Diminution of Cyclophosphamide-induced Suppression of Antitumor Immunity by an Immunomodulator PS-K and Combined Therapeutic Effects of PS-K and Cyclophosphamide on Transplanted Tumor in Rats

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ABSTRACT

An immunomodulator PS-K was shown to diminish the cyclophosphamide (CY)-induced suppression of specific antitumor transplantation resistance in WKA rats immunized with X-irradiated KMT-17 tumor cells when PS-K was given before treatment of CY. In the immunochemotherapy of transplanted KMT-17 tumor in WKA rats by a combination of CY and PS-K, an enhanced therapeutic effect was also observed when PS-K was given before treatment of CY, with different doses of CY and at different days of CY treatment. However, when PS-K was given just after treatment of CY, the therapeutic effect was rather diminished in comparison with the group having CY treatment alone. By means of the Winn assay, spleen cells obtained from KMT-17-bearing rats (TBR) treated with PS-K followed by CY inhibited the admixed tumor cells more strongly than did spleen cells obtained from TBR treated with CY alone. Recovery of thymus weight from the damage caused by CY was accelerated in TBR treated with PS-K followed by CY and was delayed in TBR treated with CY followed by PS-K. These observations suggest that diminution of CY-induced immunosuppression by PS-K possibly results in an enhanced therapeutic effect in WKA rats treated with PS-K followed by CY.

INTRODUCTION

It is well known that most anticancer drugs have suppressive effects on the antitumor immunity of the hosts. This immunosuppressive effect is becoming a major obstacle in the treatment of cancer patients by chemotherapy. It is thus very important to restore the antitumor immunity suppressed by anticancer drugs or to prevent the immunosuppression in order to achieve an improved therapeutic effect in cancer chemotherapy. We have therefore been looking for some substances which are able to diminish the anticancer drug-induced suppression of antitumor immunity. In preliminary experiments, 3 immunomodulators PS-K, lentinan and OK-432, which are presently being used clinically in Japan, were tested for their effectiveness to restore the antitumor immunity suppressed by CY.3

PS-K is a protein-bound polysaccharide isolated from Basidiomycetes (1, 9, 10, 12), lentinan is a polysaccharide isolated from Letinus edodes (Berk) Sing. (4, 8), and OK-432 is a Streptococcus pyogenes preparation (2, 11). The results showed that only PS-K was the drug which had such activity.4 In this paper, an appropriate combination timing of PS-K and CY was studied on transplanted tumors in rats and mice.

MATERIALS AND METHODS

Animals. An inbred strain of Wistar King Apektman/Hok (WKA) rats was supplied by the Experimental Animal Institute, Hokkaido University School of Medicine, Sapporo, Japan. BALB/c and C57BL/6 mice were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Japan.

Tumors. KMT-17, Meth-A, and YM-12 were transplantable fibrosarcomas induced by 3-methylcholanthrene in a WKA rat, in a BALB/c mouse, and in a C57BL/6 mouse, respectively. These tumors were maintained in the ascites form. The cell doses of KMT-17 causing 50% lethality when injected s.c. into WKA rats were 5 x 103. CY, CY supplied by Shionogi Co., Ltd., Osaka, Japan, was dissolved with sterile 0.85% NaCl solution and given i.v. through the tail vein within 30 min after the dissolution.

PS-K. PS-K in sterile 0.85% NaCl solution, supplied by Kureha Chemical Co., Ltd., Tokyo, Japan, was given i.p. according to the time schedule.

Immunization. Fifty million X-irradiated (8000 rads) KMT-17 tumor cells were inoculated once i.p. into the rats.

In Vivo Tumor-neutralizing Assay (Winn Assay). A modified form of the Winn assay was used (13). Animal spleens were aseptically removed from their hosts and teased into loose-fitting glass homogenizers. The cell suspensions were passed through gauze and washed twice in minimal essential medium. The spleen cell suspension (1 x 107; 0.1 ml) was mixed with an equal volume of 1 x 106 tumor cells, with 0.2 ml of the cell mixture being inoculated s.c. into syngeneic rats which had been irradiated with 300 rads 1 day before inoculation and immediately thereafter rescued with 1 x 107 bone marrow cells. Results were evaluated by measuring the weight of tumors on the seventh day after s.c. inoculation of the mixture because measuring tumor weight on the seventh day gave most quantitative information on the tumor inhibition by spleen cells.

Statistical Analysis. The significant differences in mean survival time and in lethal rate were tested by the t test and the χ² test, respectively.

RESULTS

Effect of PS-K on CY-induced Suppression of Antitumor Transplantation Immunity. In order to discover whether the suppression of antitumor immunity by the strongly immunosuppressive agent CY might be diminished by PS-K, the effect of...
PS-K was examined in WKA rats immunized with X-irradiated tumor cells followed by a treatment of CY. PS-K (50 mg/rat) was given i.p. on Days 3 and 4 or 6 and 7 into WKA rats immunized i.p. with $5 \times 10^5$ X-irradiated tumor cells on Day 0, followed by CY (40 mg/kg) on Day 5, while $1 \times 10^6$ KMT-17 cells were inoculated s.c. on Day 10. As shown in Table 1, when PS-K was given on Days 3 and 4 before treatment of CY, the lethal rate decreased from 100 to 78.6%, and the MSD was significantly prolonged in comparison with the group having immunization plus CY treatments alone. However, when PS-K was given on Days 6 and 7 at a dose of 50 mg/rat after treatment of CY or given 4 times at a dose of 25 mg/rat before treatment of CY, no beneficial effect was observed. These results suggest that the dose, timing, and frequency appeared to be very important factors for a beneficial immunomodulation effect observed by PS-K.

**Combined Therapeutic Effects of PS-K and CY on Transplanted Tumor.** In order to examine whether the beneficial effect of PS-K observed in the induction of antitumor transplantation resistance by X-irradiated tumor cells might also be observed in the immunochemotherapeutic procedure, a combination immunochemotherapy of PS-K and CY was performed on transplanted KMT-17 tumor in WKA rats. KMT-17 cells ($1 \times 10^5$) were inoculated s.c. on Day 0, and CY (40 mg/kg) was given i.v. on Day 5 preceded by PS-K on Days 3 and 4 after tumor inoculation (PS-K plus CY), or CY treatment was first given on Day 5 followed by PS-K on Days 6 and 7 (CY plus PS-K). PS-K treatment alone had little effect on KMT-17 tumor growth, while CY treatment alone was not effective enough to inhibit tumor growth; 16 out of 20 (80%) rats died with tumor (Table 2). When PS-K was given on Days 3 and 4 before treatment of CY, an enhanced therapeutic effect was observed; the lethal rate significantly decreased from 80% to 44.4%, although the MSD of those rats which died was not significantly different between the 2 groups. However, when PS-K was given on Days 6 and 7 after treatment of CY, the therapeutic effect was diminished in comparison with the group having CY treatment alone; the MSD was slightly but significantly shortened from 32.8 to 26.8 days. PS-K appears to be most effective when given just before treatment of CY but not just after it.

To discover furthermore whether the above beneficial combination effect might be observed when PS-K was combined with different doses of CY or at different days of CY treatment, the dose of CY was at first changed from 40 mg/kg to 60, 80, and 100 mg/kg. As shown in Table 3, an increased survival time was observed after PS-K plus the 3 higher doses of CY while a decreased tumor total was seen at the lowest CY dose. At lower doses of CY (40, 60 mg/kg), the lower incidence of macroscopic metastases in lungs, adrenal glands, and axillary lymph nodes was observed in the groups having PS-K plus CY treatments compared with the groups having CY treatment alone (data not shown).

### Table 2

<table>
<thead>
<tr>
<th>CY (40 mg/kg)* on Day 5</th>
<th>PS-K (50 mg/rat) on the following days</th>
<th>Lethal growth</th>
<th>MSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>3, 4</td>
<td>8/18 (44.4)c</td>
<td>30.1 ± 2.3d</td>
</tr>
<tr>
<td>Yes</td>
<td>6, 7</td>
<td>13/13 (100)</td>
<td>28.6 ± 1.1f</td>
</tr>
<tr>
<td>Yes</td>
<td>No</td>
<td>16/20 (80.0)</td>
<td>32.8 ± 1.8</td>
</tr>
<tr>
<td>No</td>
<td>3, 4</td>
<td>4/4 (100)</td>
<td>17.3 ± 1.4</td>
</tr>
<tr>
<td>No</td>
<td>6, 7</td>
<td>4/4 (100)</td>
<td>20.2 ± 1.4</td>
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<tr>
<td>No</td>
<td>1–6</td>
<td>4/4 (100)</td>
<td>17.0 ± 1.4</td>
</tr>
<tr>
<td>No</td>
<td>No</td>
<td>11/11 (100)</td>
<td>17.1 ± 0.8</td>
</tr>
</tbody>
</table>

*a* KMT-17 cells ($1 \times 10^5$) were inoculated s.c. on Day 0. CY (40 mg/kg) was given i.v. on Day 5, and PS-K (50 mg/rat) was given i.p.

### Table 3

<table>
<thead>
<tr>
<th>Dose of CY (mg/kg) on Day 5</th>
<th>PS-K on Days 3 and 4</th>
<th>Lethal growth</th>
<th>MSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>No</td>
<td>10/10 (100)b</td>
<td>34.7 ± 2.7c</td>
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<tr>
<td>40</td>
<td>Yes</td>
<td>5/8 (62.5)d</td>
<td>28.4 ± 2.7</td>
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<tr>
<td>60</td>
<td>No</td>
<td>9/9 (100)</td>
<td>30.0 ± 1.5</td>
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<tr>
<td>60</td>
<td>Yes</td>
<td>6/8 (75.0)</td>
<td>37.0 ± 1.8</td>
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<tr>
<td>80</td>
<td>No</td>
<td>6/6 (100)</td>
<td>32.8 ± 2.3</td>
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<tr>
<td>80</td>
<td>Yes</td>
<td>6/8 (75.0)</td>
<td>39.0 ± 1.6</td>
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<tr>
<td>100</td>
<td>No</td>
<td>9/9 (100)</td>
<td>37.1 ± 1.6</td>
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<tr>
<td>100</td>
<td>Yes</td>
<td>7/9 (77.8)</td>
<td>48.1 ± 2.5</td>
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<tr>
<td>No</td>
<td>No</td>
<td>6/6 (100)</td>
<td>16.0 ± 0.5</td>
</tr>
</tbody>
</table>

*a* KMT-17 cells ($1 \times 10^5$) were inoculated s.c. on Day 0. PS-K (50 mg/rat) was given i.p. on Days 3 and 4, and a different dose of CY was given i.v. on Day 5.

### Table 1

<table>
<thead>
<tr>
<th>Immunized on Day 0</th>
<th>CY on Day 5</th>
<th>Dose (mg/rat)</th>
<th>Days</th>
<th>Lethal growth</th>
<th>MSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>Yes</td>
<td>50</td>
<td>3, 4</td>
<td>11/14 (78.6)c,d</td>
<td>28.5 ± 2.5e,f</td>
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<td>Yes</td>
<td>50</td>
<td>6, 7</td>
<td>13/14 (92.9)</td>
<td>23.0 ± 1.8</td>
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<tr>
<td>Yes</td>
<td>Yes</td>
<td>25</td>
<td>1, 2, 3, 4</td>
<td>9/9 (100)</td>
<td>19.4 ± 0.8</td>
</tr>
<tr>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>21/21 (100)</td>
<td>20.4 ± 0.7</td>
</tr>
<tr>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>2/18 (11.1)</td>
<td>31.5</td>
</tr>
<tr>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>14/14 (100)</td>
<td>19.1 ± 1.0</td>
</tr>
</tbody>
</table>

*a* WKA rats were immunized i.p. with $5 \times 10^5$ X-irradiated KMT-17 cells on Day 0, and CY (40 mg/kg) was given i.v. on Day 5.

*b* MSD is calculated only on animals which died with tumor.

c Numbers in parentheses, percentage.

d p < 0.05 compared with the group having CY treatment alone.

*Numbers in parentheses, percentage.

* p < 0.05 compared with the group having immunization plus CY treatments alone.

*d* p < 0.01 compared with the group having CY treatment alone.

*e* Mean ± S.E.

*f* p < 0.02 compared with the group having PS-K treatment alone.

*Mean ± S.E.

*g* p < 0.02 compared with the group having CY treatment alone.

*h* p < 0.01 compared with the group having CY treatment alone.

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Next, the day of CY treatment was changed from Day 5 to Day 10. Tumor size at Day 5 was just palpable, and at Day 10 the tumor was about 24 mm in diameter. As shown in Table 4, when CY (40 mg/kg) was combined with PS-K, no enhanced therapeutic effect was observed. However, when CY (100 mg/kg) was used, a beneficial combination effect was observed. The enhanced therapeutic effect was also observed when PS-K was given on Days 18 and 19 after treatment of CY, but this was a lesser effect than when PS-K was given just before treatment of CY.

**Tumor-neutralizing Activity of Spleen Cells Obtained from TBR Treated with PS-K and/or CY.** Immune response to KMT-17 in TBR treated with PS-K and/or CY was examined by the in vivo Winn assay. Spleen cells were obtained on Days 14 and 19 from TBR which had previously been treated with PS-K plus CY (Table 5). The spleen cells were admixed with KMT-17 cells, and the mixture was inoculated s.c. We observed that spleen cells removed from TBR which were treated with PS-K and CY more strongly inhibited tumor growth than did spleen cells from TBR treated with only CY. An enhanced cytotoxicity was also observed with an in vitro 51Cr release assay using spleen cells from PS-K and CY treated animals (data not shown).

**Changes in Thymus Weight of TBR Treated with PS-K and/or CY.** In order to discover the effect of PS-K on the cytotoxicity of CY to thymus, considered to be a primary lymphoid organ, changes in thymus weight were examined in TBR treated with PS-K and/or CY. As shown in Chart 1, as the tumor grew larger, thymus weight gradually decreased. When CY (40 mg/kg) was given 5 days after inoculation of tumor cells, thymus weight rapidly decreased owing to the cytotoxicity of CY on thymus cells. Thymus weight on Day 8 was about 50% of that of untreated TBR. Although there were no differences in thymus weight on Day 8 among the groups having PS-K plus CY, CY plus PS-K, and CY treatment alone, thymus weights on Days 14 and 19 were heavier in the group having PS-K plus CY treatments than in the group having CY treatment alone. Thymus weight was rather lighter in the group having CY plus PS-K treatments. As tumor size was almost equal in all 3 groups by the 14th day, the influence of tumor burden on thymus weight seems to be excluded. When PS-K was given before treatment of CY, the recovery of thymus weight from the damage caused by CY appeared to be accelerated. On the other hand, when PS-K was given after treatment of CY, its recovery appeared to be delayed. As to the changes in spleen weight, the accelerated recovery of spleen weight from the damage caused by CY treatment was observed in TBR treated with CY plus PS-K as well as in TBR treated with PS-K plus CY. No clear relationship was observed between the changes in spleen weight and the combination therapeutic effects (data not shown).

**Effective Timing of PS-K and CY Treatments in Other Tumor Cell Lines.** To investigate whether the similar effective timing of PS-K and CY treatments is observed in other tumor cell lines, the combination therapeutic effects of PS-K and CY were examined in YM-12 and Meth-A tumor cell lines (Table 6). Meth-A tumor cells 1 x 10⁶ were inoculated i.p. into BALB/c mice, and CY (100 mg/kg) and PS-K (5 mg/mouse) were combined. MSD was significantly prolonged in comparison with the group having CY treatment alone. No clear relationship was observed between the changes in spleen weight and the combination therapeutic effects (data not shown).
YM-12 tumor cells, 5 × 10⁵, were inoculated i.p. into C57Bl/6 mice, CY treatment alone appeared to have slightly suppressive effect on this tumor. A beneficial combination therapeutic effect of PS-K and CY was observed when PS-K was given after treatment of CY as well as before treatment of it. These results suggest that the beneficial combination effect was induced in spite of tumor cell lines used, at least when PS-K was given before treatment of CY.

DISCUSSION

The chemotherapeutic effect is considered to be the sum of the effects of anticancer drugs on tumor cells themselves and on the antitumor immunity of the hosts. One of the approaches to achieve an improved therapeutic effect in cancer chemotherapy is to develop new anticancer drugs having a stronger cytotoxic effect on tumor cells and less immunosuppressive or, preferably, an immunostimulatory effect. Another approach is to develop methods to prevent or reduce the adverse effects of anticancer drugs, for example, cardiotoxicity in Adriamycin, pulmonary fibrosis in bleomycin, and immunosuppression in most anticancer drugs. We have focused in the present study on their immunosuppressive effect and tried to diminish it.

We have demonstrated that PS-K is able to diminish the immunosuppression by CY in WKA rats immunized with tumor cells and that a combination of PS-K with CY is able to improve the therapeutic effect on transplanted KMT-17 tumor. The precise mechanisms of the enhanced therapeutic effect when PS-K was given before treatment of CY are not still clear. However, the results of antitumor immune response and of changes in thymus weight may suggest that the accelerated recovery from the damage caused by CY of immunocompetent cells or their precursors occurred when PS-K was given before treatment of CY; as a result, stronger antitumor immune response was induced. The observation reported by Barbui et al. (3) that thymus weight correlated well with the thymic lymphocyte activity in surgical injury-induced immunodepressed rats may support the above explanation. The second possibility for the enhanced therapeutic effect by giving PS-K before treatment of CY is that PS-K may increase the activity of CY, for example, by affecting the drug-metabolizing enzyme (7), resulting in an increase of the cytotoxicity of CY on tumor cells.

In preliminary experiments, there were no significant differences in blood concentration of CY between TBR treated with PS-K plus CY and TBR treated with CY alone (data not shown). Therefore, this possibility seems unlikely. Another possibility is that PS-K may make tumor cells themselves more sensitive to CY. When tumor cells were transplanted from PS-K-treated and PS-K-untreated animals and then evaluated for in vivo sensitivity to CY, no differences were observed (data not shown). Furthermore, as the beneficial combination therapeutic effect was observed when PS-K was given at appropriate intervals after treatment of CY (Table 4), this possibility also seems unlikely.

Some reports have been published about the effects of PS-K on immune responses. Nomoto et al. (9) have demonstrated that PS-K restores the impaired antibody-producing capacities in ICR mice bearing Sarcoma 180 and suppresses tumor growth. Restorative activity of PS-K on delayed hypersensitivity has also been observed in tumor-bearing mice. Since the restorative effect of PS-K was not observed in normal mice, they speculated that it might compete with immunosuppressive factors produced in the tumor-bearing state. Oguchi et al. (10) have reported that PS-K restored to normal level thymus cell counts, which had decreased to 40% of the normal level in C3H/He mice bearing × 5563 tumor and that it normalized the percentage of Lyt-1⁺ and Lyt-2⁺ populations in thymuses. An unknown factor in PS-K might act on the differentiation of thymus cells.

In immunochemotherapy, the importance of the timing of the combination of chemotherapy and immunotherapy has already been reported by other investigators (1, 5, 6, 14). Akiyama et al. (1) in our laboratory have demonstrated a more critical combination timing of PS-K and CY in the KMT-17 tumor system. When PS-K was given on Day 1 followed by CY on Day 3, the highest survival rate was obtained. In their study, the question whether PS-K should be given 1 day after the inoculation of tumor cells or PS-K should be given 2 days before treatment of CY still remained unanswered. Our present results may suggest that the giving of PS-K before treatment of CY is the critical factor for showing a beneficial combination effect. We then checked the generality of the effective timing of PS-K and CY using 2 other tumor cell lines. When PS-K was given just after treatment of CY, 3 different results were ob-
tained, an adverse effect, no effect, and a beneficial effect. However, in spite of the difference in sensitivity to CY and PS-K among the 3 tumor cell lines, a beneficial combination effect was observed in all 3 tumor cell lines when PS-K was given just before treatment of CY. Experiments are in process which will attempt to further determine the optimal time of administration of PS-K with CY to achieve the desired therapeutic effect.

REFERENCES


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