Enhanced Induction of Immune Resistance by Concanavalin A-bound L1210 Vaccine and an Immunopotentiator Prepared from Coriolus versicolor

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SUMMARY

Combined administration of a vaccine consisting of a small number (2 × 10⁶) of L1210 murine leukemic cells treated with glutaraldehyde and concanavalin A and a protein-bound polysaccharide preparation of Coriolus versicolor induced synergistic resistance to L1210 leukemia in BALB/c × DBA/2Cr F₁ mice. This effect was dependent on the dose and timing of the administration of the protein-bound polysaccharide preparation, being most effective at the time of or 1 day after the second vaccination. Induced resistance was not cross-reactive with P388 murine leukemia, indicating specificity of resistance. This immunopotenti- nation by the protein-bound polysaccharide did not occur when L1210 cells treated with glutaraldehyde, but not with concanavalin A, were used as a vaccine.

INTRODUCTION

In the field of cancer immunology, substances are now being sought that might serve as immunopotentiators in the development of an immunity to a syngeneic tumor. It has been shown in several laboratories that a water extract of C. versicolor (Fr) Quel of Basidiomycetes (PSK) exerts a beneficial therapeutic effect by itself in various experimental tumors (4, 5, 8, 9). Although its mechanism is not fully understood, various effects of PSK on the host immune system have been reported (5, 6, 8). These findings suggest the possibility that PSK might act as an immunopotentiator.

To investigate its possible role as an immunopotentiator, we combined PSK with a vaccine prepared by glutaraldehyde and Con A treatment of L1210 murine leukemic cells. This vaccine is immunogenic by itself (1), but was used in this study at a dose that gave poor immunity to subsequent L1210 challenge. The immunity was strongly augmented, however, by i.p. administration of PSK as an immunopotentiator to the vaccine.

MATERIALS AND METHODS

Animals and Leukemias. Adult male BALB/c × DBA/2Cr F₁ mice (hereafter called CD2F₃,) for the experiments and DBA/2Cr mice for carrying tumors were supplied by the National Cancer Institute, NIH, Bethesda, Md. L1210 and P388 leukemias were supplied by the National Cancer Institute and have been carried in the transplantable ascites form in syngeneic DBA/2Cr mice. Leukemic cells were collected in Hanks' balanced salt solution (Kyokuto Chemicals, Tokyo, Japan) 4 or 5 days after the inoculation of 1 to 3 × 10⁵ cells. The cell suspension was centrifuged in a cold room at 800 rpm (100 × g) for 5 min in a swing-type Model CD-50 SN centrifuge (Tomy Seiko Co., Ltd., Tokyo, Japan). The sediment was resuspended in PBS and used for preparing vaccines and for challenges. All the centrifugations described below were carried out at 4°C.

Vaccine Preparation and PSK. L1210 vaccine was prepared as reported previously (1). Briefly, tumor cells (0.5 × 10⁷ cells/ml) were incubated with 0.013% glutaraldehyde on ice for 30 min. After being washed with PBS, the cells were incubated with Con A (165 μg/ml) (Miles Laboratories, Inc., Kankakee, Ill.) on ice for 1 hr. After being washed with PBS, the cells were suspended in PBS, 0.1 or 0.2 ml of the suspension, equivalent to 1 × 10⁶ cells, was administered i.p. to the animals. Except as otherwise stated (Table 4), this preparation (G-Con A-L1210) is referred to simply as the vaccine. In the experiment shown in Table 4, another vaccine (G-L1210) was used, which was prepared by treatment with glutaraldehyde but not with Con A. Systemic leukemia was never produced by inoculation with either vaccine preparation alone in control mice (1, 2).

PSK was supplied by Kureha Chemical Industries Co., Ltd., Tokyo, Japan, and was diluted with 0.85% NaCl solution if necessary.

Vaccination, PSK Administration, and Evaluation of Immune Resistance. Mice were immunized i.p. twice at 1- or 2-week intervals with 10⁶ vaccine cells and were given PSK i.p. at the indicated times. They were challenged i.p. with 10⁶ live leukemic cells 6 or more days after the last vaccination. One month later, survivors were further challenged with 10⁶ live leukemic cells, and those that survived for another month and showed no sign of tumor (i.e., increase of ascitic fluid and/or palpable tumor growth) were considered to be cured. Results were also evaluated in terms of the relative percentage of mean survival days of test animals compared with control animals dying from the tumor.

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2 The abbreviations used are: PSK, protein-bound polysaccharide preparation of Coriolus versicolor; Con A, concanavalin A; PBS, 5 mm phosphate-buffered 0.85% sodium chloride solution adjusted to pH 7.4; G-Con A-L1210, L1210 cells treated with glutaraldehyde and concanavalin A; G-L1210, L1210 cells treated with glutaraldehyde.

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Con A-bound Tumor Vaccine and an Immunopotentiator

RESULTS

Schedule Dependence of L1210 Vaccine Potentiation by PSK. PSK (250 mg/kg/day) was given i.p. for 4 consecutive days at different times during the course of the experiment to animals nonsensitized or sensitized by G-Con A-L1210 vaccine (10^6 cells), and its effect on the induction of resistance to live L1210 challenge (10^3 cells) was examined (Chart 1). Administration of the vaccine twice separated by a 2-week interval induced very few resistant mice (Group 1, 3% cure). PSK combined with the vaccine was also not very effective in producing cures when given either before a second vaccination (Group 3, 7% cure) or after challenge (Group 5, 0% cure). However, PSK produced a high cure incidence when administered either after the first vaccination (Group 2, 20% cure) or between a second vaccination and a challenge (Group 4, 39% cure). Considering the low cure incidence in PSK-treated animals (Group 6, 0% cure), high cure incidence in Group 4 was thought to be due to a synergistic effect between the vaccine and PSK. In this experimental protocol, however, it was not clear whether the PSK effect was associated with the second vaccination or with the challenge, in terms of timing. Both of these possibilities were examined. The twice vaccinated animals were given PSK i.p. starting 1 or 5 days after the last vaccination (Chart 2). Six of 10 animals survived the challenge when PSK was administered beginning 1 day after the second vaccination, and this heightened resistance lasted as long as 18 days. In contrast, 1 of 10 animals survived the challenge when PSK was administered starting 5 days after the second vaccination. This result suggests that, in terms of timing, synergistically effective administration of PSK was associated with vaccination rather than with challenge. In the following studies, therefore, the effect of PSK on the second vaccination (Chart 1, Group 4) was evaluated in more detail.

Frequency, Timing, and Dosages of PSK Administration and Enhanced Secondary Vaccination. In the above experiments, PSK was administered for 4 consecutive days. To determine whether frequent administration of PSK was essential for secondary vaccine potentiation, we sensitized groups of mice twice with i.p. G-Con A-L1210 vaccine and gave i.p. PSK (250 mg/kg/day) for 1, 2, 4, or 5 consecutive days starting 1 day after the second vaccination. They were subsequently challenged i.p. with live L1210 cells. The results (Chart 3) show that a single administration of PSK produced as many cured mice as did multiple administrations. This enabled us to examine in greater detail the timing of PSK administration associated with secondary vaccine potentiation. Groups of mice sensitized i.p. twice with G-Con A-L1210 vaccine were given i.p. PSK (250 mg/kg) once either before, at the same time as, or after the second vaccination. They were subsequently challenged i.p. with live L1210 cells (Chart 4). High cure incidences
were found when PSK was administered on the same day as or 1 day after the second vaccination (59 and 55%, respectively). Earlier (1 day before the second vaccination) or later (3 or 5 days after the second vaccination) administration of PSK was much less effective.

The effect of PSK dosage was examined, and the results are given in Table 1. Groups of mice sensitized i.p. twice with G-Con A-L1210 vaccine were given different doses of PSK i.p. on the day of or 1 day after the last vaccination and were subsequently challenged i.p. with live L1210 cells. Optimal effects with appreciable numbers of cured mice were seen at doses between 15.6 and 1000 mg/kg. No apparent drug toxicity was observed at 1000 mg/kg. Clearly, the potentiation of L1210 vaccine was dose dependent, with the maximal effect at 250 mg of PSK per kg (52% cure), although there was a wide effective dose range.

**Specificity of Secondary Vaccine Potentiation by PSK.** It was determined whether the enhanced protection against tumor challenge seen with the secondary vaccine and PSK administration was specific. Two groups of mice sensitized i.p. twice with G-Con A-L1210 vaccine and given PSK i.p. (250 mg/kg/day) for 4 consecutive days starting 1 day after the second vaccination were subsequently challenged i.p. with either live L1210 cells (106) or live P388 cells (106) (Table 2). All of the immunopotentiayed mice were killed by P388 leukemia without any prolongation of the life span, whereas a 50% cure incidence was obtained for those challenged with L1210 leukemia. Thus, the enhanced protective effect seen with secondary L1210 vaccine and PSK was specific for L1210.

**Interaction of PSK with Secondary Vaccine.** We examined the possibility that PSK, when combined, exerted its effect in vivo by making the vaccine more immunogenic. Groups of mice sensitized i.p. once with G-Con A-L1210 vaccine (106 cells) were given i.p. 1 of the 3 following inocula for a second vaccination: G-Con A-L1210 vaccine, an incubation mixture of G-Con A-L1210 vaccine and PSK, or G-Con A-L1210 vaccine incubated with PSK followed by washing with PBS to remove unbound PSK (Table 3). With the second inoculum an animal was given an injection of G-Con A-L1210 vaccine (106 cells) and PSK at a dose of 5 mg/mouse, which was equivalent to 250 mg/kg, assuming the body weight of an animal to be 20 g. Animals were subsequently challenged i.p. with live L1210 cells, and percent cure incidences were examined. An appreciable cure rate was found in the second group (50%), whereas none of the animals in the first and third groups survived the challenge. Therefore, it is unlikely that in vivo modification of secondary vaccine by PSK was involved in the enhanced induction of immune resistance by PSK.

**Vaccine Preparations and Immunopotentiation by PSK.** Throughout the above experiments, G-Con A-L1210 cells were used as the vaccine. We also examined whether synergistic protection of animals from L1210 by vaccination and PSK administration could be achieved with a simplified vaccine preparation. Two groups of mice were sensitized i.p. twice with either G-Con A-L1210 (106 cells) or G-L1210 (106 cells) and were then given PSK i.p. (250 mg/kg/day) for 4 consecutive days starting 1 day after the second vaccination. They were subsequently challenged i.p. with live L1210 cells (Table 4). None of the animals vaccinated with G-L1210 and potentiated by PSK survived the challenge or lived for a prolonged time, whereas 50% of the animals vaccinated with G-Con A-L1210 and potentiated by PSK were cured. This indicates that the synergistic effect of the vaccine and PSK is dependent on the complete tumor vaccine preparation.

**DISCUSSION**

We have demonstrated enhanced protection from L1210

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**Table 1**

<table>
<thead>
<tr>
<th>Dose of PSK (mg/kg)</th>
<th>No. of cured mice/total no. of mice</th>
<th>% cure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>10/30</td>
<td>33</td>
</tr>
<tr>
<td>250</td>
<td>15/29</td>
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<tr>
<td>62.5</td>
<td>7/19</td>
<td>37</td>
</tr>
<tr>
<td>15.6</td>
<td>9/31</td>
<td>29</td>
</tr>
<tr>
<td>3.9</td>
<td>2/20</td>
<td>10</td>
</tr>
<tr>
<td>0.98</td>
<td>0/10</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 2**

<table>
<thead>
<tr>
<th>Challenging inoculum</th>
<th>No. of cured mice</th>
<th>T/C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210 cells</td>
<td>5/10</td>
<td></td>
</tr>
<tr>
<td>P388 cells</td>
<td>0/10</td>
<td>89 ± 5.5*</td>
</tr>
</tbody>
</table>

* Relative percentage of mean survival days of test animals compared with control animals dying from the tumor.

* Mean ± S.D.

**Table 3**

<table>
<thead>
<tr>
<th>No. of cured mice/total no. of mice</th>
<th>Incubation with PSK</th>
<th>Washing with PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
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</table>
the administration of PSK. We investigated the timing of week intervals with either G-L1210 (10^6 cells) or G-Con A-L1210 vaccination. One-month survivors were rechallenged with live L1210 cells (10^6), and 2-month survivors were recorded as cured mice.

<table>
<thead>
<tr>
<th>Vaccine preparation</th>
<th>No. of cured mice/total no. of mice</th>
<th>T/C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-L1210</td>
<td>0/10</td>
<td>99 ± 7.3</td>
</tr>
<tr>
<td>G-ConA-L1210</td>
<td>5/10</td>
<td></td>
</tr>
</tbody>
</table>

* Relative percentage of mean survival days of test animals compared with control animals dying from the tumor.
* Mean ± S.D.

leukemia by a combined administration of vaccine and PSK. This enhanced protection was dependent on the preparation of the vaccine and the dosage and timing of the administration of PSK. We investigated the timing of PSK administration extensively. We found that PSK enhanced secondary vaccination more markedly than it enhanced primary vaccination. For the synergistic effect to occur, the vaccine and PSK had to be present at almost the same time. This did not indicate, however, that the vaccine was modified by PSK in vivo, resulting in increased immunogenicity of the vaccine, because when we sensitized animals with G-Con A-L1210 vaccine preincubated with PSK in vitro we could not find any increased incidence of mice resistant to the challenge of L1210 cells (Table 3).

PSK is active in enhancing both cellular and humoral immunity (5, 6, 8). In CD2F mice, inhibition of macrophage migration was nonspecifically augmented by PSK (unpublished result). We do not know how any of these immunological activities are associated with synergistically augmented immunity. PSK alone did not show any antitumor activity to L1210 leukemia (Chart 1). It was effective only when it was administered at the time of or after vaccination. This indicates that PSK amplified an immunity triggered by the vaccine. Potentiation of immunity was dependent on the composition of the vaccine preparation. G-L1210 vaccine could not substitute for G-Con A-L1210 vaccine (Table 4). G-ConA-L1210 vaccine alone was immunogenic, but G-L1210 vaccine was not (1). It is probable that, for potentiation by PSK to occur, the vaccine must be immunogenic by itself.

By investigating the role of bound Con A in G-Con A-L1210 vaccine, one might derive a hypothesis as to which kinds of immunocytes are involved in the synergistic effect of PSK. The fact that PSK induced more pronounced protection from L1210 leukemia when it was combined with the second vaccination than when it was combined with the first vaccination indicates the possibility that memory cells were involved in the observed enhancement. This concept is open to further investigation. PSK is a preparation of polysaccharide containing about 15%-protein constituent (8). Whether we can attach any significance to the protein constituent in terms of the synergistic activity of PSK is unknown. However, it is presumed that the protein constituent might endow PSK with a beneficial property that is not found in simple polysaccharide immunopotentiators, such as lentinan and zymosan. Our preliminary results suggest that p.o. PSK is active, but to a lesser extent than i.p. PSK, in inducing the synergistic effect when combined with the vaccine. This indicates that the protein constituent is important in the synergistic effect. Further characterization of the active component of PSK is needed to answer this question.

Many kinds of immunopotentiators are currently under experimental and clinical study. At present it is not clear whether PSK is superior to the others in any respect. Although we used narrowly defined experimental conditions, i.e., 10^6 G-Con A-L1210 vaccine cells and 10^3 challenging cells, to demonstrate enhancement of the vaccine-induced immune response by PSK, PSK is not necessarily immunologically effective only in this limited situation. PSK was therapeutically effective in syngeneic and allogeneic tumor systems (6, 9). We believe that PSK is less toxic than other immunopotentiators. No apparent drug toxicity was noted in mice at dosages of 1000 mg/kg once or of 250 mg/kg/day for 20 consecutive days. Thus, PSK is extremely safe. We are comparing the effects of PSK and different kinds of immunopotentiators, including Baccilus Calmette-Guérin (7), Corynebacterium parvum (3), and pyran copolymer, in producing a synergistic effect on immunity when combined with vaccine. This approach will indicate the value of the immunological potency of PSK.

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