectiveness of various doses of MoCY for the cure of mice bearing a large primary MOPC-315 tumor and extensive metastases

<table>
<thead>
<tr>
<th>Tumor bearers* treated with Drug</th>
<th>Dose (mg/kg)</th>
<th>Effectiveness of chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. of surviving/total</td>
</tr>
<tr>
<td>Saline control</td>
<td></td>
<td>0/20</td>
</tr>
<tr>
<td>MoCY 15</td>
<td></td>
<td>0/10</td>
</tr>
<tr>
<td>MoCY 45</td>
<td></td>
<td>1/20</td>
</tr>
<tr>
<td>MoCY 100</td>
<td></td>
<td>0/10</td>
</tr>
<tr>
<td>MoCY 150</td>
<td></td>
<td>51/94</td>
</tr>
<tr>
<td>MoCY 450</td>
<td></td>
<td>12/20</td>
</tr>
<tr>
<td>MoCY 1000</td>
<td></td>
<td>0/10</td>
</tr>
<tr>
<td>BiCY 15</td>
<td></td>
<td>31/43</td>
</tr>
</tbody>
</table>

* Mouse bearing a s.c. tumor with a diameter of 22 mm.

### Table 2 Comparison of the effect of MoCY and BiCY chemotherapy of tumor bearers on the ability of tumor cells from the primary tumor nodule to incorporate [3H]thymidine

<table>
<thead>
<tr>
<th>Tumor cell* from tumor bearers treated with Drug</th>
<th>Dose (mg/kg)</th>
<th>% of inhibition of [3H]thymidine incorporation (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline control</td>
<td></td>
<td>72.71 ± 1.577*</td>
</tr>
<tr>
<td>BiCY 15</td>
<td>15.0</td>
<td>426 ± 40</td>
</tr>
<tr>
<td>BiCY 5.0</td>
<td>1,869 ± 88</td>
<td>99.4</td>
</tr>
<tr>
<td>BiCY 5.0</td>
<td>5.0</td>
<td>1,794 ± 2,209</td>
</tr>
<tr>
<td>MoCY 15.0</td>
<td>150.0</td>
<td>1,425 ± 77</td>
</tr>
<tr>
<td>MoCY 50.0</td>
<td>50.0</td>
<td>32,614 ± 792</td>
</tr>
<tr>
<td>MoCY 16.7</td>
<td>63,233 ± 2,483</td>
<td>12.5</td>
</tr>
</tbody>
</table>

* Tumor cells were obtained from mice 2 h after chemotherapy.

### Table 3 Effect of ATS on the curative effectiveness of the lowest curative dose of MoCY for mice bearing a large MOPC-315 tumor burden

<table>
<thead>
<tr>
<th>Chemotherapy of tumor bearers*</th>
<th>Drug (mg/kg)</th>
<th>Treatment with ATS*</th>
<th>No. of surviving/total</th>
<th>% of survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>+</td>
<td>0/20</td>
<td>0</td>
</tr>
<tr>
<td>BiCY 15</td>
<td></td>
<td></td>
<td>0/20</td>
<td>0</td>
</tr>
<tr>
<td>MoCY 150</td>
<td></td>
<td>+</td>
<td>16/33</td>
<td>48.5</td>
</tr>
<tr>
<td>MoCY 150</td>
<td></td>
<td></td>
<td>0/20</td>
<td>0</td>
</tr>
</tbody>
</table>

* The chemotherapy (CY) was administered to mice bearing a 22-mm tumor.

* Mice were given 0.25 ml of rabbit anti-mouse thymocyte serum i.p. on Days 0, 2, and 4 postchemotherapy.
cooperation with T-cell-dependent antitumor immunity (Table 1), we have chosen to evaluate the splenic antitumor immune potential of each CY-treated tumor bearer individually (Table 4). As expected (10), in vitro immunized spleen cells from untreated tumor bearers exhibited a suppressed level of antitumor cytotoxicity compared to that exhibited by in vitro immunized normal spleen cells. Treatment of the tumor bearers with the lowest curative dose of either MoCY or BiCY enabled their spleen cells to develop upon in vitro immunization an enhanced level of antitumor cytotoxicity. However, the level of splenic antitumor immune potential exhibited by CY-treated tumor bearers varied from one mouse to another. For example, whereas the level of antitumor cytotoxicity exhibited upon in vitro immunization by spleen cells from two tumor bearers treated with MoCY greatly exceeded that of in vitro immunized spleen cells from normal mice, the level of antitumor cytotoxicity exhibited by in vitro immunized spleen cells from the third MoCY-treated tumor bearer was similar to that exhibited by in vitro immunized normal spleen cells.

The combined results of 7 separate experiments that evaluated the effect of MoCY and BiCY therapy with the lowest curative dose of drug on the antitumor immune potential of spleen cells from mice bearing a large MOPC-315 tumor are provided in a scattergram format in Fig. 2. Normal spleen cells immunized in vitro exhibited a substantial level of antitumor cytotoxicity (mean, 24.8% ⁵¹Cr release). Spleen cells from untreated tumor-bearing mice were suppressed relatively to spleen cells from normal mice in their ability to mount an antitumor cytotoxic response following in vitro immunization (mean, 7.7% ⁵¹Cr release) with spleen cells from 10 of 12 of the tumor-bearing mice exhibiting a lower level of cytotoxic activity than the mean cytotoxicity of the spleen cells from normal mice. Low dose BiCY therapy of tumor bearers enhanced the ability of their spleen cells to mount an antitumor cytotoxic response following in vitro immunization (56.3 versus 7.7% ⁵¹Cr release with spleen cells from untreated tumor bearers). In fact, spleen cells from all (11 of 11) BiCY-treated mice developed, upon in vitro immunization, a higher level of antitumor cytotoxicity than the mean cytotoxicity exhibited by in vitro immunized normal spleen cells. Within 5 days after MoCY therapy, spleen cells from most, but not all, mice were enhanced in their ability to mount an antitumor cytotoxic response following in vitro immunization; spleen cells from 12 of 13 of the treated mice exhibited a higher level of cytotoxicity than the mean cytotoxicity exhibited by in vitro immunized spleen cells from untreated tumor-bearing mice, and spleen cells from 7 of 13 of the MoCY-treated tumor bearers exhibited a substantially higher level of cytotoxicity than the mean cytotoxicity exhibited by normal spleen cells (Fig. 2). When spleen cells subjected to in vitro immunization were obtained from mice that completely eradicated a large MOPC-315 tumor following low dose MoCY therapy (i.e., 10 days or more after the chemotherapy), a very potent level of antitumor cytotoxicity (mean, 82.5% ⁵¹Cr release), greatly exceeding that of in vitro immunized spleen cells from normal mice, was observed with spleen cells from all treated mice. Thus, chemotherapy with the lowest curative dose of MoCY leads to the rapid appearance of varying degrees of enhanced antitumor immune potential in the hitherto immunosuppressed tumor bearer spleen cells.

MoCY-induced Appearance of Immunopotentiating Activity in Tumor Bearer Spleens. We have recently shown that the Sephadex G-10-adherent spleen cell population from tumor bearers treated with BiCY (15 mg/kg) when added to the in vitro immunization culture of normal spleen cell brings about the development of an augmented level of antitumor cytotoxicity (12, 13). Experiments were performed to determine whether the Sephadex G-10-adherent spleen cell population from tum-

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**Table 4. Effect of chemotherapy with the lowest curative dose of MoCY on the ability of hitherto immunosuppressed tumor bearer spleen cells to mount an antitumor cytotoxic response following in vitro immunization**

<table>
<thead>
<tr>
<th>Donors of spleen cells</th>
<th>Pretreatment of spleen cell donors</th>
<th>Antitumor cytotoxicity (% of specific ⁵¹Cr release) at an effector/target cell ratio of 100/1</th>
<th>25/1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mouse</td>
<td>None</td>
<td>12.0 ± 2.8*</td>
<td>0.1 ± 1.3</td>
</tr>
<tr>
<td>Normal mouse</td>
<td>None</td>
<td>22.5 ± 4.0</td>
<td>5.4 ± 0.6</td>
</tr>
<tr>
<td>Tumor bearer</td>
<td>None</td>
<td>10.1 ± 2.1</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>Tumor bearer</td>
<td>None</td>
<td>-3.3 ± 0.4</td>
<td>-2.3 ± 0.4</td>
</tr>
<tr>
<td>Tumor bearer</td>
<td>MoCY</td>
<td>0.3 ± 1.6</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Tumor bearer</td>
<td>MoCY</td>
<td>24.7 ± 3.0</td>
<td>ND</td>
</tr>
<tr>
<td>Tumor bearer</td>
<td>MoCY</td>
<td>85.0 ± 0.9</td>
<td>54.0 ± 8.0</td>
</tr>
<tr>
<td>Tumor bearer</td>
<td>MoCY</td>
<td>88.7 ± 1.2</td>
<td>77.8 ± 2.7</td>
</tr>
<tr>
<td>Tumor bearer</td>
<td>BiCY</td>
<td>77.9 ± 3.3</td>
<td>39.8 ± 3.6</td>
</tr>
<tr>
<td>Tumor bearer</td>
<td>BiCY</td>
<td>40.8 ± 5.0</td>
<td>ND</td>
</tr>
<tr>
<td>Tumor bearer</td>
<td>BiCY</td>
<td>64.3 ± 8.3</td>
<td>21.7 ± 3.4</td>
</tr>
</tbody>
</table>

* Spleen cells were obtained 3 days after chemotherapy of mice bearing a 21-mm tumor.
* Mean ± SE.
* One-hundred fifty mg/kg.
* ND, not done.
* Fifteen mg/kg.
tumor-bearing mice treated with MoCY (150 mg/kg) can also bring about the generation of enhanced antitumor cytotoxicity when added to the in vitro immunization culture of normal spleen cells. Mice bearing a 22-mm tumor were given a single i.p. injection of MoCY (150 mg/kg), and 10 days later, their Sephadex G-10-adherent spleen cells were obtained. Different numbers of Sephadex G-10-adherent cells were added to normal spleen cells so as to constitute between 0.1 and 10% of the total spleen cell population, and subsequently, the spleen cell mixtures were immunized in vitro with stimulator tumor cells (Table 5). An augmented level of antitumor cytotoxicity developed when as little as 1% Sephadex G-10-adherent spleen cells from MoCY-treated tumor bearers were present among the normal spleen cells (from 23 to 49% 51Cr release). However, a substantially higher level of antitumor cytotoxicity developed when the percentage of added Sephadex G-10-adherent spleen cells from MoCY-treated tumor bearers was increased to 3 or 10% (i.e., from 23 to 73 or 80% release, respectively). Thus, when as few as 1% Sephadex G-10-adherent spleen cells from MoCY-treated tumor bearers are present among normal spleen cells subjected to in vitro immunization, enhanced lytic activity is generated. To avoid cumbersome presentation of subsequent results, we will refer to the enhanced lytic activity generated in the presence of Sephadex G-10-adherent spleen cells from MoCY-treated tumor bearers as MoCY-induced immunopotentiating activity. However, as stated in the "Discussion," the enhanced lytic activity observed when normal spleen cells are immunized in vitro in the presence of Sephadex G-10-adherent splenic cells from MoCY-treated tumor-bearing mice might instead (or in addition) be the result of potent lytic activity exerted by the Sephadex G-10-adherent splenic cells from MoCY-treated tumor bearers present among the normal spleen cells and expanding over the 5-day period of the in vitro immunization culture.

Partial Characterization of the Sephadex G-10-adherent Immunopotentiating Cells Which Operate in the Spleen of Tumor Bearers Treated with MoCY. Since the Sephadex G-10-adherent splenic cell population of MoCY-treated tumor bearers consists of macrophages (~70%) as well as T-cells (~15%), we evaluated which of these cell types is responsible for the exertion of the MoCY-induced immunopotentiating activity. This was done initially by determining the effect of depleting macrophage or T-cells from the Sephadex G-10-adherent splenic cell population on the MoCY-induced immunopotentiating activity. Ten days after MoCY therapy of mice bearing a 22-mm tumor, the Sephadex G-10-adherent splenic cell population was obtained. An aliquot of the Sephadex G-10-adherent cells was depleted of macrophages by the use of carbonyl iron and magnet (Table 6) or of T-cells by treatment with monoclonal anti-Thy 1.2 plus complement (Table 7). Depletion of phagocytic cells from the Sephadex G-10-adherent cell population did not reduce and actually enhanced the immunopotentiating activity (34 versus 52% 51Cr release; Table 6). In contrast, depletion of T-cells completely abolished the immunopotentiating activity; i.e., from 78 to 36% 51Cr release which is essentially the same level of antitumor cytotoxicity as that exhibited by normal spleen cells immunized in the absence of Sephadex G-10-adherent splenic cells (Table 7). Thus, depletion experiments indicate that T-cells among the Sephadex G-10-adherent spleen cells from MoCY-treated tumor-bearing mice are responsible for bringing about the development of enhanced lytic activity when added to the in vitro immunization culture of normal spleen cells.

Next, experiments were performed to determine whether the MoCY-induced immunopotentiating activity is retained following passage of the Sephadex G-10-adherent cell population over nylon-wool columns, a fractionation procedure commonly used to enrich for T-cells (26). The Sephadex G-10-adherent splenic cell population was obtained on Day 10 post-MoCY therapy of mice bearing a 22-mm tumor. Subsequently, an aliquot of the Sephadex G-10-adherent cells was depleted of nylon-adherent cells (Table 8). Upon nylon wool fractionation of Sephadex G-10-adherent splenic cells from MoCY-treated tumor bearers, the nylon wool nonadherent cells retained the immunopotentiating activity. As a control we provide information that depletion of T-cells from another aliquot of the same batch of Sephadex G-10-adherent cells abolished the immunopotentiating activity. Thus, treatment of MOPC-315 tumor bearers with MoCY enables their Sephadex G-10-adherent, nylon wool nonadherent, splenic T-cells to bring about the generation of

<table>
<thead>
<tr>
<th>% of the added adherent cells (Table 5)</th>
<th>Antitumor cytotoxicity (% of specific 51Cr release) at an effect/target cell ratio of</th>
<th>Antitumor cytotoxicity (% of specific 51Cr release) at an effect/target cell ratio of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100/1</td>
<td>25/1</td>
</tr>
<tr>
<td>0</td>
<td>23.3 ± 1.2*</td>
<td>10.5 ± 1.4</td>
</tr>
<tr>
<td>1</td>
<td>33.5 ± 5.0</td>
<td>16.1 ± 1.6</td>
</tr>
<tr>
<td>1</td>
<td>51.6 ± 1.4</td>
<td>19.8 ± 3.3</td>
</tr>
</tbody>
</table>

* The Sephadex G-10-adherent cells were obtained 10 days after chemotherapy of mice bearing a 22-mm tumor.

* Mean ± SE.

<table>
<thead>
<tr>
<th>% of the added adherent cells (Table 7)</th>
<th>Depletion of T-cells by treatment with anti-Thy 1.2 plus complement</th>
<th>Antitumor cytotoxicity (% of specific 51Cr release) at an effect/target cell ratio of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100/1</td>
<td>25/1</td>
</tr>
<tr>
<td>0</td>
<td>30.7 ± 1.8*</td>
<td>7.8 ± 0.6</td>
</tr>
<tr>
<td>3.0</td>
<td>77.9 ± 2.8*</td>
<td>61.6 ± 1.6</td>
</tr>
<tr>
<td>3.0</td>
<td>36.4 ± 5.5</td>
<td>16.7 ± 2.5</td>
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<tr>
<td>1.0</td>
<td>61.9 ± 6.3</td>
<td>41.6 ± 2.3</td>
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<tr>
<td>1.0</td>
<td>31.3 ± 1.2</td>
<td>7.0 ± 1.7</td>
</tr>
<tr>
<td>0.3</td>
<td>62.9 ± 2.7</td>
<td>35.2 ± 0.9</td>
</tr>
<tr>
<td>0.3</td>
<td>36.1 ± 1.0</td>
<td>8.0 ± 0.6</td>
</tr>
</tbody>
</table>

* The Sephadex G-10-adherent cells were obtained 10 days after chemotherapy of mice bearing a 22-mm tumor.

* Mean ± SE.
enhanced cytotoxic activity when added to the immunization culture of normal spleen cells.

Ability of Mice Cured by MoCY to Resist a Lethal Challenge with MOPC-315 Tumor Cells. Mice cured of a 22-mm MOPC-315 tumor following therapy with the lowest curative dose of MoCY were challenged 30 days later with 1 x 10^6 MOPC-315 tumor cells (a dose which is 300-fold greater than the minimal lethal tumor dose) on the contralateral side. The challenged mice were monitored for 60 days for tumor appearance (Table 9). As a control, we provide information regarding the resistance to tumor challenge of mice cured by the lowest curative dose of BiCY. All mice cured by the MoCY were resistant to the tumor challenge, demonstrating that mice cured by the lowest curative dose of MoCY exhibit long-lasting potent antitumor immunity.

DISCUSSION

The results presented herein illustrate that mice bearing a large primary MOPC-315 tumor and extensive metastases can be cured by a single i.p. injection of either BiCY (15 mg/kg) or MoCY (150 mg/kg). As previously observed with BiCY (10, 11, 15), cure attained by the lowest curative dose of MoCY is due to cooperation between the direct antitumor toxic effect of the drug and host T-cell-dependent antitumor immunity and involves the immunomodulatory activity of the drug which shifts the balance from immunosuppression to immunopotentiation. Moreover, mice cured by either drug exhibit long-lasting, potent antitumor immunity. The need for a 10-fold higher dose of MoCY as compared with BiCY for the cure of MOPC-315 tumor bearers is due, at least in part, to the 10-fold lower direct antitumor toxicity of the monofunctional as compared with the bifunctional drug. Thus, the cross-linking property of the bifunctional alkylating agent is not essential for the immunomodulatory activity of BiCY nor for its direct tumoricidal and/or tumoristatic activity. Our results are consistent with the observation made recently by Morikawa et al. (27), that administration of the DNA strand breakee, bleomycin, to rats at an advanced stage of KMT-17 fibrosarcoma growth was effective in enhancing the antitumor immunity through the elimination of suppressor cell activity and, consequently, the enhanced antitumor immunity apparently facilitated the therapeutic effectiveness of the drug.

MoCY, like BiCY (28), is not active in its native chemical form but requires conversion by microsomal enzymes to exert its alkylating activity. The metabolism of MoCY has not been well studied, but the metabolism of a number of bifunctional analogues of BiCY has been characterized and shown to be analogous to the metabolites of BiCY (28), and therefore analogous monofunctional metabolites would be expected to be found with MoCY. Accordingly, since phosphoramide mustard, which is a bifunctional nitrogen mustard, is responsible for the bulk of the cytotoxic activity of BiCY, a monofunctional analogue of phosphoramide mustard is expected to be responsible for most of the cytotoxic activity of MoCY.

We have shown here that a dose of 150 mg of MoCY/kg is required to reduce the proliferative capacity of tumor cells from the primary tumor nodule to the same extent as that obtained following treatment of the tumor bearers with the lowest curative dose of BiCY, namely, 15 mg/kg. The reduced direct antitumor activity of the monofunctional as compared with the bifunctional alkylating agent was also observed by other investigators. For example, Sternberg et al. (29) have shown that several monofunctional mustards are 50 to 100 times less toxic than the bifunctional nitrogen mustard. The much greater direct antitumor activity of bifunctional alkylating agents has been attributed to the formation of cross-links between important macromolecules (29) [e.g., BiCY leads to dimer formation between guanine bases in DNA (30)], in contrast to monofunctional alkylating agents which cause single-strand breaks in DNA and are more mutagenic (31).

We have estimated the degree of drug-mediated inhibition of tumor cell proliferative capacity from the degree of drug-mediated reduction in [3H]thymidine incorporation. The validity of utilizing nucleotide precursor incorporation as a measure of cell proliferation has been challenged by several investigators who observed no decrease in nucleotide precursor incorporation when tested a few hours following exposure to chemotherapeutic drugs, despite complete inhibition of tumor cell growth (32, 33). However, when a longer time interval (i.e., 72 to 96 h) was allowed between exposure of the tumor cells to chemotherapeutic drugs and the measurement of nucleotide precursor incorporation, a strong concordance was found between [3H]thymidine incorporation and the ability to form colonies in soft agar (34) or to establish lethal tumors in new recipients (35). The longer time interval between exposure to drug and measurement of nucleotide precursor incorporation is apparently of sufficient duration to permit accurate evaluation of cell kill produced by agents which allow cells to complete several divisions prior to cell death (33). In our studies, we allowed 72 to 96 h to elapse after chemotherapy of tumor bearers before assaying for [3H]thymidine incorporation, and we found that with both MoCY and BiCY the lowest curative dose of drug reduces the proliferative capacity of the tumor cells by approximately 98%. The host's T-cell-dependent antitumor immunity controls the remaining 2% of the proliferative capacity which most likely represents quite a large tumor burden since, at the time of chemotherapy, the primary tumor nodule is 20 to 25 mm in diameter and contains at least 80 x 10^6 viable tumor cells (11).

We have previously shown that spleen cell suspensions from mice bearing a large MOPC-315 tumor exhibit a depressed antitumor immune potential due, in part, to the inhibitory activity of a Sephadex G-10-adherent spleen cell population (12). Here we show that a greatly augmented level of antitumor cytotoxicity developed when the Sephadex G-10-adherent cells

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Table 8 Effect of nylon-wool fractionation on the ability of Sephadex G-10-adherent splenic cells from MoCY-treated tumor bearers to bring about the generation of an augmented level of antitumor cytotoxicity when added to the immunization culture of normal spleen cells

<table>
<thead>
<tr>
<th>Treatment of Sephadex G-10-adherent splenic cells</th>
<th>% of the added adherent cells</th>
<th>Antitumor cytotoxicity (% of specific 51Cr release) at an effector/target cell ratio of 100/1</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>29.9 ± 3.6</td>
</tr>
<tr>
<td>Nylon-wool fractionation</td>
<td>3</td>
<td>67.5 ± 0.4</td>
</tr>
<tr>
<td>Anti-Thy 1.2 + complement</td>
<td>3</td>
<td>78.9 ± 1.1</td>
</tr>
</tbody>
</table>

* Sephadex G-10-adherent cells were obtained 10 days postchemotherapy of mice bearing a 22-mm tumor. * Mean ± SE.
from MoCY-treated tumor bearers that were added to the in vitro immunization culture of normal spleen cells constituted only 3% or even 1% of the spleen cell mixture. As reported previously for BiCY (13), the T-cells among the Sephadex G-10-adherent spleen cells from MoCY-treated tumor bearers are responsible for the enhanced lytic activity observed when Sephadex G-10-adherent splenic cells from MoCY-treated tumor bearers are added to the immunization culture of normal spleen cells. Since the concentration of T-cells in the adherent spleen cell population is approximately 15%, even if it is assumed that all the T-cells in the adherent population are responsible for the enhanced lytic activity observed, the adherent T-cells added to the in vitro immunization culture of normal spleen cells represent at most only 0.45% of the total spleen cell mixture.

In light of the low frequency of the adherent splenic T-cells from MoCY-treated tumor bearers in the in vitro immunization culture of normal spleen cells, it is likely that the adherent cells act as amplifier cells to enhance the generation of antitumor cytotoxicity by the normal spleen cells. However, the possibility that the added Sephadex G-10-adherent splenic cells from MoCY-treated tumor bearers expand during the 5 days of immunization culture and they themselves exert an extremely potent lytic activity should be considered. We have attempted to determine whether indeed the Sephadex G-10-adherent splenic cells from MoCY-treated tumor bearers act as amplifier cells to enhance the generation of antitumor cytotoxicity by normal spleen cells. This was done by comparing the level of antitumor cytotoxicity generated when normal spleen cells were immunized in vitro in the presence of adherent spleen cells from CY-treated tumor-bearing mice to that exhibited by in vitro immunized normal spleen cells admixed at the stage of the 51Cr release assay with in vitro immunized Sephadex G-10-adherent spleen cells from CY-treated tumor bearers. Although such mixtures, as well as in vitro immunized Sephadex G-10-adherent cells from CY-treated tumor bearers, did not exhibit a higher lytic activity than did in vitro immunized normal spleen cells assayed alone, it does not necessarily mean that the Sephadex G-10-adherent cells cannot develop a potent antitumor cytotoxicity when present among normal spleen cells subjected to in vitro immunization. The possibility exists that macrophages, which are present in a very high concentration in the immunization cultures of Sephadex G-10-adherent populations and inhibit the generation of antitumor cytotoxicity, are diluted when the Sephadex G-10-adherent cells are added to the immunization culture of normal spleen cells, thereby losing their inhibitory activity and allowing the Sephadex G-10-adherent splenic cells from MoCY-treated tumor bearers to develop a potent lytic activity of their own.

A 150-mg/kg dose of MoCY is required for the cure of most mice bearing a large MOPC-315 tumor burden. However, when the dose of MoCY administered to the tumor bearers is increased by ~7-fold (i.e., to 1000 mg/kg), all mice die within 4 days postchemotherapy from general drug toxicity. In contrast, a 15-mg/kg dose of BiCY is curative for most mice bearing a large MOPC-315 tumor, and when the dose of drug administered to tumor bearers is increased by as much as ~13-fold (i.e., to 200 mg/kg), no lethality due to drug toxicity is observed, but instead, all treated mice are cured (10, 15). Thus, MoCY is curative for mice bearing a large MOPC-315 tumor over a narrower dose range than BiCY.

Spleen cells from tumor-bearing mice are inferior to spleen cells from normal mice in their ability to mount an antitumor cytotoxic response following in vitro immunization. Enhancement in the ability of spleen cells from tumor-bearing mice to mount an antitumor cytotoxic response following in vitro immunization was observed almost always within 5 days after therapy of the tumor-bearing mice with the lowest curative dose of MoCY. However, the magnitude of MoCY-induced enhancement in the antitumor immune responsiveness of tumor bearer spleen cells varied among the treated mice. Specifically, the level of antitumor cytotoxicity exhibited upon in vitro immunization by spleen cells from 46% of the MoCY-treated tumor bearers approximated that exhibited by in vitro immunized normal spleen cells, while the level of antitumor cytotoxicity exhibited upon in vitro immunization by spleen cells from the other 54% of the MoCY-treated tumor bearers greatly exceeded the normal level. Consistent with the variable effectiveness of low-dose MoCY therapy in bringing about the appearance of potent antitumor immune responsiveness in the hitherto immunosuppressed tumor bearer spleen cells, the low dose of MoCY was curative for only 54% of the mice. The observations that the same percentage of tumor-bearing mice which exhibit greatly enhanced splenic antitumor cytotoxic responsiveness following low-dose MoCY therapy is also cured by the low dose of MoCY are consistent with the explanation that low-dose MoCY therapy is curative only for mice in which the chemotherapeutic agent efficiently stimulated the antitumor immune potential of their spleen cells.

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REFERENCES

15. Mokyr, M. B., and Dray, S. Some advantages of curing mice bearing a large


Ability of Cyclophosphamide in the Absence of Cross-Linking Activity to Exert the Immunomodulatory Effect Required for the Cure of Mice Bearing a Large MOPC-315 Tumor

Margalit B. Mokyr, Robert B. Brundrett, Michael Colvin, et al.


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