ABSTRACT

DBA/2 mice and BALB/c × DBA/2 F₁ mice were inoculated i.p. with 1 × 10⁵ L1210 leukemia cells on Day 0. Beginning with Day 1 following inoculation, the animals were given injections of 6-mercaptopurine riboside (200 mg/kg i.p.) once daily for 5 consecutive days in combination with a single s.c. injection of levamisole (LMS) (10 mg/kg). In DBA/2 mice, a marked decrease in the total peritoneal cell count was achieved when LMS was given on any day from Day -2 to 3 as compared with therapy using 6-mercaptopurine riboside alone, although the survival time was not prolonged in these mice. When LMS was given on Day 1 or 3, a decrease was observed in the in vitro growth rate of the L1210 cells obtained on Day 11 from the peritoneal cavity. In BALB/c × DBA/2 F₁ mice, no significant changes were observed in either the total peritoneal cell count or the in vitro growth rate when LMS was injected on Day 3 or daily from Day 3 to 5. Daily injection of LMS from Day 1 to 3 resulted in a conspicuous inhibition of the in vitro growth rate, although no significant changes were observed in the total peritoneal cell count. It seems reasonable to conclude from these results that LMS has a potentiating effect on the antitumor activity of 6-mercaptopurine riboside.

INTRODUCTION

With the accumulation of knowledge concerning tumor immunity, the therapeutic potentials of specific and nonspecific immunotherapy have come to be investigated. To date, several nonspecific immunostimulating compounds (3, 4) have been put to practical use and are now under clinical evaluation concerning their efficacy and limitations. However, it must be remembered that the clinical indications regarding use of these immunostimulants are not yet established, because their practical use does not depend entirely on the convincing experimental results. LMS⁵ is one of these nonspecific immunostimulants, and there have been many reports (18, 21, 24) dealing with the results of concomitant use of LMS and various anticancer agents in the treatment of various types of experimental tumors. We observed that when allogeneic mice were immunized with L1210 cells, concomitant use of LMS not only potentiated the immune response⁶ but also augmented the

transferring activity of immune RNA⁷ in the antitumor immune response to L1210 cells. On the basis of these findings, the present study was designed to assess whether LMS has a potentiating effect on the antitumor activity of 6-MPR against L1210 leukemia cells.

MATERIALS AND METHODS

Cells and Animals. DBA/2 mice, 6 to 12 weeks of age, and BALB/c × DBA/2 F₁, (hereafter referred to as CD2F,) mice, 8 to 16 weeks of age, obtained from the Animal Laboratory of Gunma University School of Medicine, were used. These F₁ animals were the most readily obtainable strain in Japan. L1210 leukemia cells were provided by Dr. Shimoyama, National Cancer Research Center of Japan.

Chemioimmunotherapy. Each experimental group consisted of 5 mice which were inoculated i.p. with 1 × 10⁵ L1210 leukemia cells on Day 0. From Day 1 (24 hr following inoculation), 6-MPR (Morishita Pharmaceutical Co., Ltd., Tokyo, Japan), diluted with 0.85% NaCl solution, was injected i.p. once daily for 5 consecutive days in doses of 100, 200, or 400 mg/kg. LMS (Kyowa Hakko Kogyo, Co., Ltd., Tokyo, Japan) diluted with 0.85% NaCl solution, was administered s.c. in a dose of 10 mg/kg to DBA/2 mice on Day -2, 0, 1, or 3, to CD2F, mice on Day 3 (Group 2), and in a daily injection from Days 1 to 3 (Group 3) or Days 3 to 5 (Group 4).

Total Cell Count and Growth Rate. Groups of 5 mice were decapitated on Day 11, and the peritoneal cavities were washed twice with 4 ml of Roswell Park Memorial Institute Medium 1640 (Nissui Seiyaku Co., Ltd., Tokyo, Japan). All washings were collected, and the number of tumor cells was counted. Subsequently, these cell suspensions were centrifuged, and the sedimented cells were washed twice with the same medium and then resuspended in Roswell Park Memorial Institute Medium 1640 supplemented with 20% heat-treated fetal bovine serum (Flow Laboratories, Rockville, Md.) penicillin G (50 units/ml), and streptomycin (50 μg/ml) to give a concentration of 1 × 10⁵ cells/ml. One hundred μl of the cell suspension were placed in a microculture plate (NUNC, Roskilde, Denmark) and incubated at 37° in a 5% CO₂ humidified incubator for 4 days. The number of viable cells was then counted using the trypan blue dye exclusion method, and the in vitro growth rate was calculated using the following formula:

Growth rate = \( \frac{\text{No. of viable cells/ml after 4 days of incubation}}{1 \times 10^5 \text{L1210 cells/ml}} \)

¹ Supported by the Cancer Research Fund from the Ministry of Health and Welfare of Japan.
² To whom requests for reprints should be addressed.
³ The abbreviations used are: LMS, levamisole; 6-MPR, 6-mercaptopurine riboside.
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Cultivation was carried out in triplicate.

**Statistical Analysis.** Means and standard deviations were calculated and the statistical significance of the differences was determined by Student's t test, p < 0.05 being considered significant.

**RESULTS**

**Effects of Chemotherapy with 6-MPR on L1210-bearing DBA/2 Mice (Table 1).** DBA/2 mice inoculated i.p. with 1 × 10⁶ L1210 leukemia cells on Day 0 were treated daily with i.p. injections of various doses of 6-MPR for 5 days beginning with Day 1 and were sacrificed on Day 11, i.e., just before death of the control mice, to determine the total cell count and the in vitro growth rate of cells in the peritoneal cavity. The total cell count of the leukemic mice was decreased significantly (p < 0.01) and the mean survival time of the leukemic mice was prolonged significantly (p < 0.01) in all groups which were treated with various doses of 6-MPR. However, the cells from animals given 6-MPR (200 and 400 mg/kg) did not show a significant decrease in their growth rate in comparison with the group not given 6-MPR. Based on these results, a 200-mg/kg dose of 6-MPR, which had resulted in a 50% increase in survival time, was selected for 6-MPR therapy in the following experiments.

**Effects of a Single Dose of LMS Combined with 6-MPR on L1210-bearing DBA/2 Mice.** In leukemic mice given 6-MPR (200 mg/kg) once daily for 5 days as described above, LMS (10 mg/kg) was injected s.c. on Day —2, 0, 1, or 3. The total peritoneal cell count and in vitro growth rate were determined on Day 11; the results are shown in Chart 1. It is evident that the additional administration of LMS on any experimental day resulted in a marked decrease (p < 0.01) in the total peritoneal cell count as compared with administration of 6-MPR alone. In mice given LMS on Day —2 or 0, no marked change in the growth rate was observed although the total peritoneal cell count was markedly decreased. Mice given LMS on Day 1, i.e., the first day of 6-MPR therapy (0.02 < p < 0.05), or Day 3 (p < 0.01), had a significant decrease in the growth rate as well as a marked decrease in the total peritoneal cell count.

**Effects of Daily Doses for 3 Consecutive Days of LMS Combined with 6-MPR on L1210-bearing CD2F, Mice.** Groups of mice were given LMS (10 mg/kg) for the first 3 days of 6-MPR therapy, i.e., from Day 1 to 3 (Group 3), or for the last 3 days of 6-MPR therapy, i.e., from Day 3 to 5 (Group 4), while one group was treated with a single dose of LMS on Day 3 (Group 2) for comparison with the results in DBA/2 mice.

**DISCUSSION**

In the present study, we have focused our attention on the treatment of L1210-bearing mice with 6-MPR and LMS, using it as a model of chemioimmunotherapy. There have been many reports dealing with unsuccessful results using combinations of LMS and various antitumor agents against L1210 leukemia cells (4, 18). Our results show that, while increase in survival time was not achieved by concomitant use of LMS, LMS demonstrated a potentiating effect on the cytoidal activity of 6-MPR. Of interest is the fact that this effect of LMS varies with the time of its administration in experimental animals, in this case, either syngeneic or semisyngeneic mice (Chart 1; Table 2).

Recent reports have indicated the existence of physicochemical and/or antigenic relationships between tumor-associated antigens and H-2 antigens (8, 17). Cytotoxic T-cells function most efficiently when target cells, especially virus-infected target cells, share some identical H-2 antigens with effector cells and stimulator cells (7, 10, 13). L1210 cells, although they are a methylcholanthrene-induced leukemia of the mouse, have been shown to produce C-type viruses which can alter a portion of the cell membrane (2, 9, 19). LMS itself has no antitumor activity, but it may exert its influence through the activation of T-cells (22). Accordingly, the differences observed in the in vitro growth rate of tumor cells from the peritoneal cavity between DBA/2 mice and CD2F, mice similarly treated.
with a single dose of LMS and daily administration of 6-MPR may be attributable to the differences between the L1210 cells and the host effector cells. T-cells activated by LMS exhibited more efficient cytotoxic activity against the L1210 cells in syngeneic DBA/2 mice than in semisynthetic CD2F1 mice. In addition, Adler et al. (1) have pointed out that a discrimination in the lymphocyte response to mitogen was observed in various animal strains despite close genetic relationships. This also supports the results observed in the present study showing a difference in the effect of LMS on in vitro growth rate of cells from 2 separate strains of mice.

In experiments designed similarly to the one presented here, LMS has been reported by some investigators to enhance the susceptibility of tumor cells to anticancer agents. It has been suggested that this enhancement might result from inhibition of alkaline phosphatase activity in 6-mercaptopurine-resistant L1210 cells (6; 23, 25). In the present study, however, it is hardly conceivable that LMS has enhanced the cytotoxic effect of 6-MPR through such a mechanism since the L1210 cells used in this experiment were 6-mercaptopurine sensitive.

It is, however, of great interest that a single dose of LMS on Day 1 or 3 produced a significant decrease both in the total peritoneal cell count and in the in vitro growth rate in DBA/2 mice and that in CD2F1 mice treated with a daily dose of LMS from Day 1 to Day 3 a significant decrease was observed only in the in vitro growth rate, there being no change in the total peritoneal cell count. The fact that macrophages with specific and nonspecific antitumor activity (11, 14, 15) could have accumulated in the peritoneal cavity must be taken into consideration (5, 16, 20, 24). An actual increase in the number of macrophages in the peritoneal cavity was observed following LMS administration in this experiment. These macrophages may inhibit the in vitro growth of the tumor cells without bringing about a significant decrease in the total cell count in the peritoneal cavity.

Suppressor cells have been reported to begin appearing 24 hr after inoculation with tumor cells (12). In the present experiments, the effects of suppressor cells and their precursors might have been abrogated by 6-MPR since treatment was instituted at the same point in time. It can be postulated that treatment with 6-MPR might have resulted in a transient immunosuppressive state and that subsequent immunorestitution might have been accelerated by combined use with LMS during the restoration period. In this case, the mechanism by which restoration of the immune response occurred in the face of 6-MPR treatment remains to be clarified.

In any event, we can conclude that the administration of LMS at an early point in time after tumor inoculation augments the cytotoxic effects of 6-MPR. However, as regards the synergistic effects of LMS on prolongation of survival time, we obtained the same inconclusive results as those of other investigators. Further studies are in progress to establish regimens that might prolong survival time by combination chemoimmunotherapy.

ACKNOWLEDGMENTS

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REFERENCES


Table 2

Effects of chemoimmunotherapy on L1210-bearing CD2F1 mice

Mice were given 6-MPR (200 mg/kg i.p.) once daily for 5 consecutive days after 1 x 10^6 L1210 cell inoculation (Day 0). LMS (10 mg/kg) was injected s.c. on Day 3, 1, 2, 3, or 3, 4, 5 after L1210 cell inoculation. The in vitro assay was made on Day 11.

<table>
<thead>
<tr>
<th>Treatment (no. of days after L1210 inoculation)</th>
<th>Total cell count of peritoneal cavity (x 10^6 cells)</th>
<th>Growth rate</th>
<th>Mean survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-MPR</td>
<td>LMS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>21.6 ± 9.7^a</td>
<td>18.6 ± 3.2</td>
<td>13.0 ± 2.8</td>
</tr>
<tr>
<td>Group 1</td>
<td>2.2 ± 0.5</td>
<td>13.5 ± 0.6</td>
<td>19.8 ± 1.6</td>
</tr>
<tr>
<td>Group 2</td>
<td>4.8 ± 2.1^b</td>
<td>11.7 ± 0.5^b</td>
<td>18.8 ± 2.9</td>
</tr>
<tr>
<td>Group 3</td>
<td>5.6 ± 0.5^b</td>
<td>0.9 ± 0.5^b</td>
<td>21.7 ± 4.9^b</td>
</tr>
<tr>
<td>Group 4</td>
<td>3.2 ± 1.3^b</td>
<td>15.2 ± 1.7^b</td>
<td>21.7 ± 5.7^b</td>
</tr>
</tbody>
</table>

^a Mean ± S.D.; each of these data is the average from 5 mice/group.  
^b Not significant.  
^c p < 0.01.
Effects of Levamisole Administration on Treatment with 6-Mercaptopurine Riboside of Mice Bearing L1210 Leukemia Cells

Takaji Miura, Eishi Nogiwa, Satonori Kurashige, et al.


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