Immunotherapeutic Response of Concanavalin A-bound L1210 Vaccine Enhanced by a Streptococcal Immunopotentiator, OK-432

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ABSTRACT

Immunotherapeutic response to concanavalin A (Con A)-bound L1210 murine leukemia vaccine and immunopotentiators was examined in histocompatible animals bearing a small burden of L1210 leukemic cells. When combined with Con A-bound vaccine, a streptococcal immunopotentiator, OK-432 (NSC B116209), prepared from Streptococcus pyogenes, was potent in antitumor therapy and resulted in a number of cured animals. Administration of either Con-A-bound vaccine or OK-432 alone did not produce any beneficial effect on leukemic animals. The enhanced therapeutic response was dependent on the effectiveness of the dose and timing of the administration of OK-432 when given after vaccination. Combined modality of Con A-bound L1210 vaccine and OK-432 was not effective in animals bearing P388 murine leukemia cells, which indicates specificity of therapeutic response.

In enhancing the therapeutic potency of Con A-bound leukemia vaccine, pyran copolymer (NSC 46015) was as effective as OK-432, whereas Bacillus Calmette-Guérin and Corynebacterium parvum were far less effective. When combined with OK-432, therapeutic response to Con A-bound L1210 vaccine was much greater than response to glutaraldehyde-, mitomycin C-, or Vibrio cholerae neuraminidase-treated L1210 vaccine.

INTRODUCTION

Recent progress in tumor immunology has made immunological modality in antitumor therapy feasible, although the therapeutic effects thus far demonstrated are far from satisfactory. One approach to further advancing this modality is to develop tumor vaccines and immunopotentiators more potent than those currently available. In this regard, we, as well as others, reported that Con A-bound tumor vaccine produced a strong immunoprophylactic response in animals (3, 5). A comparative study4 showed that Con A-bound vaccine was more potent in inducing immune resistance in animals than were other types of L1210 vaccine prepared in the presence of formaldehyde, glutaraldehyde, mitomycin C, or Vibrio cholerae neuraminidase. Nevertheless, Con A-bound tumor vaccine was not sufficiently powerful in immunotherapy of different experimental tumors to produce cured animals, although its potency was clearly demonstrated (10).

Recently, we found that Con A-bound, but not Con A-free, tumor vaccine was highly potentiated by an immunopotentiator inducing enhanced immunoprophylactic response in animals (4). This led us to examine the immunotherapeutic potency of Con A-bound tumor vaccine when combined with immunopotentiators.

In this paper, we report the following findings. Among the immunopotentiators tested for potency of enhancement of Con A-bound L1210 murine leukemia vaccine, OK-432 was most potent in inducing immunoprophylactic response. Combination of these 2 agents exerted a strong immunotherapeutic response in L1210 leukemic animals and produced many cures.

MATERIALS AND METHODS

Cells and Animals. L1210 and P388 murine leukemia cells induced in DBA/2 mice by methylcholanthrene were supplied by Division of Cancer Treatment, the National Cancer Institute, Bethesda, Md., and passaged in male DBA/2Cr mice. Male BALB/c x DBA/2Cr F1 mice used in the experiments were supplied by Simonsen Laboratories, Gilroy, Calif., and Laboratory Supply Co., Indianapolis, Ind., respectively.

Vaccine. Con A-bound L1210 leukemia vaccine was prepared according to the method reported previously (4). Briefly, L1210 cells were incubated with glutaraldehyde (0.025%) for 30 min on ice and then were incubated with Con A (165 μg/ml) for 1 hr on ice. Other types of L1210 vaccine in Table 5 were prepared according to methods reported elsewhere and the proliferation of these vaccine cells was completely suppressed in vivo as well as in vitro.5

Evaluation of Immunoprophylactic and Immunotherapeutic Response. For immunoprophylactic response, animals were challenged with 103 live L1210 cells i.p. 1 week after the last vaccination. For immunotherapeutic response, animals inoculated i.p. with 1 to 2.5 × 107 live L1210 cells were given i.p. 8 × 105 vaccine cells and/or immunopotentiators by the indicated regimen. In both situations, animals that survived for 1 month after inoculation of live leukemic cells and without any sign of tumor (i.e., increase of ascitic fluid and/or delayed palpable s.c. growth of tumor) were challenged further with the same number of, or 10 times more, live leukemic cells i.p. Those surviving the second challenge for another month and free of any sign of tumor were counted as cured animals. Mean survival times were calculated both for mice that died within 1 month and for those that survived for 1 month, although either they did bear tumors or they were killed by reinoculation of live tumor cells. Although the results are presented in summarized form, i.e., cumulated for cure incidences and averaged for
mean survival times, none of the experimental groups of one experiment is included in any other experiment. Thus, overlapping of the experimental groups was eliminated.

**Immunopotentiators and Chemicals.** Immunopotentiators used are: OK-432 (kindly supplied by Chugai Pharmaceutical Co., Ltd., Tokyo, Japan); BCG (Japan BCG Co., Ltd., Tokyo); Corynebacterium parvum (Institut Merieux, Lyon, France, kindly supplied by Dr. T. Toge); poly(l)-poly(C) (Yamasu Shoyu Co., Ltd., Choshi, Chiba Pref., Japan); levamisole (Kyowa Hakko Co., Ltd., Tokyo, Japan); and PSK (kindly supplied by Kureha Chemical Industries Co., Ltd., Tokyo, Japan). Con A (Miles Laboratories, Inc., Kankakee, Ill.), Vibrio cholerae neuramindase (Grand Island Biol. Co., Grand Island, N. Y.), and mitomycin C (Kyowa Hakko Co., Ltd., Tokyo, Japan) were used. One KE of OK-432 is equivalent to 0.1 mg of lyophilized bacteria. Doses of immunopotentiators used in this study were those that produced the highest cure incidences as assayed preliminarily by the immunoprophylactic regimen reported previously (4). Animals inoculated twice with $10^8$ Con A-bound vaccine cells at 1-week intervals and given immunopotentiators on the following day were challenged with $10^3$ live Li 210 cells 1 week later and examined for cures as defined in this paper. Optimal doses of immunopotentiators and induced cure incidences were: OK-432, 60 to 80% at 25 to 400 KE/kg; BCG, 50 to 70% at 0.5 to 50 mg/kg; C. parvum, 40 to 50% at 0.032 to 50 mg/kg; poly(l)-poly(C), 50% at 10 mg/kg; levamisole, not effective; PSK, 30 to 50% at 62.5 to 1000 mg/kg; Con A-bound vaccine without immunopotentiators produced a 15% cure incidence (6 cured mice of 54 mice tested). These results indicate that immunopotentiators had a poor dose-response relationship in this assay system. Each of these effective doses was selected for each immunopotentiator in this study.

**RESULTS**

**Enhanced Immunoprophylactic Response of Con A-bound L1210 Vaccine by Different Immunopotentiators.** Groups of animals were inoculated i.p. with Con A-bound L1210 vaccine and different immunopotentiators and were examined for induction of immune resistance by subsequent inoculation of live L1210 cells (Table 1). Single inoculation of Con A-bound L1210 vaccine did not produce a significant number of cured animals and did not prolong the life span of the animals remarkably, if at all. Combined inoculation of the vaccine and immunopotentiators at optimal doses induced varying degrees of immune resistance in animals. Poly(l)-poly(C), levamisole, and PSK did not potentiate the leukemia vaccine, whereas C. parvum, BCG, lentinan, pyran copolymer, and OK-432 potentiated the leukemia vaccine as evidenced either by cure incidence or by mean survival time, although single inoculation of any of these did not induce detectable immune resistance, (data not included). Of the effective immunopotentiators, OK-432 exerted its potency invariably in a number of experiments. In the following therapeutic experiments, the potency of Con A-bound vaccine as combined with OK-432 was examined.

**Synergistic Therapeutic Effect of Con A-bound L1210 Vaccine and OK-432 in Leukemic Animals.** Groups of animals inoculated with live L1210 cells were treated with Con A-bound vaccine on Day 1 and/or OK-432 on Day 3 (Chart 1). Inoculation of the vaccine or OK-432 alone did not produce any cured animals, although the former induced a marginal but reproducible prolongation of life span in animals. In contrast, combined inoculation of Con A-bound vaccine and OK-432 exerted a strong immunotherapeutic influence and produced 9 cured animals of 10 animals tested.

**Effect of Timing of OK-432 Administration on Immunotherapy by Con A-bound L1210 Vaccine.** Groups of animals inoculated with live L1210 cells were given Con A-bound vaccine on Day 1 and OK-432 at indicated doses on Day 3 (Chart 2). Those given OK-432 at the time of or 1 day after inoculation of live L1210 cells were not cured, whereas those given OK-432 later than 2 days after live L1210 inoculation were cured or lived for prolonged periods of time.

**Dose Response of OK-432 In Immunotherapy by Con A-bound L1210 Vaccine.** Groups of animals inoculated with live L1210 cells were given Con A-bound vaccine on Day 1 and OK-432 at indicated doses on Day 3 (Table 2). OK-432, 400 and 100 KE/kg, produced a cure rate of 50%, whereas OK-432 at lower doses induced poor responses.

**Specificity of Immunotherapy by Con A-bound L1210 Vaccine and OK-432.** Groups of animals inoculated with live L1210 or P388 cells were given Con A-bound L1210 vaccine on Day 1 and OK-432 on Day 3 (Table 3). About 60% of L1210-

![Table 1](data:image/png;base64,iVBORw0KGgoAAAANSUhEUgAAAAEAAAABCAQAAAC1HAwCAAAAC0lEQVR42mP8B8Q8AAACQgAD8VhAAA%3D%3D)

<table>
<thead>
<tr>
<th>Immunopotentiators</th>
<th>Dose/kg</th>
<th>No. of cured mice/total</th>
<th>Survival days</th>
</tr>
</thead>
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<tr>
<td>OK-432</td>
<td>100 KE</td>
<td>21/34</td>
<td>15.8 ± 2.1</td>
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<tr>
<td>Pyran copolymer</td>
<td>25 mg</td>
<td>12/15</td>
<td>22.0 ± 1.8</td>
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<tr>
<td>C. parvum</td>
<td>0.125 mg</td>
<td>5/10</td>
<td>23.9 ± 2.1</td>
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<tr>
<td>Lentinan</td>
<td>1.25 mg</td>
<td>1/10</td>
<td>19.4 ± 0.3</td>
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<td>BCG</td>
<td>0.5 mg</td>
<td>3/10</td>
<td>21.4 ± 1.6</td>
</tr>
<tr>
<td>PSK</td>
<td>250 mg</td>
<td>2/20</td>
<td>12.5 ± 0</td>
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<tr>
<td>Levamisole</td>
<td>0.38 mg</td>
<td>1/10</td>
<td>11.0 ± 0</td>
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<tr>
<td>Poly(l)-poly(C)</td>
<td>10 mg</td>
<td>1/10</td>
<td>11.0 ± 0</td>
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<tr>
<td>None</td>
<td>0/45</td>
<td>0/45</td>
<td>12.0 ± 0.8</td>
</tr>
</tbody>
</table>

* Mean survival days of nonvaccinated control mice, 10.7 ± 0.2.

* p < 0.05 by Fisher's exact test as compared with that of vaccinated mice given no immunopotentiator.

* Mean ± S.E.

Chart 1. Enhanced therapeutic effect of Con A-bound L1210 vaccine by OK-432 in L1210 leukemic animals. Groups of mice inoculated i.p. with $10^8$ live L1210 cells (1) were given i.p. $8 \times 10^5$ Con A-bound L1210 vaccine cells (2) and/or OK-432, 100 KE/kg (3), at the indicated intervals (days) and examined for survival. Summarized results of 2 experiments are presented. For definition of cure and calculation of mean survival days, see "Materials and Methods."
Leukemic Vaccine Preparations Producing Enhanced Immunotherapeutic Effect as Combined with OK-432. Groups of animals inoculated with live L1210 cells were given the indicated L1210 vaccine preparations on Day 1 and OK-432 on Day 3 (Table 5). Glutaraldehyde-, mitomycin C-, or V. cholerae neuraminidase-treated L1210 vaccine did not produce cured animals or prolonged life span of animals, whereas Con A-bound L1210 vaccine produced a 40% cure. These results indicate that Con A-bound vaccine was the most potent immunotherapeutically as combined with OK-432.

**DISCUSSION**

Con A-bound L1210 vaccine is immunoprophylactically more potent than other forms of the leukemia vaccines prepared in the presence of glutaraldehyde, formaldehyde, mitomycin C, or V. cholerae neuraminidase. Nevertheless, Con A-bound vaccine was not therapeutically effective in leukemic animals, although it marginally prolonged their life span (Chart 1). However, combination of Con A-bound vaccine with OK-432 induced a synergistic therapeutic effect in leukemic animals and produced a number of cures. The dose and timing of administration of OK-432 were important for this synergistic effect to occur.

**Table 2**

<table>
<thead>
<tr>
<th>Dose response of OK-432 in active immunotherapy of L1210 leukemia by Con A-bound L1210 vaccine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups of mice inoculated i.p. with 10^2 or 2.5 x 10^2 live L1210 cells were given i.p. 8 x 10^6 Con A-bound L1210 vaccine cells on Day 1 and OK-432 at indicated doses on Day 3 and were examined for survival. Summarized results of 3 experiments are presented.</td>
</tr>
<tr>
<td>Dose of OK-432 (KE/kg)</td>
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</tr>
<tr>
<td>400</td>
</tr>
<tr>
<td>100</td>
</tr>
<tr>
<td>25</td>
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<tr>
<td>6.25</td>
</tr>
<tr>
<td>1.56</td>
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<td>0</td>
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Mean ± S.E. of 2 experiments.

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* p < 0.02 by Fisher's exact test as compared with that of vaccinated mice given no immunopotentiator.

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bearing animals were cured, whereas none of the P388-bearing animals were cured or lived for a prolonged period of time. These results indicate that immunotherapeutic activity by Con A-bound vaccine and OK-432 was specific to L1210 leukemia.

**Immunopotentiators Producing Enhanced Immunotherapeutic Effect as Combined with Con A-bound L1210 Vaccine**

Groups of animals inoculated with live L1210 cells were given Con A-bound vaccine on Day 1 and optimal doses of different immunopotentiators on Day 2 or Day 3 (Table 4). OK-432 and pyran copolymer produced significant numbers of cured animals as compared with the number cured in the absence of immunopotentiators. In contrast, BCG and C. parvum failed to induce therapeutic effects in animals in terms of cure incidence and mean survival time.

**Table 4**

<table>
<thead>
<tr>
<th>Effect of different immunopotentiators on active immunotherapy of L1210 leukemia by Con A-bound L1210 vaccine</th>
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</thead>
<tbody>
<tr>
<td>Groups of mice inoculated i.p. with 2.5 x 10^2 live L1210 cells were given i.p. 8 x 10^6 Con A-bound L1210 vaccine cells on Day 1 and immunopotentiators of indicated doses on Day 2 or Day 3 and were examined for survival. Summarized results of 2 experiments are presented except C. parvum.</td>
</tr>
<tr>
<td>Immunopotentiators</td>
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<tr>
<td>OK-432</td>
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<tr>
<td>Pyran copolymer</td>
</tr>
<tr>
<td>BCG</td>
</tr>
<tr>
<td>C. parvum</td>
</tr>
<tr>
<td>None</td>
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</tbody>
</table>

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a Mean survival days of control mice, 12.0 ± 0.3 (L1210) and 20.3 ± 0.7 (P388), respectively.

b p < 0.01 by Fisher's exact test.

c Mean ± S.E.
vaccine was not effective (Chart 2), which suggests that the immune response triggered by the leukemia vaccine was amplified and/or supplemented by OK-432.

With regard to effectors involved in enhanced immune response by Con A-bound vaccine and OK-432, further experimentation is needed. The fact that OK-432 as well as pyran copolymer, another potentiator of Con A-bound vaccine, are activators of macrophages (1, 9) suggests the possibility that macrophages are involved in the observed enhanced immune response by Con A-bound vaccine and immunopotentiators. However, it is unlikely that macrophages are effectors in this situation because OK-432 and pyran copolymer are supposed to induce nonspecific effector macrophages, whereas effectors involved in this situation are specific to L1210 leukemia. In fact, OK-432 alone failed to produce an immunotherapeutic effect in mice bearing the leukemia (Chart 1), and OK-432 given before inoculation of Con A-bound vaccine did not induce a synergistic therapeutic effect (Chart 2).

These results lead us to think that macrophages activated by OK-432 and pyran copolymer might not be effectors; rather, they help to produce effectors. In this regard, it should be pointed out that lymphocyte blastogenic potency of Con A-bound vaccine is closely associated with the eventual in vivo production of immune resistance (2), although its detailed mechanism is yet to be clarified. The fact that macrophages are involved in blastogenic response of lymphocytes by the lectin (8) suggests the possibility that macrophages activated by OK-432 and pyran copolymer might enhance the blastogenic response of Con A-bound vaccine, leading to the eventual enhanced immune response.

Immunogenic potency of X-irradiated L1210 cells combined either with BCG (6) or with pyran copolymer (7) had been demonstrated previously. In this paper, Con A-bound L1210 vaccine combined either with BCG or with pyran copolymer induced apparently poorer therapeutic as well as immunophrophylactic responses than those demonstrated in the above reports. However, as investigators of both studies pointed out, they performed the experiments under conditions in which an allogeneic reaction was probably involved. Thus, it appears that histocompatibility would be liable for the apparent difference in induced immune response of the previous studies and the present study. Alternatively, it is possible that the regimens used in this study were not optimal for BCG and pyran copolymer although their doses were carefully selected on the basis of inducing ability of immunophrophylactic response ("Materials and Methods"; Table 1). The situation might be true with other immunopotentiators that failed to enhance the potency of Con A-bound vaccine immunotherapeutically as well as immunophrophylactically.

Combined modality of Con A-bound vaccine and OK-432 was immunotherapeutically effective when the tumor burden was small. Animals inoculated with $10^3$ live L1210 cells and treated by the combined modality did not show as remarkable a response as did animals inoculated with $10^2$ live leukemic cells.6 This does not necessarily devaluate the immunological approach to cancer therapy. A large mass of tumor would be eliminated by other modalities such as surgery, irradiation, and chemotherapy. Thus, it is quite important to develop a therapeutical approach to eliminate a residual small mass of tumor completely and to protect from recurrence. In this regard, finding that leukemic animals were cured and resistant to further inoculation of live L1210 cells after combined treatment with Con A-bound vaccine and OK-432 indicates that this immunological modality should be taken into account as one possible approach to this purpose. Much careful study including effects of injecting sites of immunopotentiators is needed before a further evaluation of this modality from the clinical viewpoint.

To advance an immunological approach furthermore, we need more potent immunopotentiators. Thus far we do not have any established way of screening immunopotentiators. Immunophrophylactic response would not necessarily screen immunopotentiators effective in immunotherapy because of differences in immunological status of tumor-free and tumor-bearing hosts. Nevertheless, we assume that the screen of tumor vaccine in this study is a useful one because it provides to immunopotentiators an immunological situation similar to that in the tumor-bearing hosts, in that a mass of tumor-associated antigen precedes the inoculation of immunopotentiators. Thus, we might expect basically the same type of immunological stimulation of immunopotentiators in a tumor-bearing host as that in a tumor-free host, although the outcome would be less remarkable in a tumor-bearing host because of many suppressing factors. In fact, this study indicates that an agent potent in inducing immunophrophylactic response would be potent in inducing immunotherapeutic response as well. Screening of immunopotentiators by this system is now under way.

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