ABSTRACT

Some of the possible mechanisms by which polyethylene glycol (PEG) augments the ability of MOPC-315 tumor bearer spleen cells to mediate in vitro antitumor cytotoxicity were evaluated. The level of antitumor cytotoxicity obtained in 5-day cultures of tumor bearer spleen cell suspensions correlated inversely with the percentage of Trinitrophenol (TNP)-rosettable cells (presumably metastatic tumor cells) present in the spleen. The kinetics of decrease in the percentage of TNP-rosettable cells coincided with the appearance of antitumor cytotoxicity. In addition, PEG was shown to interfere with the ability of viable tumor cells to suppress the in vitro generation of antitumor cytotoxicity in normal spleen cells cultured with mitomycin C-treated tumor cells. However, the decrease in the content of TNP-rosettable cells and the concurrent increase in the level of antitumor cytotoxicity were not due to direct cytotoxic and/or cytostatic effects of PEG on tumor cells. Spleen cells cultured in the presence of PEG had an increased rate of [3H]thymidine incorporation and proliferation compared to spleen cells cultured in the absence of PEG. However, the PEG-induced decrease in the percentage of TNP-rosettable cells either preceded or occurred at the same time that the PEG-induced increase in spleen cell number was observed. Therefore, spleen cell proliferation can at best explain only partially the PEG-induced decrease in the content of TNP-rosettable cells, and other mechanisms for the decrease must be considered.

INTRODUCTION

In several tumor systems, spleen cells obtained from tumor-bearing animals at early stages of tumor growth and subsequently cultured in the presence of inactivated tumor cells (in vitro immunization) were shown to exhibit substantial levels of antitumor cytotoxicity. On the other hand, spleen cells obtained from tumor-bearing animals at a more advanced stage of tumor growth exhibited much lower levels, if any, of antitumor cytotoxicity following in vitro immunization correlated with the appearance of a gradual increase in the percentage of metastatic tumor cells in the spleens of tumor-bearing mice (17). Some of the tumor cells in the spleens of tumor-bearing mice were viable and capable of proliferation upon their injection into naive susceptible mice (17). Most tumor cells from tumor bearer spleen cell suspensions were removed by passing the suspensions over DNP4-lysine/Sepharose, which takes advantage of the presence of IgA with high affinity for DNP on the surface of MOPC-315 tumor cells (17). The removal of DNP-adherent cells from tumor bearer spleen cell suspensions prior to in vitro immunization resulted in the generation of augmented levels of antitumor cytotoxicity, as compared to the levels exhibited by unfractionated, in vitro-immunized tumor bearer spleen cell suspensions. Although depletion of DNP-adherent cells led to augmentation in the level of antitumor cytotoxicity obtained upon in vitro immunization, the method has the disadvantage of low cell recovery (15 to 25%) (17).

Augmentation in the level of antitumor cytotoxicity exhibited by tumor bearer spleen cells was also accomplished when tumor bearer spleen cells were immunized in vitro in the presence of 2% PEG with apparently quite good cell recovery (22). Moreover, substantial levels of antitumor cytotoxicity were obtained when the tumor bearer spleen cell suspensions were cultured in the presence of 2% PEG, even in the absence of mitomycin C-treated tumor cells (22). In addition, MOPC-315 tumor bearer spleen cells that exhibited high levels of anti-MOPC-315 cytotoxicity following 5-day culture with PEG in the presence or absence of added stimulator tumor cells were virtually noncytotoxic to allogeneic EL4 (C57BL/6) and syngeneic blast (BALB/c) cells. Normal spleen cells cultured in the presence of PEG with or without added MOPC-315 stimulator tumor cells also exhibited augmented anti-MOPC-315 cytotoxicity, but, in contrast to tumor bearer spleen cells, they were also cytotoxic for allogeneic EL4 and syngeneic blast cells (22). The present study was designed to evaluate some of the mechanisms involved in PEG-mediated potentiation of the generation of antitumor cytotoxicity by MOPC-315 tumor bearer spleen cells.

MATERIALS AND METHODS

Spleen Cell Suspensions. 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 s.c. MOPC-315 tumors. Single-cell suspensions were prepared by me-
mechanical disruption between glass slides as described previously (14), and the viability as determined by trypan blue dye exclusion (0.4%) always exceeded 95%.

Tumor Cells. MOPC-315 cells were maintained by serial s.c. inoculation in syngeneic BALB/c mice. Single-cell suspensions were prepared by mechanical disruption (14), and the viability exceeded 85%.

In Vitro Immunization. The in vitro generation of antitumor cytotoxicity was performed by a modification (14) of the method of Burton et al. (5). Briefly, responder spleen cells (75 × 10^6) were cultured with mitomycin C-treated (50 μg/ml for 30 min) MOPC-315 stimulator cells (2.5 × 10^6) in Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 5% fetal calf serum, 1% nonessential amino acids, 50 units penicillin per ml, and 50 μg streptomycin per ml (Grand Island Biological Co., Grand Island, N.Y.). The cultures were incubated at 37° in 5% CO_2 in air for 1 to 9 days.

Addition of PEG to Cultures. PEG (Carbowax PEG 6000; Fisher Scientific Co., Pittsburgh, Pa.) was dissolved at 37° in Roswell Park Memorial Institute Tissue Culture Medium 1640 [20% (w/v) stock solution], sterilized by filtration, and added to the culture medium at a final concentration of 2% (w/v). This concentration of PEG was shown previously to be the optimum for the potentiation of in vitro generation of antitumor cytotoxicity (22).

Antitumor Cytotoxicity Assay. Cell-mediated lysis was determined as described previously (14), utilizing the 51Cr release assay (4). Mixtures of 5 × 10^4 (0.4 ml) labeled target tumor cells and 5 × 10^6 (0.4 ml) spleen cells in 15- × 75-mm tubes were incubated at 37° for 3.5 hr. At the end of the incubation period, cells were pelleted, and the supernatants were transferred to identical tubes. Both the supernatant and the pellet were counted in a γ counter, and the percentage of 51Cr release for each sample was calculated as follows.

\[
\text{% of } 51\text{Cr release} = \frac{\text{counts in supernatant}}{\text{counts in supernatant + counts in pellet}} \times 100
\]

The percentage of specific 51Cr release was calculated by the following formula:

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

where \(T\) is the percentage of lysis with test spleen cells; \(S\) is the percentage of spontaneous release, which ranged between 14 and 18%; and \(M\) is the percentage of maximal 51Cr release (ranging between 78 and 81%) obtained from cells by 3 cycles of freezing and thawing. The data shown are representative of 2 to 4 individual experiments, and the value for the percentage of 51Cr release is reported in the tests as numerical percentage representing the mean of triplicate samples. The variation in percentage of 51Cr release between individual samples rarely exceeded 5% of the mean. All points differing by 6% 51Cr release were significantly different, \(p < 0.05\) by Student’s t test.

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. TNP was coupled to SRBC by reacting 50 mg 2,4,6-trinitrobenzenesulfonic acid (Eastman Kodak Co., Rochester, N.Y.) with 1 ml packed SRBC by the method of Rittenberg and Pratt (23), as modified according to the procedure of Hannestad et al. (9). The rosetting procedure was essentially the method described by Hannestad et al. (9). Briefly, tumor cells or spleen cells (2.5 × 10^6) suspended in 0.25 ml minimal essential medium (Grand Island Biological Co.) supplemented with 10% heat-inactivated fetal calf serum were mixed with 0.25 ml 1% TNP/SRBC in modified barbital buffer, pH 7.3 to 7.4 (9). To promote cell-to-cell contact which would result in rosette formation, the mixtures were centrifuged at 250 × g for 15 sec and were subsequently allowed to stand at 4° for 30 min. At the end of the incubation period, the pellets were gently resuspended, and the percentage of rosettes was determined by counting 300 spleen cells in triplicate. With the use of this technique, at least 80% of cultured MOPC-315 tumor cells formed rosettes with TNP/SRBC.

[11]Thymidine Incorporation. Normal spleen cells were cultured in vitro, with or without added stimulator tumor cells, in the presence or absence of 2% PEG. On Days 1 through 5 after culture initiation, the rate of [11]thymidine incorporation was determined. This was done by removing 1 ml of medium containing cells from the cultures every 24 hr and either diluting it 2-fold or spinning the cells down and resuspending them at a concentration of 5 × 10^5/ml and, in both cases, seeding 0.2 ml/well. The wells were pulsed with 0.2 μCi of [11]thymidine and harvested 6 hr later onto glass-fiber filter paper (H. Reeve Angel Co., Inc., Clifton, N.J.) with a Mask III multiple harvester (Microbiological Associates, Bethesda, Md.). The filters were washed extensively with 0.15 M NaCl, and [11]H incorporation was determined by liquid scintillation counting. Similar results were obtained by both procedures.

Spleen Cell Proliferation. Normal or tumor bearer spleen cells were cultured in vitro for 1 to 9 days. On various days after culture initiation, the number of cells recovered from each culture was determined and corrected for viability (as determined by trypan blue dye exclusion). For tumor bearer spleen cells, the number of viable spleen cells was obtained by deducting from the total number of viable nucleated cells the number of tumor cells identified by rosette formation with TNP/SRBC.

RESULTS

Correlation between the Level of Antitumor Cytotoxicity and the Percentage of TNP-rosettable Cells in Suspensions of Tumor Bearer Spleen Cells Cultured for 5 Days in Medium Containing PEG. Spleen cells from mice bearing 25-mm tumors were cultured in vitro for 5 days in medium containing 2% PEG in the presence or absence of added mitomycin C-treated stimulator tumor cells. At the end of the incubation period, the spleen cell cultures were evaluated for the content of TNP-rosettable cells and for the levels of antitumor cytotoxicity (Table 1). As a control, tumor bearer spleen cells were cultured for 5 days in the absence of PEG and added tumor cells. These control spleen cells exhibited a minimal level of antitumor cytotoxicity (7% 51Cr release) and contained a high percentage of rosettable cells (26%). Culture of tumor bearer spleen cells with added tumor cells resulted in a slightly higher level of antitumor cytotoxicity and a slightly lower concentration of TNP-rosettable cells. In the presence of 2% PEG, a substantial augmentation in the level of antitumor cytotoxicity (from 7 to 54%) was obtained which was associated with a drastic decrease in the percentage of rosettable cells (from 26 to 8%). In the presence of both PEG and added tumor cells, a much higher level of antitumor cytotoxicity was obtained, which was associated with an even lower percentage of rosettable cells (similar to the background level of rosetting obtained with normal spleen cells). Thus, it appears that the level of antitumor cytotoxicity obtained at the end of the 5-day culture correlates inversely with the percentage of TNP-rosettable cells.

For purpose of comparison, we included in Table 1 results which confirm our previous work demonstrating that PEG can potentiate the in vitro generation of antitumor cytotoxicity in normal spleen cells (22). However, it should be emphasized that we have shown previously that the cytotoxic activity exhibited by tumor bearer spleen cells following culture with PEG was apparently specific, whereas the cytotoxic activity ex-
Table 1

<table>
<thead>
<tr>
<th>Source of responder spleen cells</th>
<th>Antitumor cytotoxicity (% of specific 51Cr release)</th>
<th>TNP-rosettable cells on Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor bearers</td>
<td>Control</td>
<td>7 ± 0.1^c</td>
</tr>
<tr>
<td>Tumor bearers</td>
<td>Stimulator cells</td>
<td>10.0 ± 0.7</td>
</tr>
<tr>
<td>Tumor bearers</td>
<td>2% PEG</td>
<td>54.0 ± 1.1</td>
</tr>
<tr>
<td>Tumor bearers</td>
<td>Stimulator cells and 2% PEG</td>
<td>98.0 ± 1.2</td>
</tr>
<tr>
<td>Normal mice</td>
<td>Control</td>
<td>12.0 ± 0.2</td>
</tr>
<tr>
<td>Normal mice</td>
<td>Stimulator cells</td>
<td>78.0 ± 1.0</td>
</tr>
<tr>
<td>Normal mice</td>
<td>2% PEG</td>
<td>45.0 ± 1.4</td>
</tr>
<tr>
<td>Normal mice</td>
<td>Stimulator cells and 2% PEG</td>
<td>99.0 ± 1.1</td>
</tr>
</tbody>
</table>

^c Tumor bearers were mice bearing MOPC-315 tumors with diameter of 25 ± 0.5 mm.

Effect of adding 2% PEG to tumor bearer or normal spleen cells cultured in vitro for 5 days in the presence or absence of stimulator tumor cells on the level of antitumor cytotoxicity and on the percentage of TNP-rosettable cells

Kinetics of Development of Antitumor Cytotoxicity and Its Correlation to the Percentage of TNP-rosettable Cells in Suspensions of Tumor Bearer Spleen Cells Cultured in the Presence of PEG. Spleen cells from mice bearing 25-mm tumors were cultured in vitro for 0 to 9 days in medium containing 2% PEG in the presence or absence of added tumor cells. At the end of the culture period, the spleen cell cultures were evaluated for the content of TNP-rosettable cells and for the level of antitumor cytotoxicity (Chart 1). As a control, tumor bearer spleen cells cultured in the absence of PEG with or without added tumor cells were also evaluated. In the absence of PEG and added tumor cells, the content of TNP-rosettable cells increased to about 12% after 2 days in culture; by Day 3, it increased further to about 20%, and this concentration was maintained in the remaining 6 days of culture. In the presence of added tumor cells and the absence of PEG, the increase in the content of rosettable cells was slower than that seen in the absence of both PEG and added tumor cells, but here, too, it reached about 20%. With both of the above cultures, only minimal levels of antitumor cytotoxicity were obtained. In the presence of PEG and the absence of added tumor cells, a substantial decrease in the concentration of TNP-rosettable cells was first observed on Day 5 concurrently with the appearance of antitumor cytotoxicity. In the presence of both PEG and added tumor cells, the decrease in the concentration of TNP-rosettable cells occurred on Day 3, when substantial levels of antitumor cytotoxicity were also first seen. Thus, the decrease in the percentage of TNP-rosettable cells correlated with the appearance of antitumor cytotoxicity.

Effect of PEG on the Capacity of Viable Tumor Cells to Suppress the In Vitro Generation of Antitumor Cytotoxicity upon Immunization of Normal Spleen Cells with Mitomycin C-treated Tumor Cells. In vitro immunization of normal spleen cells (75 x 10^6) with mitomycin C-treated MOPC-315 cells (2.5 x 10^6) resulted in substantial levels of antitumor cytotoxicity (83%) (Chart 2). As an increased number of viable tumor cells was added to the immunization mixture, the cytotoxicity obtained decreased and was virtually abolished when 2% or more viable tumor cells were present [in confirmation of our previous observation (22)]. In the presence of 2% PEG, much less reduction in the level of antitumor cytotoxicity was observed by normal spleen cells following culture with PEG was not specific (22).
whether the augmented levels of antitumor cytotoxicity obtained when 2% PEG is added to the cultures of tumor bearer spleen cells, with or without added tumor cells are due to cytotoxic and/or cytostatic effects of PEG on viable tumor cells. Initially, we evaluated whether PEG is directly cytotoxic to tumor cells by culturing 51Cr-labeled tumor cells with various concentrations of PEG ranging from 0 to 2% for 3.5 hr. The 51Cr released by tumor cells cultured with PEG did not differ from that released by tumor cells cultured in the absence of PEG (spontaneous release) which was 17%. These data suggest that PEG does not have a direct toxic effect for MOPC-315 tumor cells. However, this conclusion is based on the evaluation of the toxicity of PEG for tumor cells in a short-term assay, and it is possible that longer incubation of PEG with tumor cells is required for PEG to exert its toxic effects. PEG might also exert a cytostatic effect on the tumor cells. Therefore, we determined whether culture of tumor cells with PEG for longer periods of time (1 to 4 days) leads to a decrease in the number of tumor cells recovered as compared to that recovered from control flasks containing spleen cells cultured in the absence of PEG (Chart 3). Culture of tumor cells for 1 to 2 days resulted in an increase in the number of viable tumor cells followed by a decrease in the number of viable cells on Days 3 to 4. However, at any day during the culture period, culture of MOPC-315 tumor cells in medium containing 2% PEG resulted in recovery of at least as many viable tumor cells as that obtained from cultures in the absence of PEG. Thus, PEG is not cytotoxic or cytostatic for viable tumor cells. Therefore, the decrease in the percentage of TNP-rosettable cells and the concurrent increase in the level of antitumor cytotoxicity with tumor bearer spleen cells cannot be attributed to direct cytotoxic and/or cytostatic effects of PEG on tumor cells.

**Effect of Adding PEG at the Effector Stage on the Level of Antitumor Cytotoxicity Obtained with in Vitro-Immunized Spleen Cells.** Experiments were performed to determine whether the augmented levels of antitumor cytotoxicity obtained with tumor bearer spleen cells cultured with or without added tumor cells in the presence of 2% PEG are due to enhancement of the interaction between the cytotoxic cells and target tumor cells. This was done by evaluating the level of antitumor cytotoxicity obtained with in vitro-immunized spleen cells from normal mice, with or without PEG addition, at the stage of the 51Cr release assay (Chart 4). The presence of PEG in the 51Cr release assay had no effect on the level of antitumor cytotoxicity obtained at the end of the 4-hr assay nor on the rate of 51Cr release. Thus, the augmentation in the level of antitumor cytotoxicity obtained when PEG is added to culture of tumor bearer spleen cells with or without added tumor cells does not appear to be simply the result of increased interaction between effector and target cells mediated by PEG carried over into the assay stage.

**Effect of PEG on the Incorporation of [3H]Thymidine by Normal Spleen Cells.** Normal spleen cells (1 × 10^5/well) were cultured in vitro with or without added stimulator tumor cells in the presence or absence of 2% PEG. On Days 1 through 5 post-culture initiation, the rate of [3H]thymidine incorporation was much greater when PEG was present than in the absence of PEG. When tumor bearer spleen cells were cultured without added stimulator tumor cells, there also the rate of [3H]thymidine incorporation was much greater when PEG was present than in the absence of PEG. Thus, PEG leads to increased incorporation of [3H]thymidine into spleen cells.
Effect of PEG on Proliferation of Spleen Cells. Initially, 75 x 10^6 normal spleen cells were cultured with or without stimulator tumor cells in the presence or absence of 2% PEG. On Days 1 through 9 postculture initiation, the number of viable cells recovered per culture was determined (Chart 6). In the first 3 days in culture, the number of viable spleen cells per culture gradually decreased in all cultures, and no significant difference was observed between cultures maintained in the presence or absence of PEG. However, on Day 5 post-culture initiation, a substantial increase in the number of viable spleen cells was observed for cultures maintained in the presence of PEG and the absence of stimulator cells. An even greater increase was observed for cultures maintained in the presence of both PEG and stimulator tumor cells. In contrast, no significant increase in the number of viable spleen cells was observed with spleen cells cultured in the absence of PEG with or without stimulator tumor cells. A similar pattern of cell recovery was maintained through Day 9 of culture: (a) virtually no change in the recovery of cells from cultures maintained in the absence of PEG regardless of whether stimulator tumor cells were also present; (b) increased recovery of cells from cultures maintained in the presence of PEG and absence of stimulator tumor cells; and (c) further increase in the recovery when cultures were maintained in the presence of both PEG and stimulator tumor cells.

When tumor bearer spleen cells were cultured in vitro in the presence or absence of PEG with or without stimulator tumor cells, a similar pattern of cell recovery was observed (Chart 7). On Day 3 post-culture initiation, there was no significant difference between the number of cells recovered from cultures maintained in the presence compared to absence of PEG. On Day 5 post-culture initiation, there was a minimal change in the number of cells recovered from cultures maintained in the absence of PEG with or without stimulator tumor cells, but there was an increase in that recovered from cultures maintained in the presence of PEG with or without stimulator tumor cells. Accordingly, the number of cells recovered on Day 5 of culture from cultures maintained in the presence of PEG with or without tumor cells was about 2-fold greater than that recovered from cultures maintained in the absence of PEG and stimulator tumor cells. Thus, by Day 5 post-culture initiation, PEG leads to proliferation of normal or tumor bearer spleen cells and promotes proliferation of spleen cells in response to stimulator tumor cells.

DISCUSSION

We have shown previously (22) and confirmed here that PEG substantially augments the ability of MOPC-315 tumor bearer spleen cells to mediate antitumor cytotoxicity. In the present study, some of the possible mechanisms by which PEG exerts its effect have been explored. PEG was not cytotoxic and/or cytostatic to tumor cells. Nevertheless, PEG interfered with the ability of viable tumor cells to suppress the in vitro generation and/or expression of antitumor cytotoxicity. Culture of tumor bearer spleen cells in the presence of PEG (25) and/or mitomycin C-treated tumor cells (26), or in the absence of both PEG and mitomycin C-treated tumor cells (27), resulted in a drastic decrease in the frequency of TNP-rosettable cells present in the spleen, and this coincided with the appearance of antitumor cytotoxicity. The level of antitumor cytotoxicity obtained in 5-day cultures correlated inversely with the percentage of TNP-rosettable cells present. PEG promoted...
spleen cell proliferation. However, the proliferation can at best account for part of the decrease in the percentage of rosettable cells, since the decrease in the content of TNP-rosettable cells occurred either prior to or at about the same time as the increase in spleen cell number. Other possible mechanisms for PEG-induced decrease in the content of TNP-rosettable cells in tumor bearer spleen cell suspensions are considered below. PEG has been used to potentiate [3H]thymidine incorporation in response to T-cell mitogens (3, 20), weak mixed lymphocyte reactions (2), and in vitro generation of antitumor cytotoxicity in normal spleen cells (10, 22). However, the antitumor cytotoxicity generated by normal spleen cells in the presence of PEG with or without added stimulator tumor cells was nonspecific and led to lysis of the syngeneic tumor, an allogeneic unrelated tumor, and normal syngeneic blast cells (10, 22). In contrast, the cytotoxicity generated by tumor bearer spleen cells in the presence of PEG was apparently specific (22).

We have shown previously (17) that viable tumor cells are present in the spleen of MOPC-315 tumor-bearing mice and that such viable tumor cells can suppress the in vitro generation and/or expression of antitumor cytotoxicity. Depletion of DNP-adherent cells from tumor bearer spleen cell suspensions resulted in the removal of most tumor cells. Upon in vitro immunization, the DNP-nonadherent cells generated higher levels of antitumor cytotoxicity than did in vitro-immunized, unFractionated tumor bearer spleen cell suspensions. Here we show that PEG interferes with the suppressive effect of viable tumor cells and that culture of tumor bearer spleen cell suspensions in the presence of PEG results in a decrease in the percentage of TNP-rosettable cells to background levels. Thus, the addition of PEG to cultures of MOPC-315 tumor bearer spleen cell suspensions obviates the need for prior removal of TNP-rosettable cells to obtain augmented levels of antitumor cytotoxicity following in vitro immunization.

The percentage of TNP-rosettable cells present in tumor bearer spleen cell suspensions cultured with PEG dropped to background levels by the third day of culture when mitomycin C-treated tumor cells were also present and by the fifth day in the absence of mitomycin C-treated tumor cells. However, proliferation of tumor bearer spleen cells in cultures maintained in the presence of PEG was seen by Day 5 but not by Day 3 of culture initiation, regardless of whether mitomycin C-treated tumor cells were also present. Thus, dilution of TNP-rosettable cells due to PEG-induced proliferation of spleen cells might account for some of the drop in the percentage of TNP-rosettable cells in cultures maintained in the absence of but not in the presence of added stimulator cells. Possibly, the added tumor cells promoted the maturation of precytotoxic cells into cytotoxic cells, a process which does not require cell division (13, 25). Alternatively, the added tumor cells may have led to the proliferation of the specific clone of T-cells responding to MOPC-315 without a great increase in the total spleen cell number. The outcome of both possibilities would be an increased antitumor potential resulting in elimination of the remaining tumor cells.

The decrease in the percentage of TNP-rosettable cells in cultures of tumor bearer spleen cell suspensions was not due to direct cytotoxic and/or cytostatic effects of PEG on MOPC-315 tumor cells. The possibility of direct PEG potentiation of antitumor cytotoxicity seems unlikely, since the addition of PEG at the effector stage did not result in augmented levels of antitumor cytotoxicity nor in an increased rate of $^{51}$Cr release. Therefore, some other mechanisms are considered. (a) PEG might interfere with the suppressive ability of viable tumor cells. This might happen, for example, by suppressing the production and/or secretion (by tumor cells) of factors mediating the suppression of the generation and/or expression of antitumor cytotoxicity. Such factors may include prostaglandin E (6), tumor antigens (1), or MOPC-315 IgA. (b) Tumor cells might exert their suppression via another cell, and PEG might decrease the frequency and/or the responsiveness of that cell to tumor cells or tumor product(s). (c) PEG might render the target cells of tumor cell-mediated suppression more resistant to the suppression, and subsequently greater numbers of tumor cells would be required to obtain the same degree of suppression. The result of the above possibilities would be the expression of at least some of the existing antitumor immunity which might eliminate the remaining tumor cells in the course of generating a secondary type antitumor cytotoxic response.

Both in human (11) and in murine (21) lymphoid cell systems, DNA synthesis by T-lymphocytes increases by culturing them with non-T-lymphoid cells derived from the same donor. The stimulation by the non-T-lymphocytes was shown to be mediated by la antigens (20, 21), which are present on most B-cells (7) and macrophages (8). PEG was shown to promote the rate of DNA synthesis by T-lymphocytes responding to allogeneic non-T-cells (20). However, DNA synthesis does not necessarily mean cell proliferation (19). Here, we show that PEG promotes not only the rate of DNA synthesis but also cell proliferation; the number of spleen cells recovered from cultures maintained in the presence of PEG was about 2-fold greater than that recovered from cultures maintained in the absence of PEG.

Thus, our data suggest that the action of PEG in augmenting the levels of antitumor cytotoxicity exhibited by tumor bearer spleen cell suspensions cultured with PEG and added stimulator tumor cells can be divided into 2 stages. In the first stage, which lasts 3 days, the percentage of TNP-rosettable cells dropped to background levels concurrently with the appearance of a substantial level of antitumor cytotoxicity. In the second stage, first seen on Day 5, proliferation of spleen cells occurred. Since the animals had been primed to the MOPC-315 tumor antigens, the frequency of T-cells with specificity for MOPC-315 in the spleen was greater than that to other antigens. Following proliferation, the frequency of MOPC-315-specific cytotoxic T-lymphocytes should have increased preferentially, and as a consequence, the tumor bearer spleen cells would exhibit a greater level of antitumor cytotoxicity. Indeed, the level of cytotoxicity exhibited by tumor bearer spleen cells cultured with PEG and added stimulator tumor cells was much greater on Day 5 than on Day 3 postculture. Moreover, following the second stage, the cytotoxicity obtained should be specific, and indeed, tumor bearer spleen cells cultured in the presence of PEG with or without added stimulator tumor cells were shown previously to be cytotoxic to MOPC-315 but not to allogeneic EL4 or syngeneic normal target cells (22).

Although fractionation on DNP-lysine/ Sepharose effectively removes most tumor cells, which lead to the generation of augmented levels of antitumor cytotoxicity following in vitro immunization, such a procedure results in low cell recovery. Only 15 to 25% of the number of cells applied to the columns is recovered (16). When the fractionation is followed by in vitro immunization, a further drop occurs in the number of cells
subjected to fractionation on DNP-lysine-Sepharose followed by in vitro immunization, the recovery would be about 3% to 8% of the initial number of cells. When tumor bearer spleen cells are cultured in the presence of PEG, there is no need for fractionation to remove tumor cells. Moreover, the number of spleen cells recovered by the end of the 5-day culture is about 2-fold greater than that recovered from cultures maintained in the absence of PEG. Accordingly, when $7.5 \times 10^8$ cells are used to set up the in vitro immunization cultures, 40 to 60% of the initial number of cells is recovered. Thus, PEG might be a useful tool for obtaining relatively large numbers of cytotoxic cells from tumor bearer lymphoid cells.

Progression of tumor growth was also shown to be associated with an increase in the percentage of macrophages present in the spleen (14). The macrophages were shown also to be capable of suppressing the in vitro generation of antitumor cytotoxicity, and the reduction or inactivation of macrophages resulted in partial restoration of antitumor potential of tumor bearer spleen cells (18). The effect of PEG on the percentage of macrophages and/or macrophage activity requires evaluation.

Tumor bearer spleen cells cultured in the presence, but not in the absence, of PEG were shown to exhibit in vivo antitumor activity in the local adoptive transfer assay (22). More recently, it became apparent that tumor bearer spleen cells cultured in the presence of PEG, even in the absence of added tumor cells, are effective in conferring systemic antitumor immunity; however, those cultured in the presence of both PEG and added tumor cells were more effective. These results imply that, even in tumor systems in which tumor cells are not available, culture of tumor bearer lymphoid cells with just PEG might enable the lymphoid cells to confer systemic antitumor immunity. Thus, tumor bearer lymphoid cells cultured in the presence of PEG with or without added stimulator tumor cells might be useful in immunotherapeutic regimens requiring histocompatible cells that are capable of conferring systemic antitumor immunity but are devoid of reactivity against normal cells.

REFERENCES

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Potentiation of Antitumor Response by PEG

Mode of Action of Polyethylene Glycol 6000 in Potentiating the 
in Vitro Generation of Antitumor Cytotoxicity by MOPC-315 
Tumor Bearer Spleen Cells

Margalit B. Mokyr, Donna Przepiorka and Sheldon Dray