Effect of Combination Treatment with Cyclophosphamide and Nonspecific Passive Immunization on a Transplantable Tumor in WKA Rats

Eiki Gotohda, Tsugumichi Kawamura, Fujiro Sendo, Masayuki Nakayama, Jitsuo Akiyama, Tsuneyuki Oikawa, Masuo Hosokawa, Takao Kodama, and Hiroshi Kobayashi

Laboratory of Pathology, Cancer Institute, Hokkaido University School of Medicine, Sapporo [E. G., T. K., J. A., T. O., M. H., T. K., H. K.], and Department of Pathology, Yamagata University School of Medicine, Yamagata, Japan [F. S., M. N.]

SUMMARY

Combination of cyclophosphamide (CY) and passive immunization with lymphoid cells sensitized to allogeneic tumor was studied in the treatment of a methylcholanthrene-induced transplantable fibrosarcoma in WKA rats. For determination of the most effective timing of combination treatment, rats given an injection of CY on Day 3 received passive transfer of the sensitized lymphoid cells on Day 0, 1, 2, 3, 4, or 6. A remarkable therapeutic effect was observed only when the passive transfer was combined on Day 4 with CY on Day 3. Rats inoculated with tumor succumbed in all cases without any treatment. After i.v. injection of CY on Day 3, 2 of 28 rats were cured (7.1%). Passive immunization with the sensitized lymphoid cells on Day 4 resulted in no therapeutic effect. After combination of CY on Day 3 and transfer of nonsensitized normal lymphoid cells on Day 4, 2 of 15 rats survived (13.3%). However, combination of CY and passive transfer of the nonspecifically sensitized lymphoid cells resulted in 23 survivors of 29 rats (79.3%).

INTRODUCTION

The growth of transplantable syngeneic tumors in WKA rats was inhibited strongly by preimmunization with tumors or normal tissues from allogeneic Donryu rats; this phenomenon was tentatively referred to as "allogeneic cell immunity" (9). An important property of this allogeneic cell immunity is that cross-reactive antigens between tumors from WKA rats and the immunizing cells from Donryu rats were not detected by cytotoxicity and immunofluorescence tests. Allogeneic cell immunity was easily abrogated by low-dose irradiation or by antilymphocytic serum. Moreover, the inhibitory effect on tumor growth was not transferred into normal rats with syngeneic spleen and lymph node cells from rats immunized with the allogeneic tumor (19). From the above results it seems that the inhibition of tumor growth was induced by nonspecific immune mechanisms. Problems concerning the effectiveness of passive immunotherapy with nonspecifically activated lymphoid cells remain to be resolved. For instance, when cells from spleen and lymph nodes from mice that received Bacillus Calmette-Guérin were mixed with polyoma tumor cells and the mixture was implanted into syngeneic mice, tumor growth was not inhibited (13). However, in humans, s.c. tumor nodules injected with autochthonous lymphocytes activated by in vitro incubation with phytohemagglutinin regressed completely or partially (1).

This paper shows that allogeneic tumor-sensitized syngeneic lymphoid cells, which have no inhibitory effect on tumors by themselves, inhibit tumor growth when they are passively transferred in combination with CY treatment and that an appropriate timing is necessary for this combination to become therapeutically effective.

MATERIALS AND METHODS

Rat. The inbred Wistar-King-Aptekman/Mk (WKA) rats used were the offspring of parents maintained by consecutive brother-sister mating for more than 200 generations in the Experimental Animal Center directed by Dr. M. Sasaki, Faculty of Science, Hokkaido University, Sapporo, Japan. Skin transplantation succeeded in all cases. Donryu rats, used for AH-66 tumor passage, were obtained commercially from the Nippon Rat Company, Urawa, Japan.

Tumor. KMT-17 was a transplantable fibrosarcoma induced by 3-methylcholanthrene in WKA rats. This was donated by Dr. M. Aizawa, Department of Pathology, Hokkaido University School of Medicine, Sapporo, Japan. The tumor was maintained in ascites form, and the mean survival time was 16.1 days when 1 × 10⁶ cells were transplanted s.c. A characteristic property of KMT-17 tumor was recently noticed by cytotoxicity test, namely, the decrease in expression of tumor-associated surface antigen after i.p. transplantation (4).

AH-66 was a transplantable hepatoma in Donryu rats induced by feeding dimethylaminoazobenzene. This tumor was donated by Dr. H. Hirai, Department of Biochemistry, Hokkaido University School of Medicine, Sapporo, Japan. AH-66 was maintained in ascites form, and the mean survival time was 10 days.

Chemotherapy. CY, supplied by Shionogi and Co.,

Received December 1, 1975; accepted March 8, 1976.

1 The abbreviation used is: CY, cyclophosphamide.
Osaka, Japan, was dissolved in sterile 0.85% NaCl solution before i.v. injection through the tail vein.

**Passive Immunization.** WKA rats were immunized s.c. 5 to 10 times with 1 to 2 x 10⁶ AH-66 tumor cells, and spleen and submandibular and mesenteric lymph nodes were removed aseptically 7 to 10 days after the final immunization. The excised spleen and lymph nodes were separated into single cells with a loosely fitting glass homogenizer, and the cells were suspended in Eagle’s minimal essential medium. Rats were given 1 x 10⁶ lymphoid cells through the tail vein after the viable cells were counted by the trypan blue dye exclusion method. Normal spleen and lymph node cells were both prepared and transferred by the same procedures as described above.

**RESULTS**

**Effect of CY on KMT-17 Tumor in WKA Rats.** For determination of the optimal dose of CY against KMT-17 tumor in WKA rats, 1 x 10⁶ tumor cells were inoculated s.c. into the right flank of rats on Day 0, and various doses of CY were injected through the tail vein on Day 3 (Table 1). All rats succumbed to the tumor when CY was not injected or when it was injected at a dose of 20 or 30 mg/kg. Only 1 of 10 rats was cured with CY injection at the dose of 40 mg/kg, but at the dose of 50 mg/kg 1 of 4 rats was cured and the other 3 died from drug intoxication within 10 days after KMT-17 inoculation. Therefore, the dose of CY used throughout the study for i.v. injection on Day 3 was 40 mg/kg.

**Effective Timing of Combination Treatment with CY and Passive Transfer of Lymphoid Cells Sensitized to an Allogeneic Tumor.** The question was asked whether lymphoid cells from rats immunized with allogeneic AH-66 tumor had an inhibitory effect on KMT-17 tumor growth if the lymphoid cells were transferred on Day 0, when the number of tumor cells was minimal (Table 2). KMT-17 tumor cells (1 x 10⁶) were inoculated s.c. on Day 0, and CY was injected i.v. on Day 3. The sensitized or nonsensitized lymphoid cells were transferred 2 hr after tumor inoculation. All rats succumbed regardless of treatment.

The mean tumor growth curves of the 5 groups are shown in Chart 1. The growth curve of the group treated with passive transfer of the sensitized lymphoid cells is significantly different from that of the untreated group. Growth curves of groups treated with passive transfer of normal lymphoid cells plus CY or of the sensitized lymphoid cells plus CY are almost the same as that of the CY-treated group. Thus, lymphoid cells from rats that had been immunized with allogeneic AH-66 tumor did not inhibit KMT-17 tumor growth at all, despite the fact that in rats actively immunized with AH-66 tumor the growth of the KMT-17 tumor was completely inhibited (9).

Then, for determination of the best timing for the combined treatment to be effective, rats inoculated with 1 x 10⁶ KMT-17 tumor cells on Day 0 were given an injection of CY on Day 3, and, in addition, they received a transfer of sensitized lymphoid cells on Day 1, 2, 3, 4, or 6. As shown in Table 3, there were no survivors in the group treated with passive transfer on Day 1 plus CY. Only 1 of 5 rats was cured in the groups treated with passive transfer on Day 2, or on Day 3, plus CY. However, combination of passive transfer on Day 4 and CY on Day 3 increased the inhibition of KMT-17 tumor growth, and 4 of 5 rats were cured. When the sensitized lymphoid cells were transferred on Day 6 into rats that had received CY on Day 3, the inhibitory effect decreased and only 2 of 5 rats survived. The mean survival of the 3 rats that died in the last group was prolonged relative to that of the other groups.

Individual tumor growth curves in each group are shown in Chart 2. In the group treated with passive transfer on Day 3 plus CY, 3 of 4 rats were cured. These results indicate that CY is effective only when given within 3 days after KMT-17 tumor inoculation. In the group treated with CY on Day 3 plus passive transfer on Day 4, 2 of 4 rats were cured. Therefore, the effect of CY is most pronounced on Day 3.

**Table 1**

*Effect of CY on KMT-17 tumor in WKA rats*

<table>
<thead>
<tr>
<th>Doses (mg/kg)</th>
<th>Survivors/rats used</th>
<th>Mean survival days</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0/5</td>
<td>16.5</td>
</tr>
<tr>
<td>20</td>
<td>0/9</td>
<td>18.0</td>
</tr>
<tr>
<td>30</td>
<td>0/10</td>
<td>20.2</td>
</tr>
<tr>
<td>40</td>
<td>1/10</td>
<td>21.9</td>
</tr>
<tr>
<td>50</td>
<td>1/4</td>
<td>24.1</td>
</tr>
</tbody>
</table>

a Various doses of CY were injected i.v. 3 days after 1 x 10⁶ KMT-17 tumor cell inoculation.

b Three of 4 rats given an injection of cyclophosphamide, 50 mg/kg, died within 10 days after tumor inoculation because of severe infection.

**Table 2**

*Lack of inhibitory effect of lymphoid cells from rats immunized with AH-66 tumor when cells were transferred on Day 0*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survivors/rats used</th>
<th>Mean survival days</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0/5</td>
<td>16.0</td>
</tr>
<tr>
<td>WKA anti-AH-66 lymphoid cells*</td>
<td>0/5</td>
<td>16.2</td>
</tr>
<tr>
<td>CY*</td>
<td>0/5</td>
<td>21.0</td>
</tr>
<tr>
<td>WKA normal lymphoid cells* + CY*</td>
<td>0/5</td>
<td>20.0</td>
</tr>
<tr>
<td>WKA anti-AH-66 lymphoid cells* + CY*</td>
<td>0/5</td>
<td>24.0</td>
</tr>
</tbody>
</table>

* Lymphoid cells (1 x 10⁶) were transferred i.v. 2 hr after KMT-17 tumor inoculation on Day 0.

+ CY, 40 mg/kg, was injected i.v. on Day 3.
4 plus CY on Day 3, the growth curves of the 4 regressing tumors are similar and the maximal tumor diameter reached is below 20 mm. In the group treated with passive transfer on Day 6 plus CY on Day 3, regression of the tumor was observed in 4 of 5 rats, but the 2 rats in which maximal tumor diameter exceeded 30 mm died in spite of the regression of s.c. tumors. At autopsy, solitary metastases in the lung and liver and massive infiltration of KMT-17 tumor in the abdominal cavity were found in both of them.

**Effect of Combination Treatment with CY and Passive Transfer of Lymphoid Cells Sensitized to an Allogeneic Tumor.** Rats inoculated with $1 \times 10^6$ KMT-17 tumor cells were treated with CY and/or passive transfer of the lymphoid cells from rats immunized with allogeneic AH-66 tumor according to the optimal timing of combination treatment derived from the above experiments (Table 4). All untreated rats inoculated with KMT-17 tumor on Day 0 succumbed; their mean survival was 16.1 days. After CY injection alone, 2 of 28 rats were cured (7.1%); with passive transfer of the sensitized lymphoid cells on Day 4 there were no survivors. When transfer with nonsensitized lymphoid cells on Day 4 was combined with CY on Day 3, 2 of 15 rats survived (13.3%). However, the combination of CY on Day 3 and passive transfer with AH-66-sensitized lymphoid cells on Day 4 increased the inhibition of KMT-17 tumor, and 23 of 29 rats were cured (79.3%). Mean survival of the rats that died in the last group was not prolonged. Cumulative survival curves of each of these 5 groups are shown in Chart 3.

**Table 3**

*Comparison of the inhibitory effect of lymphoid cells from rats immunized with AH-66 tumor when cells were transferred on various days into WKA rats that received CY, 40 mg/kg, i.v. on Day 3*

<table>
<thead>
<tr>
<th>Lymphoid cells transferred on</th>
<th>Survivors/rats used</th>
<th>%</th>
<th>Mean survival days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>0/4</td>
<td>0</td>
<td>22.8</td>
</tr>
<tr>
<td>Day 2</td>
<td>1/5</td>
<td>20.0</td>
<td>24.0</td>
</tr>
<tr>
<td>Day 3</td>
<td>1/5</td>
<td>20.0</td>
<td>22.8</td>
</tr>
<tr>
<td>Day 4</td>
<td>4/5</td>
<td>80.0</td>
<td>23.0</td>
</tr>
<tr>
<td>Day 6</td>
<td>2/5</td>
<td>40.0</td>
<td>31.7</td>
</tr>
</tbody>
</table>

**Table 4**

*Inhibitory effect of CY, passive immunization, or combination treatment on KMT-17 tumor in WKA rats*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survivors/ rats used</th>
<th>%</th>
<th>Mean Survival days</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0/20</td>
<td>0</td>
<td>16.1</td>
<td>13-19</td>
</tr>
<tr>
<td>CY</td>
<td>2/28</td>
<td>7.1</td>
<td>22.8</td>
<td>17-30</td>
</tr>
<tr>
<td>WKA anti-AH-66 lymphoid cells</td>
<td>0/10</td>
<td>0</td>
<td>19.0</td>
<td>16-25</td>
</tr>
<tr>
<td>CY + WKA normal lymphoid cells</td>
<td>2/15</td>
<td>13.3</td>
<td>25.1</td>
<td>20-28</td>
</tr>
<tr>
<td>CY + WKA anti-AH-66 lymphoid cells</td>
<td>23/29</td>
<td>79.3</td>
<td>23.5</td>
<td>21-25</td>
</tr>
</tbody>
</table>

*a CY, 40 mg/kg, was injected i.v. on Day 3.

*b Lymphoid cells ($1 \times 10^6$) were transferred i.v. on Day 4.
DISCUSSION

Some fundamental data useful for immunotherapy may be obtained from syngeneic lymphoid cell transfer systems, although nonspecific passive immunization with syngeneic lymphoid cells may not be immediately applicable to immunotherapy of human cancer. For instance, we have already reported synergistic increases in the inhibition of tumor growth when Mitomycin C treatment (3, 6) or surgical excision (8) was combined with specific active immunization with rat tumor cells that had been artificially infected with mouse leukemia viruses (10-12). In one of these studies (3), passive transfer of syngeneic lymphoid cells from rats immunized with leukemia virus-induced rat tumor, but not with the tumor itself, enhanced the therapeutic effect of active immunization with "xenogenized" tumor cells, which possessed both tumor-associated and virus-associated antigen. It was supposed that this kind of passive immunization had accelerated the appearance of, or strengthened, the immunizing effect induced by active immunization of the xenogenized tumor.

In the present study, it was found that syngeneic lymphoid cells from rats immunized with allogeneic AH-66 tumor inhibited strongly the growth of tumor when the passive immunization was combined with CY, despite the fact that lymphoid cells per se had no inhibitory effect on the growth of tumor. Furthermore, appropriate timing of combination treatment was required to obtain the most marked therapeutic effect; this was obtained only when passive transfer on Day 4 was combined with CY on Day 3. When the passive transfer on Day 6 was combined with CY on Day 3, metastases in the lung and liver and massive infiltration in the abdominal cavity were observed in 2 rats in which s.c. tumors were regressing. These results suggest, therefore, that immunotherapy, especially nonspecific passive immunotherapy, may be useful or harmful depending on timing of application relative to drug treatment.

Although the mechanisms responsible for allogeneic cell immunity and for the effectiveness of transfer of lymphoid cells from rats immunized with allogeneic tumor in combination with CY have not been elucidated, we recently obtained some suggestive results in this respect. The growth of KMT-17 tumor was inhibited by admixed AH-66-sensitized lymphoid cells with the use of an in vivo neutralization test (Winn's assay), provided that recipients were preirradiated, whereas inhibition of KMT-17 tumor was not observed in untreated normal rats (17). Even in untreated rats the growth of KMT-17 tumor was inhibited by AH-66-sensitized lymphoid cells when inactivated AH-66 tumor cells were further added to the cell mixture (19). These results suggest that allogeneic tumor-sensitized lymphoid cells, which had no inhibitory effect on tumor growth, had become effective in immunosuppressed hosts or following activation of the host lymphoid cells by antigenic stimulation. In the combination treatment reported here, CY may not directly influence the transferred lymphoid cells, since CY given 1 day prior to transfer of lymphoid cells may be eliminated from the hosts (16). Therefore, it is possible that the lymphoid cells may become effective in inhibiting tumor growth in hosts immunosuppressed by treatment with CY.

CY has been recently considered to be a selective immunosuppressant since its injection induced depletion of cells within B-cell-dependent areas of lymphoid tissues (21) and suppressed humoral antibody production (7, 14). In contrast, CY induced proportional increases of ß-carrying cells (18) and augmented a delayed hypersensitivity reaction against sheep RBC (15). In preliminary experiments with CY at the dose of 40 mg/kg in WKA rats, when the drug was given on the same day as or 3 days after immunization with sheep RBC, it suppressed antibody production but not the delayed hypersensitivity reaction against dinitrochlorobenzene. Moreover, lymphoid cells from WKA rats given CY injection 10 days previously caused more graft-versus-host reaction than did untreated normal lymphoid cells (2). These results indicate that CY may suppress the production of blocking factor (5, 20) but may enhance cell-mediated immunity. Thus, this kind of immunological imbalance caused by CY may also activate the passively transferred lymphoid cells from rats immunized with allogeneic AH-66 tumor. This interaction may be at the basis of the synergistic therapeutic effects reported herein.

REFERENCES

Effect of Combination Treatment with Cyclophosphamide and Nonspecific Passive Immunization on a Transplantable Tumor in WKA Rats


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/36/7_Part_1/2119

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.