Enhancement of the Effectiveness of Lyt-2+ T-Cells for Adoptive Chemoimmunotherapy by Short-Term Exposure of Tumor-bearer Spleen Cells to Polyethylene Glycol and/or Melphalan

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ABSTRACT

Uncultured tumor-infiltrated spleen cells (TISpC) from mice bearing large (20–22 mm) s.c. MOPC-315 plasmacytomas were previously shown to be ineffective in bringing about the cure of mice bearing a nonpalpable (Day 4) tumor that had been treated with a subcutaneous dose (10 mg/kg) of cyclophosphamide (i.e., adoptive chemoimmunotherapy, ACIT) (M. B. Mokyr, J. C. D. Hengst, and S. Dray, Cancer Res., 42: 974–979, 1982). Here we show that TISpC cultured for 5 days in the presence of inactivated MOPC-315 stimulator cells acquire some effectiveness in curing mice by ACIT, and this effectiveness is greatly enhanced if polyethylene glycol 6000 (PEG) is also added to the culture. The Lyt 2+ T-cells, and not the L3T4+ T-cells, are responsible for the effectiveness of the cultured TISpC in ACIT. In fact, the L3T4+ T-cells are apparently not required even during culture of TISpC for the generation of Lyt 2+ T-cells effective in ACIT. Although the TISpC cultured with MOPC-315 cells and PEG contained approximately twice as many Lyt 2+ cells as did TISpC cultured without PEG, the increase in the activity of the former cells is not due simply to the increase in the percentage of Lyt 2+ cells, but is most likely due to an increase in the percentage and/or activity of Lyt 2+ cells with specificity for MOPC-315-associated antigens. The effectiveness of TISpC cultured with MOPC-315 stimulator cells and PEG in ACIT can be enhanced even further by pretreatment of these cells with the immunomodulating agent melphalan (0.5 nmol/ml) prior to culture initiation. Thus, the above methods of culture render ineffective lymphoid cells effective in ACIT and are suitable for evaluation in protocols for human cancer therapy.

INTRODUCTION

Many investigators, employing various tumor models, have been successful in curing animals bearing small established tumors by the use of chemotherapy in conjunction with adoptive transfer of T-cells (i.e., by ACIT) (1–6). The T-cells employed in most of these studies were derived from donors that were highly immune to their tumor (1–5), e.g., mice that had been cured of their tumors and were resistant to a challenge with the same tumor (6). In the MOPC-315 tumor model, the donors of the highly immune lymphoid cells were mice that were in the process of eradicating a large s.c. MOPC-315 tumor as a consequence of low-dose CY therapy (6). The high potency of antitumor immunity in these CY-treated MOPC-315 tumor bearers was illustrated by the fact that the antitumor immunity was responsible for the eradication of a very large tumor burden remaining after clearance of the drug and its active metabolites from the circulation (7).

In the MOPC-315 tumor model, like in many other tumor models (8–10), lymphocytes from untreated tumor bearers are not effective in ACIT (11). In the present study, methods were sought to render spleen cells from MOPC-315 tumor bearers effective in ACIT. For this purpose, we subjected the TISpC to short-term culture with inactivated MOPC-315 tumor cells in the presence of the hydrophilic polymer PEG. PEG was selected for this investigation because of previous observations that the presence of PEG during short-term culture of MOPC-315 tumor bearer spleen cells with MOPC-315 tumor cells enhances the effectiveness of the TISpC in neutralizing MOPC-315 tumor growth in the local adoptive transfer assay (12).

In an attempt to further enhance the effectiveness of TISpC in ACIT, we also exposed the TISpC to the immunomodulating agent melphalan prior to their culture with MOPC-315 tumor cells and PEG. The rationale for selecting melphalan for this investigation was the possibility that the ineffectiveness of spleen cells from mice bearing a large tumor in ACIT was due to active immunosuppression (9, 13). Accordingly, if suppressor cells are responsible for the ineffectiveness of spleen cells from MOPC-315 tumor bearers in ACIT, then melphalan may be capable of shifting the balance from immunosuppression in favor of generating antitumor immunity effective in tumor eradication. The immunomodulatory potential of melphalan for spleen cells from MOPC-315 tumor bearers is supported by previous observations that pretreatment of TISpC from MOPC-315 tumor bearers rendered the spleen cells capable of generating potent antitumor immunity as judged from the magnitude of MOPC-315 tumor cell lysis in the 4-h 51Cr release assay (14).

The emphasis in this report is placed on determining: (a) the effectiveness of TISpC in ACIT upon short term culture in the presence or absence of stimulator tumor cells and/or PEG; (b) the stage during tumor progression for obtaining spleen cells which upon short term culture can be rendered effective in ACIT; (c) the phenotype of the T-cell subpopulation(s) that mediate(s) the cure of recipient mice by ACIT; and (d) the T-cell subpopulation(s) which are necessary during culture for the generation of effective cells. Finally, we tested the ability of melphalan to potentiate the effectiveness in ACIT of TISpC cultured with MOPC-315 tumor cells in the presence of PEG.

MATERIALS AND METHODS

Animals and Tumors. The MOPC-315 plasmacytoma was maintained in female BALB/c mice (6–8 weeks old, Charles River Laboratory, Wilmington, MA) by s.c. passage as described previously (15). MOPC-315 tumor-bearing mice were routinely prepared by s.c. injection of 1 × 10⁶ MOPC-315 tumor cells. This inoculum is at least 300 times the minimal lethal tumor dose and it leads to the development of a palpable tumor by Day 5, to a 20–23 mm diameter s.c. tumor by Day 10–11, and to the death of the mice in 14.8 ± 0.7 (mean ± SE) days.

Spleen Cell Suspensions. Unless otherwise stated, the spleen cells used in ACIT were derived from mice bearing 20–22 mm s.c. MOPC-315 tumors resulting from inoculation with 1 × 10⁶ tumor cells. The
spleens from such mice contained metastatic tumor cells comprising approximately 6-10% of the spleen cell population (16). Single cell suspensions from the spleens of normal and tumor-bearing mice were prepared by mechanical disruption between glass slides. The spleen cell viability as determined by trypan blue dye exclusion (0.4%) always exceeded 95%. In each individual experiment, pooled spleen cells from at least 10 mice were used.

Short Term Spleen Cell Culture (in Vitro Immunization). The in vitro culture of lymphoid cells used in ACIT was done according to the methods we have described previously for the generation of an antitumor cytotoxic response (12, 15). Briefly, 40 x 10^6 spleen cells were cultured in the presence of 1.3 x 10^6 MOPC-315 stimulator tumor cells and 2% w/v PEG-6000 (BDH Chemicals, Poole, England) in 25-cm² tissue culture flasks (Corning Plastics, Corning, NY) at 37°C for 5 days in 20 ml of RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum (FBS), 1% nonessential amino acids, 50 units/ml penicillin, 50 µg/ml streptomycin (GIBCO; Grand Island Biological Co, Grand Island, NY) and 5 x 10^-5 M 2-mercaptoethanol. The stimulator tumor cells were inactivated by treatment with 50 µg/ml mitomycin C (SIGMA; Sigma Chemical Co., St. Louis, MO) at 37°C for 30 min. In some experimental groups, the PEG and/or the stimulator tumor cells were omitted from the culture medium as indicated. The number of cells recovered when TISpC were cultured for 5 days in the presence of PEG and stimulator tumor cells was approximately 20 x 10^6 cells and this was 1.5- to 2-fold greater than that recovered when the PEG was omitted from the culture.

In Vitro Exposure to Melphalan. TISpC were exposed to the alkylating agent melphalan (1-phenylalanine mustard; Burroughs Wellcome Co., Triangle Park, NC) under conditions that we have described previously as optimal for rendering immunosuppressed TISpC capable of developing a strong anti-MOPC-315 cytotoxic response upon short term culture with MOPC-315 stimulator cells (14). Briefly, melphalan was dissolved in sterile acid-alcohol (95% ethanol and 2% HCl at a ratio of 5/1) and further diluted in RPMI 1640 medium. Spleen cells at a concentration of 10 x 10^6 cells/ml were exposed to 0.5 nmol melphalan/ml at 37°C for 1 h. The cells were subsequently washed and put into short term culture.

ACIT. Recipient mice were injected s.c. with 1 x 10^6 MOPC-315 tumor cells on Day 0, and 4 days later were injected i.p. with a subcuterue dose of CY, 10 mg/kg (Cytovax; Mead Johnson Co., Evansville, IN). Donor spleen cells were administered i.v. to the mice by tail vein injection on Day 5. Mice were considered "cured" if they survived an observation period of 45 or 60 days as indicated and were tumor free.

In Vitro Depletion of T-Cells or T-Cell Subsets. Cultured or uncultured TISpC were depleted of T-cells or T-cell subsets by the use of monoclonal antibodies plus complement as described previously (17). Briefly, 1.5 x 10^7 spleen cells/ml were incubated at 4°C for 45 min with an equal volume of either anti-Thy 1.2 [supernatant of hybridoma 30-H12 (18)], anti-L3T4 [supernatant of hybridoma GK 1.5, rat IgG (19)], or anti-Lyt 2.2 [supernatant of hybridoma 3.155 (20)] antibody. Subsequently, low toxicity rabbit complement (Pel Freez, Brown Deer, WI) was added and the cells were incubated at 37°C for 45 min. The effectiveness and the selectivity of each treatment was verified by determining the percentage of Thy 1*, L3T4*, and Lyt 2* cells in a population of 10,000 viable cells by flow cytometric analysis (EPIC V; Coulter Electronics Inc., Hialeah, FL). In all experiments performed, the depletions with the monoclonal antibodies plus complement were effective and selective. In some experiments we also stained TISpC that had been treated with anti-L3T4 antibody and complement with FITC-conjugated goat anti-rat IgG antibody (Boehringer Mannheim, Indianapolis, IN) to determine if the L3T4* T-cells were actually eliminated or just coated by the antibody used for the depletion.

Statistical Analysis. To determine the significance of differences in the fraction of surviving animals between groups in ACIT experiments, the G test of independence (21) was used. To determine the significance of differences in the percentage of T-cells or their subsets among TISpC cultured under different conditions, Student's t test was employed. A P value of less than 0.05 was considered significant in both tests.

RESULTS

Effect of Adding PEG and MOPC-315 Stimulator Tumor Cells to Short Term Cultures of TISpC on their Ability to Cure Mice Bearing a MOPC-315 Tumor by ACIT. TISpC from mice bearing Day 10 (≥20 mm) MOPC-315 tumors or spleen cells from normal mice were cultured for 5 days with or without MOPC-315 stimulator tumor cells in the presence or absence of 2% PEG. The effectiveness of the spleen cells in ACIT was assessed by their ability to aid in the cure of an established MOPC-315 tumor in conjunction with a subcutaneous dose of CY (Table 1). Specifically, mice bearing a Day 4 (0-5 mm) s.c. MOPC-315 tumor were given a single i.p. injection of 10 mg/kg CY, and 1 day later the mice received an i.v. injection of either 50 x 10^6 or 10 x 10^6 cultured spleen cells. TISpC cultured with stimulator tumor cells were more effective in ACIT than normal spleen cells cultured under the same conditions (e.g., with 50 x 10^6 cells, 45 versus 14% cure). Inclusion of 2% PEG in cultures of TISpC and MOPC-315 tumor cells led to a substantial increase in cure rate compared to the same number of TISpC cultured in its absence (e.g., with 50 x 10^6 cells, 92 versus 45% cure). In fact, 10 x 10^6 TISpC cultured with MOPC-315 and PEG were at least, and even possibly more, effective than 50 x 10^6 TISpC cultured with stimulator cells alone (68 versus 45% cure). Inclusion of PEG in cultures of normal spleen cells and MOPC-315 stimulator cells did not significantly enhance the effectiveness of the spleen cells in curing mice by ACIT (e.g., with 50 x 10^6 cells, 21 versus 14% cure). In the absence of stimulator tumor cells, the presence of 2% PEG in the TISpC cultures did not significantly enhance the effectiveness of the TISpC in ACIT compared to TISpC cultured alone (e.g., with 50 x 10^6 cells, 20 versus 10% cure). In fact, the cure rate observed with TISpC cultured with PEG only was not significantly higher than that observed without adoptive cell
ADOPTIVE CHEMOIMMUNOTHERAPY

Transfer (e.g., with 50 x 10^6 cells, 20 versus 7% cure). Thus, short term culture of TISpC with stimulator tumor cells enhances their ability to cure recipient MOPC-315 tumor bearers in ACIT and the addition of PEG to the culture enhances this ability much further.

Effect of Tumor Progression on the Potential of Spleen Cells to Become Effective in ACIT upon Short Term Culture with MOPC-315 Tumor Cells and PEG. Experiments were performed to determine the stage of MOPC-315 tumor progression that was optimal for the generation of splenic cells effective in ACIT. Initially, spleen cells obtained from mice on various days after inoculation of 1 x 10^6 MOPC-315 tumor cells were cultured for 5 days with MOPC-315 stimulator cells and 2% PEG, and were tested for their effectiveness in ACIT (Table 2). Spleen cells obtained from mice 10 days after their inoculation with 1 x 10^6 tumor cells and bearing a 22-mm tumor were, upon short term culture, optimally effective in ACIT (92% cure). By Day 13, when animals were at terminal stages of tumor progression, their spleen cells were, upon short term culture, much less effective in ACIT (29% cure).

To determine the relative importance of tumor size versus the time elapsed since tumor inoculation to the effectiveness of cultured tumor bearer spleen cells for ACIT, donor mice were inoculated with fewer MOPC-315 tumor cells to delay tumor growth. In mice receiving 5 x 10^4 tumor cells, the development of palpable tumors was delayed by 3–4 days and the survival of the mice was prolonged by approximately 3 days when compared to mice injected with 1 x 10^6 tumor cells [i.e., from 14.8 ± 0.7 (SE) days to 18.2 ± 0.6 days]. Cultured spleen cells from mice inoculated with the smaller tumor inoculum were optimally effective when obtained 13 days after tumor inoculation when the mice had a 20 mm tumor (91% cure), and this effectiveness declined by Day 16 (Table 2). Thus, the extent of an animal’s tumor load, as measured by the size of the primary tumor was a better indicator than the time elapsed since tumor inoculation for optimal effectiveness of their cultured spleen cells in ACIT.

Effect of Depleting T-Cell Subsets after Short Term Culture of TISpC on their Subsequent Effectiveness in ACIT. Experiments were performed to determine the surface phenotype of the cell responsible for the effectiveness of short term cultured TISpC in ACIT. After being cultured for 5 days with MOPC-315 stimulator cells and 2% PEG, TISpC were depleted of T-cells or their subsets by treatment with monoclonal antibodies and complement before the spleen cells were tested for their ability to cure recipient mice in ACIT. With each monoclonal antibody treatment we had first established its effectiveness and selectivity in depleting the corresponding population of lymphocytes. Specifically, anti-Thy 1.2 antibody treatment was found to reduce the percentage of T-cells among the cultured TISpC to less than 2% (e.g., from 32.4 to 1.8%). Treatment of TISpC with anti-Lyt 2.2 or anti-L3T4 monoclonal antibodies led to an almost complete elimination of the corresponding T-cell subset (e.g., from 12.8 to 1.5% with anti-L3T4 antibody and from 18.2 to 1.8% with anti-Lyt 2 antibody), without reducing, and possibly enriching the other T-cell subset. As seen in Table 3, treatment of cultured TISpC with anti-Thy 1.2 or anti-Lyt 2.2 antibody plus complement abolished the ability of these cells to aid in the cure of the recipient mice. In contrast, treatment of the cultured TISpC with anti-L3T4 antibody plus complement did not reduce their effectiveness for ACIT.

Since treatment of cultured TISpC with anti-L3T4 antibody plus complement did not alter the effectiveness of spleen cells in ACIT, experiments were performed to determine whether the L3T4+ T-cells among TISpC treated with anti-L3T4 monoclonal antibody were actually eliminated, or were just coated by the anti-L3T4 antibody used for the depletion, thereby blocking the binding of the phycoerythrin-conjugated anti-L3T4 antibody used for their detection. For this purpose, we stained the putatively L3T4-depleted TISpC with FITC-conjugated goat anti-rat IgG antibody to detect any remaining L3T4+ cells that were coated with the unconjugated rat IgG anti-L3T4 antibody used to deplete the L3T4+ population. The percentage of cells staining positively with FITC-conjugated goat anti-rat IgG among the TISpC treated with unconjugated anti-L3T4 antibody and complement was found not to differ from the percentage of cells stained with the secondary antibody among the medium-treated TISpC (1.3 versus 1.1%, respectively). Thus, the treatment of the TISpC with monoclonal anti-L3T4 antibody plus complement did not mask but actually eliminated essentially all of the L3T4+ T-cell subset. Taken together, these results show that T-cells of the Lyt 2, and not the L3T4, phenotype are responsible for the effectiveness of TISpC cultured with stimulator tumor cells and PEG in ACIT.

Effect of Depleting T-Cell Subsets Prior to Short Term Culture of TISpC on their Subsequent Effectiveness in ACIT. We investigated whether L3T4+ cells were necessary during short term culture for the generation of Lyt 2+ T-cells effective in ACIT. For this purpose, TISpC were treated with anti-L3T4 monoclonal antibody plus complement before being cultured with

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**Table 2. Effectiveness of spleen cells in ACIT upon short term culture with MOPC-315 stimulator tumor cells and PEG when the spleen cells were obtained from mice bearing different sizes of sc MOPC-315 tumors that had resulted from the inoculation with 1 x 10^6 or 5 x 10^6 tumor cells**

<table>
<thead>
<tr>
<th>Number of tumor cells inoculated</th>
<th>Days after tumor inoculation</th>
<th>Tumor size (mm ± SE)</th>
<th>Cured/total</th>
<th>Cured (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size</td>
<td>1 x 10^6</td>
<td>3</td>
<td>Nonpalpable</td>
<td>3/14</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>13.0 ± 0.9</td>
<td>7/13</td>
<td>53.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>22.4 ± 1.0</td>
<td>12/13</td>
<td>92.3</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>29.4 ± 0.6</td>
<td>14/26</td>
<td>52.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Nonpalpable</td>
<td>2/15</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Nonpalpable</td>
<td>4/17</td>
<td>23.5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>11.6 ± 1.2</td>
<td>13/24</td>
<td>54.2</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>19.5 ± 0.7</td>
<td>19/21</td>
<td>90.5</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>26.6 ± 0.9</td>
<td>9/16</td>
<td>56.3</td>
</tr>
</tbody>
</table>

* Cultured TISpC (50 x 10^6) were administered to Day 5 MOPC-315 tumor bearers one day after they received 10 mg/kg CY.

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**Table 3. Effect of depleting T-cells or their subsets from TISpC either before or after short term culture in the presence of MOPC-315 stimulator tumor cells and PEG on the ability of the TISpC to cure recipient mice in ACIT**

<table>
<thead>
<tr>
<th>TISpC treatment</th>
<th>Antibody used</th>
<th>Effectiveness of therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>After culture</td>
<td>None</td>
<td>15/20</td>
</tr>
<tr>
<td>Anti-Thy 1.2</td>
<td>2/20</td>
<td>10.0</td>
</tr>
<tr>
<td>Anti-L3T4</td>
<td>16/20</td>
<td>80.0</td>
</tr>
<tr>
<td>Anti-Lyt 2.2</td>
<td>2/20</td>
<td>10.0</td>
</tr>
<tr>
<td>Before culture</td>
<td>None</td>
<td>23/31</td>
</tr>
<tr>
<td>Anti-L3T4</td>
<td>21/28</td>
<td>75.0</td>
</tr>
<tr>
<td>Anti-Lyt 2.2</td>
<td>5/32</td>
<td>15.6</td>
</tr>
</tbody>
</table>

* Of mice receiving CY only, 1 of 27 (3.7%) were cured.

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MOPC-315 stimulator cells and 2% PEG. Initially, we established the effectiveness of the depletion procedure by demonstrating that L3T4* cells among the TISpC treated with anti-L3T4 antibody plus complement did not expand significantly during the 5-day culture (i.e., 1.5% before culture, 2.2% after culture). As seen in Table 3, TISpC depleted of L3T4* T-cells prior to culture retained their effectiveness in ACIT. As a control, we show that depleting the Lyt 2+ cells among the TISpC before subjecting them to short term culture abolished the effectiveness of the TISpC in ACIT. Thus, the L3T4* T-cells are apparently not required, but if required, then only in extremely small numbers, for the generation during short term culture of Lyt 2+ T-cells effective in ACIT.

Effect of Short Term Culture of TISpC with Stimulator Tumor Cells and PEG on the Percentage of Lyt 2+ T-Cells and on the Antitumor Reactivity of This Subset. Experiments were performed to determine whether the increase in the antitumor reactivity of TISpC cultured in the presence of MOPC-315 tumor cells and PEG was associated with an increase in the percentage of cells of Lyt 2 phenotype relative to cells cultured with MOPC-315 tumor cells only (Table 4). The percentage of cells of the Lyt 2+ phenotype approximately doubled when the TISpC were cultured with MOPC-315 stimulator tumor cells (i.e., 18 versus 9% Lyt 2+ cells) and almost quadrupled when PEG was also present in the culture (i.e., 32 versus 9% Lyt 2+ cells). On the other hand, the percentage of L3T4+ cells did not significantly increase during the culture of TISpC with MOPC-315 stimulator tumor cells alone or with MOPC-315 and PEG.

Next, we examined whether the superiority for ACIT of TISpC cultured with MOPC-315 tumor cells and PEG compared to TISpC cultured only with MOPC-315 tumor cells was due to the fact that the former contained almost twice the percentage of Lyt 2+ T-cells as did the latter. For this purpose, we adoptively transferred twofold dilutions of TISpC cultured with MOPC-315 stimulator cells in the presence or absence of PEG to recipient mice in order to compare the effectiveness of ACIT of cultured TISpC containing similar numbers of Lyt 2+ T-cells (Table 5). Although 50 x 10^6 TISpC cultured without PEG contained at least as many Lyt 2+ T-cells as did 25 x 10^6 TISpC cultured with PEG, the TISpC cultured without PEG were substantially less effective for ACIT than the TISpC cultured with PEG (56 versus 94% cure). Similarly, although 25 x 10^6 TISpC cultured without PEG contained at least as many Lyt 2+ T-cells as did 12.5 x 10^6 TISpC cultured with PEG, the former were less effective than the latter in ACIT (31 versus 69% cure). Thus, Lyt 2+ T-cells from TISpC cultured with MOPC-315 stimulator cells in the presence of PEG were more effective for ACIT than a comparable number of Lyt 2+ cells from TISpC cultured in the absence of PEG. Consequently, the increased effectiveness of TISpC cultured with PEG compared to TISpC cultured without PEG cannot be explained solely by a PEG-induced increase in the percentage of Lyt 2+ T-cells.

Effect of in Vitro Melphalan Pretreatment and Subsequent Culture with MOPC-315 Tumor Cells and PEG on the Ability of TISpC to Cure Recipient Mice by ACIT. Experiments were performed to determine whether exposure of TISpC to the immunomodulating agent melphalan prior to their short term culture with MOPC-315 stimulator cells plus PEG can further enhance the effectiveness of the TISpC in ACIT (Table 6). Pretreatment of TISpC with melphalan prior to 5 days of culture with MOPC-315 stimulator cells and PEG increased the effectiveness of TISpC in ACIT (i.e., with 10 x 10^6 cells, from 52 to 82% cure). Although short term culture of melphalan pretreated TISpC with MOPC-315 stimulator cells in the absence of PEG also increased the effectiveness of these spleen cells in ACIT (47 versus 17% cure), this effectiveness was not as great as when both MOPC-315 and PEG were present (47 versus 82% cure). Melphalan pretreated TISpC cultured with PEG in the absence of MOPC-315 stimulator tumor cells also had some effectiveness in ACIT (21% cure). However, such cells cultured in the absence of stimulator cells and PEG, or transferred immediately after exposure to melphalan (data not available).

### Table 4 Relative percentage of T-cells or their subsets among uncultured normal spleen cells, uncultured TISpC, and TISpC cultured with MOPC-315 tumor cells in the presence or absence of PEG

<table>
<thead>
<tr>
<th>Phenotype of T-cells*</th>
<th>Spleen cells analyzed (%)</th>
<th>Thy 1+ cells (% SE)</th>
<th>L3T4* cells (% SE)</th>
<th>Lyt 2* cells (% SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal, uncultured</td>
<td></td>
<td>31.5 ± 2.0</td>
<td>21.4 ± 1.5</td>
<td>11.7 ± 0.7</td>
</tr>
<tr>
<td>TISpC, uncultured</td>
<td></td>
<td>24.0 ± 3.3</td>
<td>33.4 ± 1.3</td>
<td>8.5 ± 6.1</td>
</tr>
<tr>
<td>TISpC cultured with MOPC-315</td>
<td></td>
<td>33.4 ± 1.3</td>
<td>18.2 ± 4.0</td>
<td>17.6 ± 2.1</td>
</tr>
<tr>
<td>TISpC cultured with MOPC-315 and PEG</td>
<td></td>
<td>43.4 ± 3.2</td>
<td>13.7 ± 1.2</td>
<td>31.5 ± 2.8*</td>
</tr>
</tbody>
</table>

* Mean of three separate determinations.
* P < 0.05 for this percentage versus the percentage of cells of the same phenotype among uncultured TISpC.
* P < 0.05 for this percentage versus the percentage of Lyt 2+ cells in TISpC cultured with stimulator cells in the absence of PEG.

### Table 5 Correlation between the effectiveness in ACIT and the number of Lyt 2+ T-cells among the adoptively transferred TISpC cultured with MOPC-315 stimulator cells in the presence versus the absence of PEG

<table>
<thead>
<tr>
<th>TISpC culture</th>
<th>Number of spleen cells transferred (x 10^6)</th>
<th>Number of Lyt 2+ T-cells among the transferred TISpC (x 10^6)</th>
<th>Effectiveness of therapy*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG absent</td>
<td>50.0</td>
<td>8.8</td>
<td>9/16*</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
<td>4.4</td>
<td>5/16</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>2.2</td>
<td>3/14</td>
</tr>
<tr>
<td>PEG present</td>
<td>50.0</td>
<td>15.8</td>
<td>14/14*</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
<td>7.9</td>
<td>15/16*</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>3.9</td>
<td>11/16*</td>
</tr>
</tbody>
</table>

* Number of Lyt 2+ T-cells calculated using the percentages of these cells from Table 4.
* Of mice receiving CY only, one of 11 (9.1%) were cured.
* Fraction of mice surviving 60 days following tumor inoculation.
* P < 0.05 for these mice versus mice receiving CY only.
* P < 0.05 for these mice versus mice receiving the same number of TISpC cultured with stimulator cells in the absence of PEG.

### Table 6 Effect of in vitro melphalan treatment and subsequent culture of TISpC with MOPC-315 stimulator cells in the presence or absence of PEG on the effectiveness of the TISpC in ACIT

<table>
<thead>
<tr>
<th>Drug pretreatment*</th>
<th>Tumor bearer spleen cells cultured in the presence (+) or absence (−) of melphalan (%)</th>
<th>Effectiveness of therapy*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>− +</td>
<td>5/29*</td>
</tr>
<tr>
<td>Melphalan</td>
<td>− +</td>
<td>16/34*</td>
</tr>
<tr>
<td></td>
<td>− +</td>
<td>3/14</td>
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<td></td>
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<td>28/34*</td>
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* TISpC were pretreated with melphalan (0.5 mmol/ml) or medium at 37°C for 1 h.
* Of mice receiving CY only, 2 of 27 mice (7.4%) were cured.
* Fraction of mice surviving 60 days following tumor inoculation.
* P < 0.05 for these mice versus mice receiving CY only.
* P < 0.05 for these mice versus mice receiving melphalan-treated TISpC cultured with stimulator cells in the absence of PEG.
shown), did not display enhanced effectiveness in ACIT. Thus, melphalan pretreatment of TISpC substantially enhanced the ability of these spleen cells to develop effectiveness in ACIT upon short term culture with MOPC-315 stimulator cells and PEG.

**DISCUSSION**

We had shown previously that fresh TISpC are not effective in curing mice bearing small MOPC-315 tumors by ACIT (11). Here we show that TISpC cultured for 5 days in the presence of MOPC-315 stimulator cells acquire some effectiveness in ACIT, and this effectiveness is greatly enhanced by the additional presence of PEG during the culture. The Lyt 2+ T-cell subset and not the L3T4+ T-cell subset is responsible for the effectiveness of the cultured TISpC in ACIT. In fact, the L3T4 subset is apparently not even required during culture for the generation of Lyt 2+ T-cells effective in ACIT. The superiority for ACIT of TISpC cultured with MOPC-315 cells and PEG relative to TISpC cultured with MOPC-315 tumor cells only is most likely the result of a selective increase in the percentage and/or activity of Lyt 2+ cells with specificity for MOPC-315 tumor-associated antigens. Finally, we show in this investigation that the effectiveness in ACIT of TISpC cultured with MOPC-315 tumor cells and PEG can be enhanced even further by pretreating the TISpC with the immunomodulating agent melphalan.

Many investigators have successfully used adoptively transferred lymphoid cells for the treatment, with or without chemotherapy, of mice bearing established syngeneic tumors (1–6). The majority of these studies employed spleen cells from highly immune donors. However, since such cells are not available for the therapy of cancer patients, it is important to develop adoptive immunotherapy (AIT) and chemoimmunotherapy (ACIT) models in animals which utilize cells from tumor bearers rather than from tumor-immune donors. Thus far, only very few studies have effectively utilized lymphoid cells from tumor-bearing animals for AIT or ACIT. For example, North and his associates (9, 22, 23) employing a highly immunogenic tumor, have shown that tumor-bearing spleen cells during intermediate stages of tumor growth can be effective in conjunction with cytoreductive therapy in the cure of tumor-bearing mice. However, the therapeutic effectiveness of the tumor-bearing spleen cells decayed at later stages of tumor growth due to an increase in suppressor T-cell activity. Fresh tumor-bearing lymphoid cells obtained at advanced stages of tumor growth were also not effective for AIT or ACIT in a variety of other tumor models (8, 10, 11). In order to effectively utilize immunosuppressed spleen cells for AIT or ACIT, methods are needed to manipulate these cells in vitro to overcome the immunosuppression and generate potent antitumor immunity. Recently, Shu et al. (10) were able to render tumor-bearing lymphoid cells, which were ineffective for AIT, effective for AIT by subjecting the spleen cells to short term culture with stimulator tumor cells in the presence of IL-2. Similarly, Rosenberg et al. (24) have shown that tumor-infiltrating lymphocytes cultured in the presence of IL-2 were effective in AIT and ACIT.

We have introduced a new approach to render ineffective tumor-bearing lymphoid cells highly effective in ACIT. Specifically, we show here that ineffective TISpC can be rendered highly effective in ACIT by subjecting such cells to short term culture with autochthonous stimulator tumor cells and PEG. In preliminary studies (25) we have found that short term culture of TISpC with MOPC-315 tumor cells and PEG renders the spleen cells at least as effective in curing mice by ACIT as culturing with rIL-2 plus MOPC-315 tumor cells. In addition, we found that the presence of optimal concentrations of both rIL-2 and PEG led to a much greater effectiveness of the TISpC for curing mice in ACIT than the presence of either rIL-2 or PEG. Thus, the existing protocols using rIL-2 in culture of tumor-bearing lymphocytes to generate cells effective in cancer therapy may benefit by the additional presence of PEG.

The effectiveness in ACIT of MOPC-315 tumor-bearing spleen cells cultured with MOPC-315 stimulator cells and PEG increased as the primary tumor progressed to 20–22 mm in diameter, regardless of whether 1 x 10^6 or 5 x 10^4 tumor cells were used to inoculate the mice. The fact that spleen cells from MOPC-315 tumor bearers exhibit greater antitumor reactivity at a later, rather than at an earlier stage of tumor progression is reminiscent of previous observations in this tumor model that a low dose of CY or melphalan, which is dependent on the participation of T-cells for its curative effectiveness, is most effective in curing mice that bear a Day 10–12, 20–22 mm tumor than mice bearing smaller tumors (26, 27). The increase in antitumor immune potential during tumor growth is not limited to the MOPC-315 tumor model (10, 22, 23, 28, 29).

Since progression of MOPC-315 tumor growth is associated with an increase in the frequency of metastatic tumor cells in the spleen (16), the optimal effectiveness in ACIT of spleen cells from mice bearing a 20–22 mm s.c. tumor may be the result of the presence of an optimal number of metastatic cells in the spleen to provide in situ antigenic stimulation. The role of the metastatic tumor cells, however, is not necessarily limited to in situ stimulation of the spleen cells. The metastatic tumor cells may also provide a needed stimulatory signal to the spleen cells during culture with the mitomycin C-inactivated MOPC-315 tumor cells and PEG.

As the MOPC-315 tumor reaches very late, terminal stages of tumor growth and the primary tumor is greater than 25 mm in diameter, the spleen cells from these mice are no longer optimal in ACIT upon culture with MOPC-315 cells and PEG. This observation could be due to: (a) the presence in the spleens of these mice of too many metastatic tumor cells which may inhibit the generation of antitumor reactivity; (b) an increase in suppressor cell activity (13, 30, 31); or (c) the egression of tumor reactive Lyt 2+ T-cells from the spleen. The fact that inactivated stimulator tumor cells must be present in the culture for splenic cells to become effective in ACIT indicates that the ability of metastatic tumor cells to act as a source of antigen during culture is not optimal. Accordingly, the metastatic tumor cells may not be sufficiently immunogenic [e.g., as a result of too few cells or loss of some surface antigens (32)] or may exert some immunosuppressive activity (13, 30, 31). Studies to evaluate the role of splenic metastases in rendering MOPC-315 TISpC effective in ACIT would have to await the finding of a subline of the tumor that does not metastasize to the spleen. Still, from our findings, it is clear that even in the presence of the potential immunosuppressive activity of MOPC-315 tumor metastases, culture of these spleen cells with mitomycin C-treated MOPC-315 stimulator cells and PEG renders the spleen cells highly effective in ACIT.

We have identified T-cells of the Lyt 2 phenotypes as responsible for the effectiveness of cultured tumor-bearing lymphoid cells in ACIT. Cells of the same phenotype were found to be effective in vivo in curing mice of a large MOPC-315 tumor by eradicating the tumor cells remaining in these mice after treatment with a low dose of melphalan (33). The resistance of mice
cured of a large MOPC-315 tumor by a low dose of melphalan to a challenge with MOPC-315 tumor cells is also mediated by Lyt 2+ T-cells and this resistance to tumor challenge has been shown to be much more effective for the MOPC-315 tumor than for the antigenically related MOPC-104E tumor (17). Although the Lyt 2+ cells among the TISpC cultured with MOPC-315 cells and PEG are responsible for the effectiveness of the spleen cells in ACIT, the mechanism by which they exert their effect is not known. Accordingly, although culture of TISpC with MOPC-315 cells and PEG increases the anti-MOPC-315 cytotoxic T-cell activity mediated by their Lyt 2+ T-cells (12), this does not necessarily mean that the Lyt 2+ cells mediate the effectiveness in ACIT solely through a direct specific or nonspecific cytotoxic effect. The possibility exists that the Lyt 2+ T-cells recruit other cells, such as macrophages, to aid in tumor eradication (5, 34–35).

Cells of the L3T4 phenotype are not required for the effectiveness in ACIT of TISpC cultured with MOPC-315 stimulator cells and PEG. Moreover, L3T4+ cells are apparently not required even for the generation of this ability, since depletion of this cell population from TISpC prior to short term culture with MOPC-315 and PEG does not reduce the effectiveness of the spleen cells in ACIT. The observation that L3T4+ cells are not needed for the generation during culture of Lyt 2+ cells effective in ACIT may be due to the fact that the culture represents a secondary antigenic stimulation (36–38), and is consistent with the fact that the generation of secondary immune responses requires less T-cell help than is required for the generation of primary immune responses (39). Although the generation of Lyt 2+ cells effective in ACIT does not appear to require T-cell help from the L3T4+ cells during culture, the L3T4+ cells most likely play a role in providing in situ priming of Lyt 2+ cells that occurs as the tumor progresses (33). The possibility should also be considered that T-cells of the Lyt 2 phenotype provide the needed help during the culture period for the generation of Lyt 2+ T-cells effective in ACIT (40).

At this time, we can only speculate how PEG enhances the effectiveness of cultured TISpC in ACIT. Accordingly, possible mechanisms may include: (a) an increase in cell-cell contact causing greater antigen recognition and subsequent proliferation of tumor antigen specific T-cells, (b) an increase in the release of growth factors such as IL-2, which would lead to greater proliferation of tumor antigen-specific T-cells, and/or (c) a decrease in suppressor cell activity thereby allowing the expression of existing antitumor immunity as well as the generation of additional immunity in response to in vitro stimulation.

To further enhance the effectiveness of TISpC in ACIT, the spleen cells were exposed in vitro to melphalan prior to their culture with MOPC-315 stimulator tumor cells and PEG. One reason for selecting melphalan for this purpose stems from a previous observation that in vitro exposure of TISpC to melphalan results in enhancement in the ability of these spleen cells to generate anti-MOPC-315 cytotoxicity following short term culture with MOPC-315 stimulator cells (14). This effect of melphalan was shown to be due to elimination of suppressor cell activity thereby allowing the expression and enhancement of pre-existing antitumor immunity during the 5 days of culture (14). Another reason for selecting melphalan is that as a consequence of melphalan therapy of mice bearing a late stage MOPC-315 tumor, cells of the Lyt 2 phenotype acquire the ability to eradicate in vivo a large tumor burden (33). In other words, melphalan therapy leads to the appearance of potent in vivo antitumor immunity in cells of the same phenotype, i.e., Lyt 2, as those shown here to be effective in ACIT. Indeed, exposure of TISpC to melphalan prior to their culture with MOPC-315 stimulator cells and PEG is shown here to enhance the effectiveness of the spleen cells in ACIT. The superiority in ACIT of the TISpC exposed to melphalan prior to culture with MOPC-315 tumor cells and PEG relative to those cultured without exposure to melphalan may be due to the following: (a) melphalan may eliminate suppressor cell activity not eliminated by PEG, (b) melphalan may eliminate suppressor cell activity more effectively than PEG, and/or (c) melphalan may decrease suppressor cell activity at an earlier time (i.e., prior to culture) so that antitumor immunity has more time to develop.

Thus far we have used melphalan to enhance the effectiveness of TISpC from mice bearing a Day 10 MOPC-315 tumor (resulting from the inoculation with 1 x 10⁶ tumor cells). TISpC obtained at this stage of tumor growth have been found to be more effective in ACIT than TISpC obtained at a later stage of tumor growth. Since the reduced effectiveness of TISpC obtained at a later stage of tumor growth is most likely due to down-regulation by suppressor cell activity (9, 13), it would be of interest to determine whether melphalan pretreatment can also enhance the ability of such spleen cells to become effective in ACIT upon short term culture with MOPC-315 tumor cells and PEG.

The methods of short term culture presented in this paper could be adapted for use in ongoing human protocols which use the patient’s lymphoid cells after culture with biological response modifiers such as IL-2 (41). The use of PEG in similar studies should be considered since, as shown here, PEG is an effective and nontoxic additive to short term cultures of murine lymphoid cells. Similarly, treatment of a patient’s lymphoid cells prior to culture with a drug such as melphalan at an appropriate concentration may serve to selectively inactivate tumor cells and suppressor cells so as to allow for the more efficient generation of effector cells in culture. Thus, the culture of human lymphoid cells with PEG, pretreatment of such cells with an immunomodulating drug such as melphalan, or combinations of these and other modalities such as IL-2 appear to be promising avenues for improving protocols which combine cell therapy and chemotherapy for the treatment of cancer patients.

ACKNOWLEDGMENTS

We would like to thank Katherine Siessman for her outstanding technical assistance. The Coulter Epics V Flow cytometer and the technical expertise of Dr. Karen Hagen were available through the Flow Cytometry Laboratory of the University of Illinois at Chicago Research Centers.

REFERENCES

6. Mokyr, M. B., Hengst, J. C. D., and Dray, S. Role of antitumor immunity...
ADOPTIVE CHEMOIMMUNOTHERAPY


Enhancement of the Effectiveness of Lyt-2+ T-Cells for Adoptive Chemoimmunotherapy by Short-Term Exposure of Tumor-bearer Spleen Cells to Polyethylene Glycol and/or Melphalan

James A. Wise, Margalit B. Mokyr and Sheldon Dray


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