Importance of Timing in Cyclophosphamide Therapy of MOPC-315 Tumor-bearing Mice

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ABSTRACT

The timing of cyclophosphamide (CY) administration after tumor inoculation was found to be critical for successful therapy of MOPC-315 tumor-bearing mice. Following inoculation with 3.5 × 10⁶ viable tumor cells, a single i.p. injection of CY (15 mg/kg) into mice bearing 10- to 25-mm (Days 8 to 14) tumors cured most mice, whereas injection into mice bearing nonpalpable tumors (Day 4) cured only a few of the mice. The time interval between tumor inoculation and CY administration rather than the tumor size was critical for successful therapy, since mice bearing nonpalpable tumors 12 to 13 days postinoculation with 10⁶ viable tumor cells were cured by CY therapy. Furthermore, CY therapy of mice bearing large (19-mm) tumors was not curative for mice that had been treated previously when their tumors were nonpalpable. A curative injection of CY into mice bearing large tumors resulted in an augmented ability of their spleen cells to mount a cytotoxic antitumor response upon in vitro immunization with mitomycin C-treated stimulator tumor cells. This was not further augmented by depletion of glass-adherent cells and was accompanied by a decrease in the percentage of cells bearing surface MOPC-315 myeloma protein in the spleen. The level of antitumor cytotoxicity exhibited by in vitro-immunized spleen cells from CY-treated mice was equivalent to that exhibited by in vitro-immunized spleen cells from untreated tumor-bearing mice that prior to in vitro immunization were depleted of glass-adherent cells. Since depletion of glass-adherent cells from tumor bearer spleen cells prior to in vitro immunization was shown to result in greater augmentation of antitumor cytotoxicity than that obtained by depletion of tumor cells (25), the present data suggest that in addition to the drug's tumoricidal activity, it also eliminates other suppressor elements in the spleen. Mice cured of tumors following CY therapy exhibited a high degree of antitumor immunity as judged in vivo by their ability to reject a large tumor challenge and in vitro by the ability of their spleen cells to mount a "secondary type" antitumor response upon in vitro immunization.

INTRODUCTION

A number of reports demonstrate the necessity for an active antitumor response for the eradication of established tumors by CY therapy (7, 19, 26, 28). Several mechanisms for synergism between chemotherapy and host antitumor immunity have been suggested. Thus, the drug might (a) reduce tumor burden to a level whereby existing host antitumor immunity can eliminate residual tumor cells (28), (b) slow tumor growth long enough to allow development of a potent antitumor response (7), (c) render residual tumor cells more susceptible to immune lysis (3), and (d) potentiate cytotoxic immunity by elimination of suppressor elements that interfere with an effective antitumor immune response (12).

Progression of tumor growth can be attributed to either inefficient in vivo sensitization or the appearance of suppressor elements that interfere with an effective antitumor response. Such suppressor elements were first described in the serum of tumor-bearing hosts and included blocking antibodies, free antigen shed by tumor cells, or antigen-antibody complexes (1, 16). More recently, evidence has accumulated regarding the appearance of θ-positive (11) as well as θ-negative (13) suppressor lymphoid cells during progressive tumor growth. We have shown that in comparison to normal spleen cells, spleen cells from mice bearing large MOPC-315 tumors contain metastatic tumor cells as well as an increased percentage of macrophages and upon in vitro immunization exhibit reduced levels of antitumor cytotoxicity (22-24). Both cell types were implicated as suppressor cells in the MOPC-315 tumor system since depletion of tumor cells from tumor bearer spleen cells prior to in vitro immunization resulted in less augmentation of antitumor cytotoxicity than that obtained by depletion of glass-adherent cells which included the removal of most tumor cells and macrophages (25) and possibly suppressor T-cells (10).

In the present study, we evaluated the importance of timing CY therapy for its effectiveness in curing mice bearing MOPC-315 tumors. In addition, we evaluated whether augmentation in the antitumor potential of spleen cells observed following CY treatment of mice bearing large tumors can be attributed to the elimination of suppressor elements.

MATERIALS AND METHODS

Spleen Cell Suspensions. Spleen cell suspensions were prepared from spleens of normal female BALB/c mice (8 to 12 weeks old; Laboratory Supply Co., Indianapolis, Ind.) or from BALB/c mice bearing various sizes of s.c. MOPC-315 tumors. In any individual experiment performed, the spleens used in each group were obtained from at least 3 but usually 5 to 7 mice. Single-cell suspensions were prepared by mechanical disruption between glass slides as previously described (24), and the viability as determined by trypan blue dye exclusion (0.4%) always exceeded 95%.

Target Cells. Target cells used in the ⁵¹Cr release assay included MOPC-315 plasmacytoma, EL4 leukemia, and normal BALB/c spleen cells. MOPC-315 was maintained by serial s.c. inoculation in syngeneic BALB/c mice. Single-cell suspensions...
were prepared by mechanical disruption (24), and the viability as determined by trypan blue dye exclusion (0.4%) always exceeded 85%. EL4 was maintained in ascitic form by weekly i.p. injection into C57BL/6 mice (8 to 12 weeks old; Charles River Breeding Labs., Inc., Wilmington, Mass.). Normal BALB/c target cells were blast cells induced by stimulation with concanavalin A (5 μg/ml) for 48 to 72 hr as previously described (22).

Chemotherapy. CY (Cytoxan, Mead Johnson & Co., Evansville, Ind.) was dissolved in sterile distilled water (to a concentration of 20 mg/ml) and further diluted in MEM (Grand Island Biological Co., Grand Island, N. Y.). Various doses of the drug ranging from 5 to 300 mg/kg were injected i.p. into MOPC-315 tumor-bearing mice. Mice were dosed 3 times weekly for the presence of tumors, and mice that had no detectable tumors 60 days after therapy were considered to be cured.

In Vitro Immunization. The in vitro method for generating antitumor cytotoxicity in lymphoid cells was described previously (6, 24). Briefly, responder spleen cells (75 × 10⁶) were cultured with or without mitomycin C-treated MOPC-315 stimulator tumor cells (2.5 × 10⁶) at 37 °C in a humidified atmosphere of 5% CO₂ in air for 5 days unless otherwise specified.

Antitumor Cytotoxicity Assay. The cell-mediated lysis was determined as we have previously described (24) utilizing the 3.5-hr in vitro ⁵¹Cr release assay (5). The percentage of specific ⁵¹Cr release was assessed by the following formula:

\[
\text{% of specific } ^{51}\text{Cr release} = \frac{T - C}{M - C} \times 100
\]

where T is the percentage of release with test spleen cells; C is the percentage of spontaneous release by the target tumor cells alone which ranged between 14 and 18%; and M is the percentage of maximum ⁵¹Cr release obtained by 3 cycles of freezing and thawing, which ranged between 78 and 81%. Each experiment was performed 2 to 4 times. We have observed, as have others (6, 21), that the level of antitumor cytotoxicity obtained with spleen cells immunized in vitro under identical conditions may vary substantially from one experiment to another. Still, the effect of treating tumor-bearing mice with CY was consistent. Therefore, in order to obtain a more accurate comparison of the levels of antitumor cytotoxicity exhibited by in vitro-immunized spleen cells at different times following CY therapy, the level of antitumor cytotoxicity exhibited by experimental groups was compared with respect to that of parallel cultures of in vitro-immunized normal spleen cells. The following formula was used:

\[
\text{% of corrected } ^{51}\text{Cr release} = \frac{E}{N} \times \bar{N}
\]

where E is the percentage of specific release with test spleen cells; N is the percentage of specific release with normal spleen cells immunized in parallel; and \( \bar{N} \) is the average percentage of specific release with all the in vitro-immunized normal groups in the serial study. The level of antitumor cytotoxicity is presented as the mean ⁵¹Cr release of triplicate samples ± the S.E. Variations in the ⁵¹Cr release between individual samples rarely exceed 9% of the mean. All points that differed by 5% release or more from the level exhibited by in vitro-immunized normal spleen cells were judged to be significantly different by the Student's t test (\( p = 0.05 \)).

Enumeration of Cells Rosettable with TNP-SRBC. Since MOPC-315 tumor cells possess high-affinity surface IgA with specificity for nitrophenyl compounds, we enumerated tumor cells in tumor bearer spleen cells by utilizing their ability to rosette with TNP-SRBC. Trinitrophenol was coupled to sheep RBC by the method of Rittenberg and Pratt (29) as modified by Hannestad et al. (15). The rosetting procedure used was essentially the method described by Hannestad et al. (15). The percentage of rosettes in spleen cell suspensions was determined by counting triplicate samples of 300 spleen cells.

Depletion of Cells Adherent to Glass Wool. Tumor bearer spleen cells (8 × 10⁶) suspended in 30 ml warmed MEM supplemented with 20% fetal calf serum were applied to individual 50-ml sterile glass wool columns. Columns were incubated at 37 °C for 45 min with occasional rotation. Nonadherent spleen cells were eluted in 100 ml of warmed MEM. The yield of cells recovered from the columns was about 15 to 25%.

Determination of the Percentage of Macrophages. The percentage of macrophages in spleen cell suspensions was determined by morphological criteria by counting at least 2500 but usually 5000 nucleated cells on Wright-stained smears. Enumeration of macrophages by functional criteria was done by determining the percentage of cells that ingested latex beads (Difco Laboratories, Detroit, Mich.) according to the method of Rosenstreich et al. (30). More than 90% of the cells identified as macrophages by morphological criteria phagocytized latex beads (22).

RESULTS

Determination of the Minimum Dose of CY Required for the Cure of BALB/c Mice Bearing Large MOPC-315 Tumors. Mice bearing 20-mm tumors were given a single i.p. injection of various doses of CY ranging from 5 to 300 mg/kg (Table 1). Treatment of mice with 5 mg/kg cured only 17% of the mice, whereas treatment of mice with 15 to 200 mg/kg cured most mice. Treatment of mice with 300 mg/kg resulted in toxicity, and although some reduction in tumor size was observed following CY treatment, 67% of the mice died. Therefore, in further experiments, we have treated tumor-bearing mice with CY (15 mg/kg) which was determined to be the lowest curative dose.

Effectiveness of CY Therapy for Mice Bearing Various Sizes of MOPC-315 Tumors. Initially, mice bearing either nonpalpable (Day 4 tumor bearers) or large (19-mm, Days 10 to 14) s.c. MOPC-315 tumors were treated with a single i.p. injection of CY (15 mg/kg) (Table 2). Inoculation of 3.5 × 10⁶
viable MOPC-315 tumor cells into normal BALB/c mice resulted in progressively growing tumors that killed the mice in about 21 days. Treatment of mice bearing nonpalpable tumors cured 12% of the mice and extended the survival of the remainder by 12 days, whereas treatment of mice bearing large tumors cured 81% of the mice and extended the survival of the remainder by 21 days. Large tumors regressed within 10 to 14 days after CY treatment and were nondetectable during the 60 days posttherapy. Next, the effectiveness of a single i.p. injection of CY (15 mg/kg) for curing mice bearing various sizes of s.c. MOPC-315 tumors was evaluated (Table 3). As above, treatment of mice bearing nonpalpable (Day 4) tumors cured only relatively few mice (7%). Treatment was progressively more curative when administered to mice bearing increasingly larger tumors such that treatment of mice bearing 5-mm tumors cured 47% of the mice, whereas treatment of mice bearing 25-mm tumors cured 92%. Thus, CY therapy with the selected dose was more successful in curing mice bearing larger MOPC-315 tumors than those bearing smaller tumors.

### Effect of Time Interval between Tumor Inoculation and CY Administration on the Success of Therapy

Experiments were performed to determine whether the increasing effectiveness of CY therapy with progression of tumor growth could be attributed to differences in the time of drug therapy after tumor inoculation or whether the effectiveness could be attributed to the tumor size at the time of therapy (Table 4). BALB/c mice were inoculated with various numbers of MOPC-315 tumor cells ranging from $1 \times 10^5$ to $3.5 \times 10^6$, and 12 to 13 days later when the resulting tumor sizes ranged from nonpalpable to 24 mm, they received a single i.p. injection of CY (15 mg/kg). Whereas CY therapy of mice bearing nonpalpable tumors 4 days after tumor inoculation was essentially noncurative, therapy of mice bearing nonpalpable tumors 12 to 13 days after inoculation with $10^6$ tumor cells cured most mice (7% versus 87%). Furthermore, CY therapy given to mice bearing 11-, 17-, or 24-mm tumors 12 to 13 days post-tumor inoculation was equally curative (87, 87, and 100% cured, respectively). Thus, the effectiveness of CY therapy can be attributed to the time of CY administration post-tumor inoculation rather than to the tumor size at the time of therapy.

### Effectiveness of CY Therapy for Mice Bearing Large MOPC-315 Tumors That Were Not Cured by Previous Therapy when They Had Nonpalpable Tumors

Since CY therapy was relatively curative for mice bearing 10- to 25-mm tumors but not for mice bearing nonpalpable (Day 4) tumors, experiments were performed to determine whether mice not cured by CY when they had nonpalpable tumors would be cured by a second injection of CY (15 mg/kg) when their tumors reached 19 mm (Table 5). As above, CY treatment of mice bearing 19-mm tumors cured most mice (76%), whereas treatment of mice bearing 19-mm tumors that had received CY treatment when they had nonpalpable tumors cured only 7% of the mice. Thus, therapy of mice bearing large MOPC-315 tumors with CY is curative only if the mice were not treated with the drug at early stages after tumor inoculation.

### Effect of CY Therapy of Mice Bearing Large MOPC-315 Tumors on the in Vitro Antitumor Potential of Their Spleen Cells

Mice bearing 20-mm tumors were treated with CY, and 14 days later when the tumors had completely regressed, spleen cells were obtained. The spleen cells were immunized in vitro with mitomycin C-treated tumor cells for 5 days and subsequently assayed for in vitro antitumor cytotoxicity. The level of cytotoxicity obtained was compared to that exhibited by in vitro-immunized spleen cells from normal mice or from untreated mice bearing 20-mm tumors (Table 6). In vitro-immunized spleen cells from untreated tumor-bearing mice exhibited a lower level of antitumor cytotoxicity than that exhibited by in vitro-immunized spleen cells from normal mice (32% versus 48%) (in confirmation of our previous studies (21–23).

#### Table 2

<table>
<thead>
<tr>
<th>Time of therapy</th>
<th>Effect of therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days post-tumor inoculation</td>
<td>Size of tumors (mm ± S.E.)</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Untreated</td>
<td>0</td>
</tr>
<tr>
<td>4 Nonpalpable</td>
<td>12</td>
</tr>
<tr>
<td>10-14</td>
<td>19.1 ± 2.4</td>
</tr>
</tbody>
</table>

#### Table 3

<table>
<thead>
<tr>
<th>Size of tumors at therapy (mm ± S.E.)</th>
<th>Cured (%)</th>
<th>No. of cured/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor bearer controls</td>
<td>0</td>
<td>0/15</td>
</tr>
<tr>
<td>Nonpalpable</td>
<td>7</td>
<td>1/15</td>
</tr>
<tr>
<td>4.9 ± 0.2</td>
<td>47</td>
<td>7/15</td>
</tr>
<tr>
<td>9.9 ± 0.2</td>
<td>73</td>
<td>11/15</td>
</tr>
<tr>
<td>17.2 ± 0.2</td>
<td>77</td>
<td>10/13</td>
</tr>
<tr>
<td>25.2 ± 0.3</td>
<td>92</td>
<td>12/13</td>
</tr>
</tbody>
</table>

#### Table 4

<p>| Effectiveness of CY therapy for mice bearing various sizes of s.c. tumors |
|-------------------------------------|------------------|------------------|</p>
<table>
<thead>
<tr>
<th>No. of tumor cells inoculated</th>
<th>Days post-tumor inoculation</th>
<th>Sizes of tumors (mm ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x 10^5</td>
<td>12-13</td>
<td>Nonpalpable</td>
</tr>
<tr>
<td>5 x 10^5</td>
<td>12-13</td>
<td>10.8 ± 0.5</td>
</tr>
<tr>
<td>1 x 10^5</td>
<td>12-13</td>
<td>17.1 ± 0.5</td>
</tr>
<tr>
<td>3.5 x 10^6</td>
<td>12-13</td>
<td>23.3 ± 0.4</td>
</tr>
<tr>
<td>3.5 x 10^6</td>
<td>4</td>
<td>Nonpalpable</td>
</tr>
</tbody>
</table>

#### Table 5

| Effectiveness of CY therapy for mice bearing large MOPC-315 tumors not cured by previous therapy when they had nonpalpable tumors |
|-------------------------------------|------------------|------------------|
| Experimental groups | Cured (%) | No. of cured/total |
| Untreated tumor bearers | 0 | 0/15 |
| Treated large-tumor bearers | 76 | 19/25 |
| Treated nonpalpable-tumor bearers | 7 | 1/15 |
| Double-treated tumor bearers | 7 | 1/15 |

---

*a CY (15 mg/kg) i.p.*

*b Mice treated once when tumors were 19.4 ± 0.6 mm.

*c Mice treated once 4 days after inoculation with tumor cells.

*d Mice were treated 4 days after inoculation with tumor cells, and those not cured were treated again when tumors reached 19.3 ± 0.4 mm.
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Table 6

<table>
<thead>
<tr>
<th>In vitro-immunized spleen cells from</th>
<th>Antitumor cytotoxicity (%) of specific (^{51}Cr) release ± S.E.¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mice</td>
<td>47.9 ± 0.9</td>
</tr>
<tr>
<td>Untreated tumor bearers²</td>
<td>31.6 ± 0.3</td>
</tr>
<tr>
<td>Treated tumor bearers³</td>
<td>93.9 ± 0.3</td>
</tr>
</tbody>
</table>

² At an effector/target cell ratio of 100/1.
³ Mice bearing s.c. tumors with diameters of 19.8 ± 0.3 mm.
⁴ Mice bearing s.c. tumors with diameters of 19.8 ± 0.3 mm received injections of CY (15 mg/kg) i.p. 14 days prior to in vitro immunization.

23].) CY treatment of tumor-bearing mice enabled their spleen cells to develop upon in vitro immunization a much higher level of antitumor cytotoxicity than that exhibited by either in vitro-immunized spleen cells from untreated tumor-bearing mice or in vitro-immunized spleen cells from normal mice (94% versus 32% and 48%, respectively). Thus, CY treatment of mice bearing large tumors results in augmented antitumor potential of their spleen cells.

Determination of the Optimal Duration of In Vitro Immunization for the Development of In Vitro Antitumor Cytotoxicity in Spleen Cells from Mice That Rejected Large Tumors following CY Treatment. Mice bearing 19-mm tumors were treated with CY, and 21 days later when their tumors had completely regressed ("cured" mice), spleen cells were obtained. These spleen cells or spleen cells from normal mice were evaluated for antitumor cytotoxicity prior to, or 1 to 7 days after, the initiation of in vitro culturing in the presence or absence of stimulator tumor cells (Chart 1). Freshly prepared spleen cells from normal mice were noncytotoxic, whereas those from "cured" mice exhibited some cytotoxicity (0% versus 7%) which was maintained during the 7 days of culturing in the absence of stimulator tumor cells. In vitro immunization of normal spleen cells resulted in the appearance of antitumor cytotoxicity on the fourth day of culture (31%) which reached a peak on the fifth day and was nondetectable by the seventh day (<5%) [as we had previously shown (23)]. In vitro immunization of spleen cells from "cured" mice resulted in a substantial augmentation of antitumor cytotoxicity (as compared to that exhibited by unimmunized spleen cells) as early as the second day of in vitro immunization (25% versus 8%) which reached maximal levels on the fourth day (100%) and was maintained at high levels through the seventh day (85 to 100%).

On Days 2 through 7 of the immunization process, the levels of antitumor cytotoxicity exhibited by in vitro-immunized spleen cells from "cured" mice when tested at an effector/target cell ratio of 100/1 or 20/1 were much higher than those exhibited by in vitro-immunized normal spleen cells tested at 100/1. Thus, spleen cells from mice that following CY therapy rejected large tumors respond to in vitro stimulation with tumor cells in a manner similar to a secondary response. It is also clear from these data that the optimal length of in vitro immunization for the development of antitumor cytotoxicity with spleen cells from normal mice is 5 days and with spleen cells from "cured" mice is 4 to 7 days. Therefore, in further experiments, in vitro immunization was performed for 5 days to allow a better comparison of the levels of antitumor cytotoxicity exhibited by in vitro-immunized spleen cells from CY-treated mice with those exhibited by in vitro-immunized normal spleen cells.

Effect of CY Therapy of Mice Bearing Large Tumors on the Presence of Suppressor Elements in the Spleens. Experiments were performed to determine how soon after CY therapy of tumor-bearing mice their spleen cells exhibit augmented antitumor potential and whether this augmented potential is due to elimination or inactivation of suppressor elements. Spleen cells were obtained from normal mice, mice bearing 24-mm tumors, or mice that had 24-mm tumors when treated with CY and their spleens removed 2, 4, or 7 days posttreatment (Table 7). Spleen cells were examined for the percentage of macrophages and tumor cells to determine whether the augmented cytotoxic potential correlates with a decrease in the percentage of such cells. In addition, we evaluated the effect of removing glass-adherent cells from the spleens of untreated or CY-treated tumor-bearing mice on the level of antitumor cytotoxicity obtained upon in vitro immunization. Identification of tumor cells was done utilizing their ability to form rosettes with TNP-SRBC. Normal spleen cells which contained 3.6% macrophages and 2.8% rosettable cells exhibited a substantial level of antitumor cytotoxicity upon in vitro immunization (81%). Spleen cells from untreated tumor-bearing mice which contained a higher percentage of macrophages (11%) and rosettable cells (11%) exhibited lower levels of antitumor cytotoxicity obtained with unfractionated or glass-nonadherent spleen cells from similar mice.

Chart 1. The levels of antitumor cytotoxicity exhibited by spleen cells from normal mice or CY-cured mice upon culturing in the presence or absence of stimulator tumor cells.

Table 7

<table>
<thead>
<tr>
<th>Days post-therapy</th>
<th>% of rosettable cells ± S.E.</th>
<th>% of macrophages</th>
<th>Unfractionated spleen cells</th>
<th>Glass-nonadherent spleen cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.6 ± 0.4</td>
<td>10.8 ± 0.9</td>
<td>23.5 ± 0.6</td>
<td>91.4 ± 1.1</td>
</tr>
<tr>
<td>2</td>
<td>2.6 ± 0.4</td>
<td>11.4 ± 0.9</td>
<td>59.9 ± 0.5</td>
<td>57.4 ± 1.1</td>
</tr>
<tr>
<td>4</td>
<td>3.2 ± 0.4</td>
<td>9.6 ± 0.6</td>
<td>85.7 ± 0.7</td>
<td>85.7 ± 1.1</td>
</tr>
<tr>
<td>7</td>
<td>3.0 ± 0.6</td>
<td>7.4 ± 0.1</td>
<td>100.3 ± 1.6</td>
<td>60.7 ± 1.8</td>
</tr>
</tbody>
</table>

a CY (15 mg/kg) i.p.
b At an effector/target cell ratio of 100/1.

cytotoxic release ± S.E.
antitumor cytotoxicity upon in vitro immunization (24%). As early as 2 days post-CY treatment, the percentage of rosettable cells dropped to background levels (2.6%) while the percentage of macrophages was virtually unchanged (11%), and upon in vitro immunization of the spleen cells, augmented levels of antitumor cytotoxicity were obtained (60% versus 24%). Between Days 2 and 7 post-CY treatment, further augmentation of the antitumor potential of tumor bearer spleen cells was observed, yet a drop in the percentage of macrophages was first noted on Day 7 (7% versus 11%).

Removal of suppressor elements from the spleens of MOPC-315 tumor-bearing mice was achieved in our previous studies by depletion of glass-adherent cells (22). Here, depletion of glass-adherent cells prior to in vitro immunization resulted in a higher level of antitumor cytotoxicity than that exhibited by unfraccionated spleen cells when spleen cells from untreated but not CY-treated tumor-bearing mice were used. Moreover, when glass-adherent cells were depleted from the spleens of mice treated with CY 7 days prior to in vitro immunization, the level of antitumor cytotoxicity was lower than that exhibited by the unfraccionated in vitro-immunized spleen cells (61% versus 100%). Thus, CY treatment results in the elimination of the glass-adherent suppressor elements that operate in the spleens of MOPC-315 tumor-bearing mice.

In Vitro Cytotoxicity of in Vitro-Immunized or Unimmunized Spleen Cells from Mice Cured of Large Tumors following CY Therapy when Evaluated against MOPC-315, EL4, or Normal BALB/c Target Cells. Mice that following CY therapy rejected 20-mm tumors were used as spleen cell donors 21 days after the initiation of treatment. The spleen cells were cultured in vitro in the presence or absence of stimulator tumor cells and subsequently were tested for their cytotoxicity against MOPC-315, EL4 of C57BL/6 origin, or normal BALB/c target cells (Table 8). The normal BALB/c target cells used were blast cells induced by stimulation with concanavalin A and were susceptible to lysis by C57BL/6 spleen cells immunized against MOPC-315 but resistant to lysis by either C57BL/6 or BALB/c spleen cells immunized with EL4 (data not shown). Unimmunized spleen cells from ‘cured’ mice exhibited appreciable levels of cytotoxicity against MOPC-315 (15%) but no cytotoxicity against EL4 or normal BALB/c target cells (less than 3%). In vitro immunization of spleen cells from ‘cured’ mice resulted in augmented anti-MOPC-315 cytotoxicity (95%) and virtually no cytotoxicity against EL4 or normal BALB/c blast cells (1% or 4%, respectively).

Ability of Mice ‘Cured’ by CY of Large MOPC-315 Tumors to Reject a Lethal Challenge with Viable MOPC-315 Tumor Cells. Mice that following CY treatment rejected 21-mm tumors were challenged 21 to 54 days later with $3.5 \times 10^6$ viable MOPC-315 tumor cells (Table 9). The challenge dose is 350-fold greater than the minimum dose required for the appearance of lethal tumors in all mice receiving injections (4). None of the mice that were ‘cured’ of large tumor by CY developed tumors following challenge with viable tumor cells. Thus, animals that following CY treatment were ‘cured’ of MOPC-315 tumor exhibit an augmented potential to mount an in vivo anti-MOPC-315 tumor response.

DISCUSSION

In the present study, we have shown that the timing of CY administration to MOPC-315 tumor-bearing mice is critical for successful therapy. Following inoculation with $3.5 \times 10^6$ viable tumor cells, a single i.p. injection of CY (15 mg/kg) into mice bearing nonpalpable (Day 4) tumors extended their survival but cured only a few of the mice, whereas injection of the same dose of CY into mice bearing 10- to 25-mm (Days 8 to 14) tumors cured most of the mice and extended the survival of the remainder. The time interval between tumor inoculation and CY administration, rather than the size of the tumor, was found to be critical for successful therapy since mice bearing nonpalpable tumors 12 to 13 days postinoculation with $10^6$ viable tumor cells were cured by CY (15 mg/kg). In addition, CY therapy of mice bearing large tumors was not successful if the mice had been treated previously with CY when their tumors were nonpalpable. A curative injection of CY into mice bearing large tumors resulted in an augmented ability of their spleen cells to mount a cytotoxic antitumor response upon in vitro immunization. This was not further augmented by depletion of glass-adherent cells and was accompanied by a decrease in the percentage of cells bearing surface MOPC-315 myeloma protein in the spleen. The level of antitumor cytotoxicity exhibited by in vitro-immunized spleen cells from CY-treated mice was equivalent to that exhibited by spleen cells from untreated tumor-bearing mice that were depleted of glass-adherent cells prior to in vitro immunization. Following tumor regression, mice exhibited a high degree of antitumor immunity in vivo by the ability to reject a large tumor challenge and in vitro by the ability of their spleen cells to mount a ‘secondary type’ anti-tumor response upon in vitro immunization.

A single injection of at least 15.5 mg of CY per kg was required to cure mice bearing large MOPC-315 tumors. This remarkable sensitivity to relatively low doses of CY is not confined to the MOPC-315 plasmacytoma since the MOPC-104E (18), the Adj PC-5 (27), and the Adj PC-6 (9) plasmacytomas are also extremely sensitive to CY therapy.

CY therapy of mice shortly after tumor inoculation was less curative than was therapy of mice at a later time. It seems

| Source of spleen cells | Cytotoxicity (% of specific $^{51}$Cr release ± S.E.)
|------------------------|----------------------
|                        | MOPC-315 | EL4 | Normal
| Treated mice$^a$       | None      | 15.3 ± 1.3 | 0.5 ± 0.7 | 1.0 ± 0.3
| Treated mice$^b$       | MOPC-315  | 94.8 ± 2.8 | 0.6 ± 0.7 | 3.7 ± 1.1

$^a$ At an effector/target cell ratio of 100/1.
$^b$ As normal target cells, concanavalin A-induced blast cells were used.
$^c$ Mice bearing s.c. tumors with diameters of 20.1 ± 0.5 mm were given injections of CY (15 mg/kg) i.p. 19 days prior to in vitro immunization.

| Ability of mice ‘cured’$^a$ by CY of 21-mm tumors to reject a lethal challenge with $3.5 \times 10^6$ viable MOPC-315 tumor cells$^b$
|-----------------|--------|----------------|
| Mice            | Survival (%) | No. of survivors/total
| Normal          | 0/15   |
| ‘Cured’ mice$^a$| 100/15 |

$^a$ 21 days to 54 days after CY therapy of 15 mg/kg i.p.
$^b$ Three hundred fifty-fold greater than the minimum tumor dose required for the appearance of lethal tumors in all mice inoculated.

Table 9
unlikely that tumor regression in mice bearing large tumors is caused by a direct tumoricidal action of the drug alone. An alternative and more probable explanation is that at late stages of tumor growth, tumor bearers have mounted an antitumor response that facilitates the effectiveness of CY therapy. As well, the possibility that the ineffectiveness of CY therapy for mice bearing nonpalpable tumors is due to the presence of CY-resistant suppressor cell(s) or weak antitumor potential should be considered. The requirement for an active antitumor response to facilitate the action of CY has been reported by others (7, 8, 19, 26, 28). CY was more effective when administered to tumor-bearing animals that exhibited some antitumor immunity due to previous immunization than to unimmunized tumor-bearing counterparts (7, 26). Furthermore, CY treatment of tumor bearers that were immunosuppressed [by anti-thymocyte serum (14, 28), X-irradiation (19, 28), or high doses of the drug (20)] has been reported to be less effective than similar treatment of immunocompetent tumor bearers.

We have previously shown that in vitro-immunized spleen cells from mice bearing large MOPC-315 tumors exhibited much lower levels of in vitro antitumor cytotoxicity than did in vitro-immunized spleen cells from normal mice (22–24). We have suggested (25) that this reduced ability of tumor bearer spleen cells to respond in vitro to stimulator tumor cells may be attributed to the action of: (a) metastatic tumor cells in the spleen capable of inhibiting both the generation and expression (by cold target inhibition) of antitumor cytotoxicity; (b) an increased percentage of splenic macrophages capable of inhibiting the generation of antitumor cytotoxicity; and (c) possibly suppressor T-lymphocytes. Removal of such cells from MOPC-315 tumor bearer spleen cells was achieved by fractionation of the spleen cells on glass wool prior to in vitro immunization (22, 23, 25). Thus, in the MOPC-315 tumor system, the elimination of such cells appears to be required for CY therapy to act synergistically with a cytotoxic antitumor response. Indeed, CY therapy of mice bearing large tumors resulted in a rapid disappearance of metastatic tumor cells in the spleen which was accompanied by augmentation of antitumor potential that could not be further augmented by fractionation of the spleen cells on glass wool. These data indicate that glass-adherent cells had been eliminated during the course of CY therapy.

Therapy of mice bearing large MOPC-315 tumors with CY was curative only if the mice were not treated with the drug at early stages after tumor inoculation. Preliminary data5 suggest that one effect of the administration of CY to mice bearing Day 4, nonpalpable tumors may be the selection of CY-resistant tumor cells, and this might explain the ineffectiveness of the second dose of CY when the tumors are large. This question is currently under further investigation.

The importance of the time of CY administration relative to immunization with allogeneic (2) or syngeneic (12) tumor cells on the strength of the antitumor response obtained was evaluated by other investigators. Administration of CY prior to immunization resulted in the elimination of antigen-nonspecific suppressor cells and led to the development of stronger antitumor responses than those exhibited by untreated, immunized counterparts (12). On the other hand, administration of CY to animals 2 to 6 days after immunization led to elimination of antigen-stimulated prekiller or killer cells and resulted in weaker antitumor responses than those exhibited by immunized, untreated counterparts (2, 12). In these studies, the effect of administering CY to animals later than 6 days after immunization was not evaluated. Such experiments should be performed since it is possible that administration of CY to immunized animals at a stage when the immune response is being turned off and when suppressor cells are actively proliferating will result in potentiation of this response due to elimination of suppressor cells.

In our previous studies, the reduced ability of in vitro-immunized spleen cells from MOPC-315 tumor-bearing mice to mediate antitumor cytotoxicity correlated with the appearance of metastatic tumor cells and an increase in the percentage of macrophages in the spleen (25). Since we have found that depletion of tumor cells from tumor bearer spleen cells prior to in vitro immunization resulted in less augmentation in the level of antitumor cytotoxicity than that obtained by depletion of glass-adherent cells, which included the removal of most macrophages and tumor cells, both cells were implicated as suppressor cells in the generation of antitumor cytotoxicity in the MOPC-315 tumor system (25). Further evidence for the contribution of the tumor cell to the reduced levels of antitumor cytotoxicity of in vitro-immunized tumor bearer spleen cells is illustrated here by the correlation between the rapid disappearance of cells bearing MOPC-315 myeloma protein from the spleen and rapid augmentation of antitumor potential in spleen cells from tumor-bearing mice following CY therapy. In addition, we have previously shown that depletion of viable tumor cells to the in vitro immunization culture of normal spleen cells inhibits the generation of antitumor cytotoxicity (25). However, the macrophage may not be involved in suppression since (a) the percentage of macrophages began to decline only on Day 7 post-CY treatment when the antitumor potential of tumor bearer spleen cells is almost fully developed, and (b) depletion of glass-adherent cells from the spleens of mice treated 2 days prior to the initiation of in vitro immunization did not augment the antitumor cytotoxicity beyond that obtained with the unfractionated, in vitro-immunized cells. Nevertheless, the possibility that the macrophage requires the presence of viable tumor cells to be suppressive cannot be ruled out as suggested by Kolb et al. (17) for the inhibition of primary antibody responses by suppressor macrophages, the appearance and expression of which are induced by plasmacytoma cells. This is supported by preliminary data5 that suggest that addition of tumor bearer macrophages but not of normal macrophages can inhibit the generation of antitumor cytotoxicity by normal spleen cells upon in vitro immunization. In addition, the possibility that glass-adherent suppressor T-lymphocytes operate in the MOPC-315 tumor system should be considered (10). Thus, the effectiveness of CY therapy for curing mice bearing large MOPC-315 tumors appears to be due, at least in part, to elimination or inactivation of tumor cells and macrophages. Metastatic tumor cells are apparently eliminated by a tumoricidal action of the drug, and macrophages and perhaps other cells are probably rendered inactive by either a toxic effect of the drug or their dependence on viable tumor cells for activity.

We have shown that a drug given at the “wrong” time did not cure mice with relatively small (nonpalpable) tumors and leaves them resistant to further treatment by the drug when the tumor enlarges. Yet, the same dose of drug given at the “right”

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time cures mice with relatively large tumors and leaves them immune to further tumor challenge. This phenomenon apparently has an immunological basis in that the observations correlated with the ability of spleen cells from these mice to mount an *in vitro* cytotoxic immune response to the tumor. In view of these findings, it seems of immediate interest to investigate how timing of drug therapy affects the immunological response and cure rates in other tumor systems.

REFERENCES


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Importance of Timing in Cyclophosphamide Therapy of MOPC-315 Tumor-bearing Mice

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