ABSTRACT

Treatment with both L1210 murine leukemia cell vaccine (L1210 vaccine) and 6-mercaptopurine (6-MP) induced antitumor effector cells in the spleen and peritoneal cavity of L1210-bearing mice. The in vivo neutralization test showed that the spleen cells and peritoneal cells of mice treated with both agents, but not with either agent alone, prolonged the life span of animals simultaneously inoculated i.p. with live L1210 cells. These results indicate that these antitumor cells were associated with the augmented therapeutic response in L1210-bearing mice treated with both agents.

The neutralizing activity of peritoneal cells was located to a fraction not adhering to plastic flasks and abolished by the treatment of anti-Thy 1.2 antibody and complement, indicating that they were T-cells. The in vitro antiproliferation test confirmed these observations. The spleen cells and peritoneal T-cells of these mice suppressed L1210 proliferation. Their activity was tumor specific since they suppressed the in vitro proliferation of L1210 but not P388 and L5178Y cells. The in vivo association of antitumor T-cells with the augmented therapeutic effect was substantiated by the finding that rabbit anti-mouse thymocyte globulin abolished the induced therapeutic effect.

INTRODUCTION

Most chemotherapeutic agents suppress host antitumor immunity (1). However, some of them augmented host antitumor immunity under specific experimental conditions. It was noted that the augmentation was achieved by agents directly interacting with DNA molecules such as cyclophosphamide (5, 6, 14, 16, 17, 20), busulfan (19), Adriamycin (3, 15, 17, 20), daunomycin (13), mitomycin C (13), and one of nitrosourea derivatives (13). Since all these agents are supposed to kill host cells without selectivity, they exerted the augmenting effect under limited experimental conditions specified by species and strains of animals and tumor species. In this regard, the antimitoblastic agents exerting their antitumor effect in association with cell physiology may be considered as an alternative. In fact, under the limited experimental conditions, 6-MP1 has long been known to augment the humoral and cell-mediated immune responses (2, 18). However, in antitumor immunity, it is not clarified that any of antimitoblastic chemotherapeutic agents including 6-MP augments the host antitumor immunity in immunoprophylactic and immunotherapeutic conditions.

Recently, we found that, in combination, 6-MP, an antimitoblastic agent, and L1210 murine leukemia cell vaccine produced an augmented therapeutic effect in L1210-bearing mice (8). A series of in vivo studies showed that 6-MP-induced reduction of tumor burden was only partially responsible for the augmented therapeutic response. Further study showed that the augmented therapeutic response was dependent on tumor cell vaccine as evidenced by the findings that it was achieved by the immunogenic (Con A-bound), but not by less immunogenic (Con A-free) vaccine and that it was tumor specific. These results suggested that the antitumor immunity induced in tumor-bearing mice was associated with the augmented therapeutic response. The present study is to substantiate this hypothesis and to show that 6-MP augmented host T-cell-mediated antitumor immunity.

MATERIALS AND METHODS

Cells and Animals. L1210 and P388 murine leukemia cells supplied by the National Cancer Institute (Bethesda, MD) were collected from the ascites of male syngeneic DBA/2Cr mice. L5178Y murine lymphoma cells were kindly supplied by Dr. Y. Yahara and were made i.p. transplantable in BALB/c x DBA/2Cr F1 (hereafter called CD2F1) mice. Male CD2F1 mice, histocompatible with DBA/2Cr mice, were used in the experiments. They were obtained from Charles River Japan, Inc. (Kanagawa, Japan).

Vaccination. Con A-bound L1210 vaccine was prepared as reported previously (12). Briefly, L1210 cells were incubated first for 1 h at 37°C with mitomycin C (200 µg/ml; Kyowa Hakko Co., Ltd., Tokyo, Japan) and then for 30 min on ice with Con A (165 µg/ml; Miles Laboratories, Kankakee, IL). After one washing, they were inoculated i.p. In experiments using Con A-free vaccine, the cells were incubated with mitomycin C but not with Con A.

Immunocytes. Single-cell suspensions of spleen and peritoneal cavity were prepared as reported (11), except that the spleens were either squeezed manually in a sterilized cellulose tube (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) for the in vitro test or homogenized in a rubber-coated glass homogenizer for the i.p. transfer test. Fractions of cells not adhering to plastic flasks were prepared as reported (11). In L5178Y, mice sensitized with 10⁶ Con A-bound L5178Y vaccine and pyran copolymer according to the protocol used in L1210 (11) were inoculated i.p. with 10⁶ live L5178Y cells. About 2 weeks later, they were reinoculated i.p. with 10⁶ live L5178Y cells, and the peritoneal cells of surviving mice were used as a source of anti-L5178Y immunocytes. In Table 1, peritoneal cells of mice inoculated i.p. with 1 ml of 12% sodium caseinate in 0.85% sodium chloride solution (Difco Laboratories, Detroit, MI) 2 days before were also used as a source of effector cells.

Antitumor Activity Assay of Immunocytes. For this, we used the in vivo neutralization and in vitro antiproliferation tests as reported previously (11). In the in vitro antiproliferation test, spleen or peritoneal cells (10⁵/ml) were incubated at 37°C with 10⁵ tumor cells/ml in a CO₂ incubator (Hirasawa Works, Tokyo, Japan) (Stage 1). After 3 days, 50-µl aliquots of the mixture containing surviving tumor cells were removed and mixed with 1 ml RPMI 1640 (Grand Island Biological Co., Grand Island, NY) containing 10% fetal bovine serum (Grand Island Biological Research Center, Vol. 45 July 1985

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1 The abbreviations used are: 6-MP, 6-mercaptopurine; Con A, concanavalin A.
2 Recipient of the Award of the Society for the Promotion of Cancer Research, Japan. To whom all correspondence should be addressed.
3 The research was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare (56-4); by the Ministry of Education, Science and Culture, Japan; and also by the Princess Takamatsu Cancer Research Foundation. This is Paper 19 of a series on murine leukemia vaccine.

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Tokyo, Japan). After a further 3-day incubation, tumor cell concentrations were determined on a Model ZB Coulter Counter (Coulter Electronics, Inc., Hialeah, FL) (Stage 2). With immunocytes of L5178Y-sensitized mice, 10% fetal bovine serum was substituted for by 2% normal CD2F1 mouse male mouse serum in Stage 1.

Treatment of Immunocytes with Anti-Thy 1.2 Antibody and Complement. Immunocytes (10⁷/ml) were incubated for 1 h on ice in the presence or absence of monoclonal anti-Thy 1.2 antibody (12,000 to 1:7,000; Olac 1976, Ltd., Oxon, England). They were then centrifuged and incubated at 37°C for 30 min in the presence of rabbit Low-Tox-M complement (Cedarlane Laboratories, Ltd., London, Ontario, Canada) diluted to 1:10 to 1:20. The trypan blue exclusion test showed that, under these experimental conditions, about 90% of CD2F1 mouse thymocytes were lysed (9). Throughout this treatment, cytotoxicity medium (Cedarlane) was used as the incubation and diluting medium.

Other Agents. 6-MP (Sigma Chemical Co., St. Louis, MO), normal rabbit globulin, and rabbit anti-mouse thymocyte globulin (Microbiological Associates, Walkersville, MD) were used. The globulins were reconstituted by adding distilled water as suggested by the supplier; 0.25-ml quantities were injected i.p. into the mice.

Evaluation of the Results. The results were analyzed for significance using the t test for the in vitro antiproliferation test and, for life span prolongation in multiple experiments with small numbers of mice, the paired t test was used (Tables 1 and 3). The survival of mice in experiments using more than 6 mice/group was analyzed by the Mann-Whitney test. The in vivo experiments were terminated at Day 45. Under the present experimental conditions, 45-day survivors free of tumor signs (i.e., increased ascitic fluid and/or delayed palpable s.c. tumor growth) did not develop leukemia later and were considered cured.

RESULTS

Antitumor Cells in L1210-bearing Mice Treated with 6-MP and L1210 Vaccine. L1210-bearing mice were treated with L1210 vaccine and/or 6-MP, and the antitumor activity of their spleen cells and peritoneal cells was examined by the tumor neutralization test in the peritoneal cavity of recipient mice. Because donors treated with L1210 vaccine alone died within 15 days of the inoculation or, alternatively, their immunocytes were heavily contaminated with L1210 cells, the activity of cells from these animals was not assayed.

As shown in Table 1, the i.p. injection of spleen cells from L1210-bearing donors treated with 6-MP alone shortened the life span of the recipient mice. This adverse effect is ascribable to L1210 cell contamination of the spleen; the administration of 6-MP alone to L1210-bearing donors was not curative. In contrast, the transfer of spleen cells from L1210-bearing donors treated with both 6-MP and L1210 vaccine prolonged the life span of the recipients, although marginally, indicating that these spleen cells were antitumor effector cells.

Similar observations were made when peritoneal cells were transferred. Cells from L1210-bearing donors treated with 6-MP alone resulted in a shortening of the recipients’ life span, whereas the transfer of peritoneal cells from donors treated with both 6-MP and L1210 vaccine prolonged the life span of the recipients (Table 1). These results suggest that 6-MP augmented the production of antitumor immunocytes by L1210 vaccine, leading to an augmentation of the therapeutic effect. Peritoneal cells (5 to 7 x 10⁷) from donors treated with both agents were more effective than (Experiments 1 and 3) or as effective as (Experiments 2 and 4) their spleen cells (5 to 6 x 10⁷) in prolonging the life span of L1210-bearing recipients.

Characterization of Antitumor Cells. We characterized the antitumor effector cells by testing their in vitro antiproliferation activity against L1210 cells. As shown in Table 2, spleen cells from L1210-bearing donors treated with 6-MP and vaccine, but not with 6-MP alone, suppressed the in vitro proliferation of L1210 cells. When donor spleen cells were treated with anti-Thy 1.2 antibody plus complement but not with complement alone, the suppression of L1210 cell proliferation was completely abolished. This finding indicates the splenic effector cells to be T-cells.

Peritoneal cells from L1210-bearing donors treated with 6-MP and L1210 vaccine were fractionated according to their adherence to plastic flasks. Nonadherent populations treated with complement alone suppressed L1210 proliferation to a greater degree than did those treated with anti-Thy 1.2 plus complement, and the suppression of L1210 cell proliferation was completely abolished. This finding indicates the splenic effector cells to be T-cells.

Table 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. of cured mice/total</th>
<th>Survival days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0/5</td>
<td>12 ± 0</td>
</tr>
<tr>
<td>2</td>
<td>0/5</td>
<td>10 ± 0</td>
</tr>
<tr>
<td>3</td>
<td>0/10</td>
<td>11 ± 0</td>
</tr>
<tr>
<td>4</td>
<td>0/10</td>
<td>12 ± 0</td>
</tr>
</tbody>
</table>

Spleen cells of donors treated with

6-MP

6-MP + vaccine

Sodium caseinate

Peritoneal cells of donors treated with

6-MP

6-MP + vaccine

Sodium caseinate

Mean ± SD.

Donor mice were naive mice not inoculated with L1210 but inoculated with sodium caseinate by the protocol indicated in "Materials and Methods."
T-CELL IMMUNITY AUGMENTED BY 6-MP

In vivo antitumor immunity of peritoneal T-cells vs. 6-MP and L1210 vaccine

Table 2

<table>
<thead>
<tr>
<th>Immunocyte donors treated with</th>
<th>L1210 vaccine</th>
<th>In vitro treatment of immunocytes</th>
<th>% of control L1210 cell concentration in the presence of immunocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>– +</td>
<td>None</td>
<td>118 ± 4.3&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>+ +</td>
<td>None</td>
<td>69.0 ± 12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>+ +</td>
<td>Complement</td>
<td>68.8 ± 4.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>+ +</td>
<td>Anti-Thy 1.2 antibody + complement</td>
<td>99.3 ± 11.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peritoneal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>+ +</td>
<td>Complement</td>
<td>66.8 ± 5.0&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>+ +</td>
<td>Anti-Thy 1.2 antibody + complement</td>
<td>80.4 ± 2.9&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>+ +</td>
<td>Complement</td>
<td>71.2 ± 3.8&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>+ +</td>
<td>Anti-Thy 1.2 antibody + complement</td>
<td>100 ± 11.8&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean ± SD of quadruplicate determinations.
<sup>b,c,d,e</sup>Statistically significant at P ≤ 0.05 by t test, respectively.

Table 3

<table>
<thead>
<tr>
<th>Transferred peritoneal cells treated with</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Experiment 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>15 ± 0&lt;sup&gt;b&lt;/sup&gt; (1/6)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15 ± 3 (0/7)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>14 ± 1 (0/8)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>14 ± 3 (0/8)&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Complement</td>
<td>10 ± 0 (0/6)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>12 ± 0 (0/8)&lt;sup&gt;h&lt;/sup&gt;</td>
<td>10 ± 0 (0/6)&lt;sup&gt;i&lt;/sup&gt;</td>
<td>11 ± 0 (0/6)&lt;sup&gt;j&lt;/sup&gt;</td>
</tr>
<tr>
<td>Anti-Thy 1.2 antibody + complement</td>
<td>10 ± 0 (0/6)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>12 ± 0 (0/8)&lt;sup&gt;h&lt;/sup&gt;</td>
<td>10 ± 0 (0/6)&lt;sup&gt;i&lt;/sup&gt;</td>
<td>11 ± 0 (0/6)&lt;sup&gt;j&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Survival days of L1210-bearing mice. Survival of control mice was 11 ± 0 (Experiment 1), 12 ± 0 (Experiment 2), 12 ± 0 (Experiment 3), and 12 ± 0 (Experiment 4) days, respectively.
<sup>b</sup>Mean ± SD.
<sup>c</sup>Numbers in parentheses, number of cured mice/total.

In some experiments, however, the treatment with anti-Thy 1.2 antibody plus complement abolished the antitumor activity of peritoneal T-cells incompletely (Table 2, Experiment 1). Thus, we tested by the in vivo neutralization test whether these antitumor peritoneal T-cells were effective in vivo (Table 3). Peritoneal cells of L1210-bearing mice treated with 6-MP and vaccine, nonfractionated (Table 3, Experiments 1, 2, and 4) or populations not adhering to a plastic flask, were untreated or were treated with anti-Thy 1.2 antibody and/or complement. These immunocytes (5 x 10⁶/mouse in Experiments 1 and 2, 2 x 10⁶/mouse in Experiment 3; 10⁷/mouse in Experiment 4) were transferred i.p. to recipients simultaneously inoculated i.p. with live L1210 cells (10² in Experiments 1 and 4, 2 x 10² in Experiments 2 and 3). The prolongation of survival time of the complement group was statistically supported at P < 0.05 by the paired t test as compared with the anti-Thy 1.2 antibody + complement-treated and control groups.

The above results show that antitumor T-cells were induced in L1210-bearing mice by the present therapeutic regimen. We next examined whether these antitumor T-cells were responsible for an in situ enhancement of the therapeutic effect in donor mice per se.

Effect of Anti-Mouse Thymocyte Globulin on the Therapeutic Enhancement. L1210-bearing mice treated with both 6-MP and L1210 vaccine were inoculated i.p. with rabbit anti-mouse thymocyte globulin. As shown in Table 5, the latter treatment decreased their survival time. In addition, they survived for a...
shorter period than did L1210-bearing mice treated with 6-MP alone (mean survival days, 15 versus 19). In contrast, the injection of normal rabbit globulin did not affect the therapeutic effect, confirming that antitumor T-cells were responsible for the enhanced therapeutic effect in donor mice.

**DISCUSSION**

Our previous investigation has demonstrated that treatment of L1210-bearing mice with L1210 vaccine and 6-MP resulted in an augmented therapeutic effect (8). In the present study, we examined the immunological consequence of this effect. The i.p. neutralization test showed that antitumor effector cells in the spleen and peritoneal cavity of L1210-bearing mice treated with L1210 vaccine and 6-MP, but not with either agent alone, participated in the induced therapeutic effect. We stress that, in terms of donor antitumor activity, our i.p. transfer test provides more reliable information than do the i.m. or s.c. transfer tests in which tumor cells contact immunocytes and in which the tumor surveillance process is not included (11).

The antitumor effector cells in the spleen and peritoneal cavity were identified as T-cells because their antiproliferation and neutralizing activity against L1210 cells was abolished upon their treatment with anti-Thy 1.2 antibody and complement (Tables 2 and 3). Their selectivity for L1210 cells (Table 4) and the association of antitumor activity with the nonadherent cell fraction support our conclusion. However, the present results do not eliminate the involvement of other antitumor immunocytes such as natural killer cells in the observed antitumor activity.

While the in vivo neutralization and the in vitro antiproliferation test results identified the antitumor cells to be T-cells (Tables 1 to 3), they did not prove unequivocally that T-cells eliminated L1210 cells in donors in situ. However, our finding that the inoculation of rabbit anti-mouse thymocyte globulin into L1210-bearing mice nullified the therapeutic effect induced by L1210 vaccine and 6-MP strongly suggests that T-cells participated importantly in the manifestation of this therapeutic effect.

It is not clear what role is played by the site of origin of the antitumor T-cells. The antitumor activity of peritoneal cells was greater than that of spleen cells on a per cell basis (Table 1). This observation was confirmed by the results of the in vitro antiproliferation test; at a 100:1 to 500:1 ratio of peritoneal effector cells to L1210 cells, L1210 proliferation was suppressed to the same extent as when the ratio of splenic effector cells to L1210 cells was 1000:1. Although the enriched peritoneal cells not adhering to the plastic flask were used in the in vitro study, the nonadherence may not affect the observed superiority of peritoneal cells over spleen cells in antitumor activity since the nonadherent fraction of peritoneal cells occupies more than 50% of the whole population. This observation may be relevant to experimental situations in which live L1210 cells are inoculated i.p. so that the antitumor effector cells proliferate in the peritoneal cavity and/or come into the peritoneal cavity from other parts of the body, i.e., the spleen, and then react with L1210 cells (4).

It is not clear at present how 6-MP was associated with the production of antitumor T-cells. The elimination of L1210 cells by 6-MP could have relieved the host from immunological suppression because tumor-derived suppressive factors inhibit host antitumor immunity (7,21). However, this would be only partially, if at all, responsible for the augmentation of host antitumor immunity since other chemotherapeutic agents, including cyclophosphamide, did not induce an augmented therapeutic effect, although they did eliminate L1210 cells to an extent similar to or greater than that of 6-MP (8).

On the other hand, we found that suppressor macrophages induced in the peritoneal cavity of L1210-bearing mice and capable of suppressing the anti-L1210 immunity in L1210 vaccine-primed mice were inhibited by 6-MP but not by the other chemotherapeutic agents including cyclophosphamide (10). These results suggest that the augmented antitumor immunity was induced in L1210-bearing mice by inhibiting the suppressor macrophages in a 6-MP-dependent fashion resulting in the augmentation of L1210 vaccine-dependent antitumor immunity.

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Production of Antitumor T-Cells in Tumor-bearing Mice Treated with Tumor Vaccine and 6-Mercaptopurine

Tateshi Kataoka and Fujiko Oh-hashii


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