Effect of Antineoplastic Agents on the Induction of Suppressor Macrophages by Concanavalin A-bound Tumor Vaccine

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ABSTRACT

The present study was undertaken to identify tumor vaccine-induced suppressor cells and to examine the effect of antineoplastic agents on their production and suppressor activity. Primary and secondary sensitization with concanavalin A (Con A)-bound L1210 murine leukemia cell vaccine induced suppressor cells in the peritoneal cavity of histocompatible BALB/c × DBA/2Cr F1 mice. These cells phagocytized latex beads and adhered to plastic vessels. Their suppressor cell activity, determined by polyclonal in vitro spleen cell blastogenesis, was abrogated by silica but not by rabbit anti-mouse brain antiserum and complement. This evidence led to the conclusion that the suppressor cells were macrophages.

The production of suppressor macrophages depended on both the Con A-free vaccine cells and vaccine-bound Con A molecules since, singly, neither induced the corresponding suppressor cell activity. Although the induced suppressor cells were not abrogated by mitomycin C in vitro and cyclophosphamide in vivo, the increase of peritoneal suppressor cells following the second vaccination and/or their suppressor activity was significantly inhibited by antineoplastic agents including cyclophosphamide, mitomycin C, and 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea hydrochloride. The finding that vinblastine preferentially inhibited the production of peritoneal suppressor cells suggested that these agents interfered with the proliferation of suppressor cells (and/or their progenitor cells) in response to the second vaccination.

The close correlation between the potency of these antineoplastic agents in inhibiting the production of suppressor cells and the induction of enhanced antitumor immunity by Con A-bound tumor vaccine supports the feasibility of the proposed combination of chemotherapy and immunotherapy in which antineoplastic agents are dually effective by killing tumor cells and eliminating the vaccine-associated suppressor cells.

INTRODUCTION

Immunotherapy is a modality currently under extensive experimental investigation. One of the immunological maneuvers is the use of tumor cell vaccines which induce tumor-specific antitumor activity in the host. However, X-irradiated tumor cells, used as a source of vaccine, have been reported to enhance tumor growth, suggesting the involvement of suppressor cells induced by the tumor vaccine (1). Thus, to realize the benefit of tumor vaccines, maneuvers which inhibit the production of and/or eliminate those suppressor cells are needed.

Our previous investigation revealed that the Con A-bound tumor vaccine induced tumor-specific antitumor immunity in mice (7). Furthermore immunoprophylactic study showed that Con A-bound tumor vaccine was potentiated by any of 3 antiamacrophage agents, carrageenan, silica, and trypan blue. A series of experimental results suggested but did not prove that suppressor macrophages induced by Con A-bound tumor vaccine were abrogated by these agents, resulting in the production of antitumor immunity in the host (5). Immunoprophylactic experiments also showed that antineoplastic agents potentiate the Con A-bound tumor vaccine. Although the mechanism of this potentiation remains unclear, we proposed that these agents eliminated and/or inhibited the production of suppressor cells with Con A-bound tumor vaccine (2).

The present study furthers the understanding of those earlier observations. We now report that Con A-bound tumor vaccine induces suppressor macrophages in mice, as determined by in vitro polyclonal spleen cell blastogenesis and that these macrophages are eliminated and their production is suppressed by different antineoplastic agents. Our findings provide a theoretical basis for improving the immunotherapeutic effect of tumor vaccines. In fact, we have already reported that the combination of Con A-bound tumor vaccine and antineoplastic agents, capable of immunoprophylactically potentiating the vaccine, produced an enhanced therapeutic response in tumor-bearing mice (3).

MATERIALS AND METHODS

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

Vaccine. Con A-free and Con A-bound L1210 leukemia vaccines were prepared according to our previously reported method (2).

Preparation of Peritoneal Cells. The peritoneal cavity of unsensitized mice or mice sensitized with L1210 vaccine was washed twice with more than 5 ml Hanks' balanced salt solution (Kyokuto Chemicals, Tokyo, Japan) (6). There was no further induction by agents such as thioglycollate. The recovered cells were suspended in RPMI Medium 1640 and used as a source of suppressor cells.

Separation of Adherent and Nonadherent Peritoneal Cells. To

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2 To whom requests for reprints should be addressed.

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DECEMBER 1981

5151
prepare nonadherent cells, 16 ml of peritoneal cells (2 × 10^6/ml) were incubated in plastic flasks (No. 3024F; Falcon Plastics, Oxnard, Calif.) at 37°C in a CO2 incubator. After 1 hr, the flasks were inverted and incubated for another hr under the same conditions. Cells that were floating after vigorous manual shaking were used as a source of nonadherent cells. To obtain functional adherent cells, peritoneal cells (2 × 10^6/ml) were incubated in plastic tubes (No. 2054; Falcon) for 2 hr in a CO2 incubator. After manual shaking, floating cells were discarded. The tubes were washed once with 1 ml of chilled RPMI Medium 1640, and adhering cells were assayed in situ for their suppressor activity by adding the assay components of the in vitro spleen cell blastogenesis.

Treatment with Anti-Mouse Brain Antiserum and Complement. For this treatment, RPMI Medium 1640 containing 0.3% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) and 25 mg 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.3 (Flow Laboratories, Rockville, Md.), was used. Peritoneal cells (5 × 10^6/ml) were incubated for 30 min on ice with or without final 8-fold dilutions of anti-mouse brain antisera (Cedarlane Laboratories, Ltd., London, Ontario, Canada). They were subsequently treated with rabbit sera (Low-Tox; Cedarlane) as a source of complement (6, 9).

Suppressor Activity Assayed by in Vitro Blastogenesis. A splenic single-cell suspension in RPMI Medium 1640 was prepared according to our previously reported method (4). These cells (1 × 10^6/ml) were mixed with Con A-bound L1210 vaccine cells (1 × 10^6/ml) and/or peritoneal cells, representing the source of suppressor cells. Blastoid cells were counted on a Model ZP Coulter Counter (1/µm aperture current at 1, 1/amplification at 1, lower threshold at 25; Coulter Electronics, Inc., Hialeah, Fla.) (4, 9). The blastogenesis index was calculated from the formula

\[
\frac{\text{Count of spleen cells incubated with vaccine cells and suppressor cells}}{\text{Count of spleen cells incubated without vaccine or suppressor cells}}
\]

For unknown reasons, the count of spleen cells incubated without vaccine fluctuated, leading to deviations in the blastogenesis index. To eliminate the contribution of this effect, the results of some experiments were expressed as relative percentages of blastoid cell formation in the test (suppressor cells present) and control (suppressor cells absent) mixtures (Charts 1 to 3). The calculation was

\[
\% \text{ of blastogenesis} = \left( \frac{\text{Blastogenesis index of test mixture containing spleen cells, vaccine cells, and suppressor cells}}{\text{Blastogenesis index of control mixture containing spleen cells and vaccine cells}} \right) - 1
\]

\[
\times 100
\]

For suppressor cell assay, vaccine-bound but not unbound Con A was used as the polyclonal stimulant. Our previous finding that the in vivo immunogenic potency of Con A-bound vaccine correlated well with its potency to induce in vitro spleen cell blastogenesis (4) was thought to attach further significance to the present suppressor activity assay. Blastogenesis by Con A-bound vaccine was dependent on vaccine-bound Con A, since Con A-free vaccine was not blastogenic in the present assay (4). Thus, the induced blastogenic response was considered polyclonal.

Digesting Activity of Peritoneal Cells. Peritoneal cells (2.5 × 10^6/ml) were incubated for 2 days with or without Con A-bound L1210 vaccine cells (1 × 10^6/ml) in a CO2 incubator. The mixture was diluted to 1:20 with Isoton and counted on a Coulter Counter under the same settings as described previously. More than 95% of the vaccine cells were counted under these conditions. Percentage of digestion was calculated from the formula

\[
\% \text{ of digestion} = \left( \frac{\text{Count of vaccine cells incubated with peritoneal cells}}{\text{Count of vaccine cells}} \right) - 1 \times 100
\]

Uptake of Latex Beads. This was determined as described previously (5). In brief, peritoneal cells adhering to a No. 5220 plastic dish (Lux Scientific Corp. Newbury Park, Calif.) were incubated at 37°C for 2 hr in the presence of latex beads (500 µg/ml RPMI Medium 1640; final concentration of Bacto-Latex, 0.81; Difco Laboratories, Detroit, Mich.) and, after 2 gentle washings with 5 ml phosphate-buffered saline (pH 7.2), those cells which under a phase-contrast microscope were seen to have taken up more than 3 beads were recorded as phagocytic cells.

Chemicals. The following antineoplastic agents were used: CY and vindblatine (Shionogi Pharmaceutical Co., Osaka, Japan); MMC (Kyowa Hakko Co., Ltd., Tokyo, Japan); daunomycin (Meiji Seika Kaisha, Ltd., Tokyo, Japan); ACNU (Sankyo Co., Ltd., Tokyo, Japan); ara-C (Asahi Chemical Co., Ltd., Shizuoka, Japan); 6-mercaptopurine (Sigma). Silica (0.007 µm suspended in RPMI Medium 1640; Sigma) was used as an antimacrophage agent.

RESULTS

Induction of Peritoneal Suppressor Cells in L1210 Vaccine-sensitized Mice. The peritoneal cells of mice sensitized with Con A-free or Con A-bound L1210 vaccine were counted and examined for their potency to suppress in vitro polyclonal spleen cell blastogenesis in the presence of L1210 vaccine-bound Con A (Table 1). Peritoneal cells of nonsensitized animals suppressed blastoid cell formation marginally as compared with the control. In contrast, peritoneal cells of animals sensitized once or twice with Con A-bound vaccine were strongly suppressive; their number was increased significantly, especially 1 day after the second vaccination. Vaccine-bound Con A was associated with the induction of suppressor cells, since peritoneal cells of animals sensitized with Con A-free L1210 vaccine were much less suppressive and fewer in number than in mice sensitized with Con A-bound L1210 vaccine.

Digesting Potency of Vaccine-induced Peritoneal Suppressor Cells. In the present in vitro assay of suppressor cells, the observed suppression could have resulted from the clearance of the stimulant, Con A-bound L1210 vaccine cells, by phagocytic peritoneal macrophages. To rule out this possibility, we examined the digesting potency of peritoneal cells by counting the Con A-bound L1210 vaccine cells remaining in the incubation mixture after their cocultivation with peritoneal cells (Table 2). Peritoneal cells of mice sensitized with Con A-bound L1210 vaccine were less digestive than those of mice that had not been sensitized or that had been sensitized with Con A-free vaccine. This indicates that the digestion of Con A-bound L1210 vaccine cells, which might favor decreased blastogenic stimulation, was not responsible for the suppression noted in Table 1.

Characterization of Vaccine-induced Suppressor Cells. Peritoneal cells of mice sensitized with Con A-bound L1210 vaccine once or twice were separated according to their adherence or nonadherence to plastic. Separated and unseparated cells were examined for their potency in suppressing spleen cell blastogenesis in the presence of vaccine-bound Con A (Table 3). Unseparated and adherent cell populations.
DECEMBER 1981

5153

Tumor Vaccine, Suppressor Macrophages, and Chemotherapy

Table 1

Number and suppressor activity of peritoneal cells from L1210-vaccinated mice

Mice were given i.p. injections of 10^6 Con A-free or Con A-bound L1210 vaccine cells at the indicated times. On Day 0, the peritoneal cells of these and nonsensitized mice were counted on a per-animal basis and assayed for their potency to suppress in vitro blastogenesis of normal spleen cells by Con A-bound vaccine cells. The results are expressed as the ratio of blastoid cells produced in the test mixture to those in the control mixture containing no Con A-bound vaccine. For further details, see "Materials and Methods."

<table>
<thead>
<tr>
<th>Sensitization with</th>
<th>Recovery of peritoneal cells (x 10^6/animal)</th>
<th>Blastoqenesis index of spleen cells in the presence of vaccine-bound Con A and peritoneal cells (2.5 x 10^6/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>None</td>
<td>3.2 ± 0.5^a</td>
<td>7.3 ± 0.5^b</td>
</tr>
<tr>
<td>Con A-free vaccine (Day —6)</td>
<td>4.3 ± 1.4^c</td>
<td>7.0 ± 0.2</td>
</tr>
<tr>
<td>Con A-bound vaccine (Day —6)</td>
<td>6.1 ± 0.8^e</td>
<td>3.0 ± 1.0^f</td>
</tr>
<tr>
<td>Con A-bound vaccine (Days —8 and —1)</td>
<td>10.2 ± 2.9^i</td>
<td>NT</td>
</tr>
<tr>
<td>No peritoneal cells added (control)</td>
<td>9.0 ± 0.8</td>
<td>8.5 ± 0.8</td>
</tr>
</tbody>
</table>

^a Mean ± S.D. of more than 4 experiments.
^b Mean ± S.D. of quadruplicate determinations.
^c Significant at p < 0.05 by the f test.
^d NT, not tested.
^e Significant at p < 0.01 by the f test.
^f Significant at p < 0.05 by the f test as compared with the control (no peritoneal cells present) and any other test groups.

Table 2

Digestion of Con A-bound L1210 vaccine cells by peritoneal cells of L1210-vaccinated mice

Groups of more than 3 mice were given i.p. injections of 10^6 Con A-free or Con A-bound L1210 vaccine cells. Six days later, their peritoneal cells (2.5 x 10^6 cells/ml) were cocultured for 2 days at 37°C with Con A-bound L1210 vaccine (1 x 10^6 cells/ml), and the digestion of vaccine cells by peritoneal cells was examined. For calculation of percent digestion, see "Materials and Methods."

<table>
<thead>
<tr>
<th>Sensitization with</th>
<th>% of digestion of Con A-bound vaccine cells</th>
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<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>None</td>
<td>81.7 ± 7.8^a</td>
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<tr>
<td>Con A-free vaccine (Day —6)</td>
<td>83.7 ± 4.8</td>
</tr>
<tr>
<td>Con A-bound vaccine (Day —6)</td>
<td>52.1 ± 3.3^d</td>
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^a Mean ± S.D. of quadruplicate determinations.
^b Significant at p < 0.05 by the f test as compared with other test mixtures.

(about 40% of total) manifested suppressor activity, while nonadherent cells did not. Furthermore, we found that more than 90% of the adherent cells from mice sensitized once and more than 50% of the adherent cells from mice sensitized twice took up latex beads during the first 2 hr of incubation.

Based on these findings, the effect of silica on the peritoneal suppressor cells from these mice was examined (Chart 1). Plastic-adhering peritoneal cells were incubated with silica (0 to 40 μg/ml) and then assayed for their potency in suppressing spleen cell blastogenesis in the presence of vaccine-bound Con A. At 10 to 40 μg/ml, silica abrogated peritoneal suppressor cells of either singly or doubly vaccinated mice. This resulted in the restoration of spleen cell blastogenesis; at 40 μg/ml, silica did not affect the efficacy of the present assay. This evidence indicates that the induced suppressor cells were macrophages.

Since the induction of suppressor T-cells is frequently noted under a variety of experimental and clinical situations, we examined whether the observed suppression by peritoneal cells involved T-cells. Peritoneal cells from mice sensitized once or twice were incubated first with anti-mouse brain antiserum and then with rabbit serum as a source of complement.

These and peritoneal cells incubated in the absence of anti-mouse brain antiserum and rabbit serum were assayed for their suppression of spleen cell blastogenesis in the presence of vaccine-bound Con A (Table 4). At 2 different concentrations, the suppressor activity of peritoneal cells from singly or doubly sensitized mice was not abrogated by these treatments, although the efficacy of the present experimental conditions in killing T-cell populations has already been established (6). This result indicates that suppressor T-cells are not responsible for the observed suppression by peritoneal cells.

Dependence of Suppression of Spleen Cell Blastogenesis on Peritoneal Cell Concentration. Peritoneal cells from mice not sensitized or sensitized once with Con A-bound L1210 vaccine were separated according to their adherence or nonadherence to the plastic tubes. Adherent cells (about 40% of total population) were assayed for their potency in suppressing spleen cell blastogenesis in the presence of vaccine-bound
Peritoneal cells were incubated in the presence or absence of anti-mouse brain antisera and rabbit sera as a source of complement. These cells were assayed with Con A.

Did Con A-bound L1210 vaccine, suggesting the involvement of free L1210 vaccine induced much less suppressor activity than Con A-bound L1210 vaccine (Table 5, Experiment 1). This indicates that suppressor cell induction by Con A-bound L1210 vaccine was due to the combined, rather than the single, stimulation of vaccine-bound Con A and Con A-free L1210 vaccine cells. It remains to be clarified whether the separate administration of the vaccine and Con A produces a Con A-bound L1210 vaccine complex (4).

Chart 1. In vitro abrogation of tumor vaccine-induced suppressor cells by silica. Mice were given i.p. injections of $10^6$ Con A-bound L1210 vaccine cells either once 6 days before (○, △) or twice 8 and 1 day before (□, ■) their peritoneal cells ($2.5 \times 10^5$/tube) were incubated in plastic tubes for 2 hr at 37°C. Adhering cells were incubated further in situ for 24 hr at 37°C with the indicated concentrations of silica and then assayed for their potency in suppressing in vitro spleen cell blastogenesis by vaccine-bound Con A. The results are expressed as relative percentages of blastoid cell formation in the test (○, △) and control (no suppressor cells present) mixtures. The values of mixtures containing silica but no suppressor cells are also shown (□, ■). Bars, S.D.

Table 4

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Peritoneal cells of vaccinated mice</th>
<th>Peritoneal cells concentration (x $10^5$/ml)</th>
<th>Without further treatment</th>
<th>Treated with anti-mouse brain antisera and complement</th>
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<td>1</td>
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*Mean ± S.D. of quadruplicate determinations.

Con A (Chart 2). At 1.25 x $10^5$/ml, peritoneal cells from sensitized mice suppressed blastoid cell formation by about 50%; further suppression was noted at higher concentrations. Peritoneal cells from nonsensitized mice suppressed blastoid cell formation to a lesser extent, although the suppression noted at 5 x $10^5$/ml was statistically significant ($p < 0.05$ by the t test). We also noted that, at 1.25 x $10^5$/ml, peritoneal cells from nonsensitized mice enhanced rather than suppressed blastoid cell formation (significant at $p < 0.05$ by the t test as compared with the control).

**Dependence of Induction of Suppressor Cells on L1210 Vaccine Cells and Bound Con A.** Table 1 showed that Con A-free L1210 vaccine induced much less suppressor activity than did Con A-bound L1210 vaccine, suggesting the involvement of Con A molecules in the induction of suppressor cells. In Table 5, the association of Con A with suppressor cell induction is further characterized. Con A-bound L1210 vaccine induced strong suppressor activity as assayed by spleen cell blastogenesis at either of the examined peritoneal cell concentrations. In contrast, Con A-free L1210 vaccine and Con A, corresponding to the amount bound to $10^6$ L1210 vaccine cells

(4), induced much less suppressor activity at the 2 peritoneal cell concentrations (Table 5, Experiment 1). The administration of Con A-free vaccine and Con A at a 3-hr interval induced the same suppressor activity as Con A-bound L1210 vaccine (Table 5, Experiment 2). This indicates that suppressor cell induction by Con A-bound L1210 vaccine was due to the combined, rather than the single, stimulation of vaccine-bound Con A and Con A-free L1210 vaccine cells. It remains to be clarified whether the separate administration of the vaccine and Con A produces a Con A-bound L1210 vaccine complex (4).

Chart 2. Dose response of vaccine-induced peritoneal cells in suppressing in vitro blastogenesis of spleen cells by vaccine-bound Con A. Mice were given one i.p. injection of $10^6$ Con A-bound or Con A-free L1210 vaccine cells or 0.5 μg Con A, which corresponds to the amount bound to $10^6$ L1210 vaccine cells. Six days later, their peritoneal cells were assayed for their potency in suppressing in vitro spleen cell blastogenesis by vaccine-bound Con A. The results are expressed as relative percentages of blastoid cell formation in the test (peritoneal cells present) and control (peritoneal cells absent) mixtures. Bars, S.D.

Table 5

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Peritoneal cells of animal given</th>
<th>Peritoneal cells concentration (x $10^5$/ml)</th>
<th>Without further treatment</th>
<th>Treated with anti-mouse brain antisera and complement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Con A-bound L1210 vaccine</td>
<td>$5.89 \pm 0.93$</td>
<td>$15.8 \pm 0.83$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Con A-free L1210 vaccine</td>
<td>$8.56 \pm 0.92$</td>
<td>$21.7 \pm 1.00$</td>
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</tr>
<tr>
<td></td>
<td>Con A</td>
<td>$12.5 \pm 1.59$</td>
<td>$21.8 \pm 2.49$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No peritoneal cell added</td>
<td></td>
<td>$20.6 \pm 2.30$</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Con A-bound L1210 vaccine</td>
<td>$7.97 \pm 0.93$</td>
<td>$19.8 \pm 0.95$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Con A plus Con A-free L1210 vaccine</td>
<td>$7.19 \pm 0.41$</td>
<td>$18.4 \pm 1.22$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No peritoneal cells added</td>
<td></td>
<td>$22.1 \pm 1.19$</td>
<td></td>
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</table>

*Mean ± S.D. of quadruplicate determinations.

Significant at $p < 0.05$ by the t test as compared with any of the other groups.

Significant at $p < 0.05$ by the t test as compared with the control (no peritoneal cells added).
Kinetics of Vaccine-induced Peritoneal Suppressor Cell Production. Peritoneal cells were recovered at the indicated times from mice given i.p. injections of Con A-bound L1210 vaccine on Days 0 and 7, and their number and suppressor cell activity were examined (Chart 3). One day after the first vaccination, the number and suppressor activity of the peritoneal cells increased. Thereafter, their number gradually decreased; however, their suppressor activity increased further on Day 2 and then stayed stable until the second vaccination, although it somewhat decreased on Day 6 in this particular experiment. Upon the second vaccination, the number of peritoneal cells almost doubled by the next day; the suppressor activity of these cells, on a per-cell basis, was unchanged. By the third day after the second vaccination, the number of peritoneal cells decreased; however, their suppressor cell activity remained stable.

Based on the above kinetics of peritoneal suppressor cells, we tested the hypothesis that antineoplastic agents potentiate the Con A-bound L1210 vaccine by inhibiting the increase in peritoneal suppressor cells.

Inhibition of Vaccine-associated Suppressor Cell Production by Antineoplastic Agents Leading to Potentiation of the Vaccine. Mice were twice given injections of the Con A-bound L1210 vaccine with a 7-day interval, and 1 day before the second vaccination they received an i.p. injection of an antineoplastic agent, capable or incapable of potentiating the vaccine (Table 6). Peritoneal cells were recovered 1 day after the last vaccination, and their number and suppressor activity were examined. The antineoplastic agents previously reported to be effective in potentiating Con A-bound L1210 vaccine (2), i.e., CY, MMC, daunomycin, and ACNU, inhibited the increase in peritoneal cells, although statistical difference was not attached to daunomycin. Furthermore, the suppressor activity of the peritoneal cells was abrogated in daunomycin- and ACNU-treated mice. Of the 2 agents previously reported to be ineffective in potentiating the vaccine (2), 6-mercaptopurine increased rather than decreased the number of peritoneal suppressor cells. These observations were consistent with the hypothesis that antineoplastic agents potentiate the Con A-bound vaccine by inhibiting the increase in peritoneal suppressor cells.

Effect of Vinblastine on Increase in Peritoneal Suppressor Cells. The above study disclosed that antineoplastic agents inhibited the increase in the number of vaccine-associated peritoneal suppressor cells. However, it was not clear how this came about. The antineoplastic agents may have inhibited the proliferation of suppressor cells and/or the influx of these cells into the peritoneal cavity if they were not produced there. Therefore, the effect of vinblastine on peritoneal suppressor cell production was examined since this agent selectively interferes with cell division.

The number and suppressor activity of peritoneal cells from mice, given injections of Con A-bound vaccine with a 7-day interval and either further treated or not treated with vinblastine 1 day before, or on the day of, the second vaccination, were examined (Chart 4). Peritoneal cells of mice sensitized with the vaccine and treated with vinblastine on the day of, but not the day preceding, the last vaccination were much less suppressive than those of mice only sensitized with the vaccine, although there was no significant difference in the peritoneal cell number (Experiment 1). By the same protocol (Experiment 2), however, CY did not inhibit the vaccine-induced suppressor cell production (blastogenesis index: Group 1 versus Group 4, not significant; but Group 1 versus Group 3, significant at p < 0.05), although CY as well as vinblastine did not inhibit the accumulation of cells in the peritoneal cavity. These results substantiate the vinblastine-induced inhibition of suppressor cell production as a consequence of selective interference of suppressor cell division and indicate that the vaccine induced the uniform influx of host cells into the peritoneal cavity, possibly without local proliferation except that of suppressor cells which had accu-

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**Table 6**

<table>
<thead>
<tr>
<th>Mice given injections of Con A-bound vaccine and</th>
<th>Peritoneal cells recovered (×10^7/animal)</th>
<th>Spleen cell blastogenesis index in the presence of peritoneal cells (1.9×10^3/ml)</th>
<th>Potentiation of Con A-bound vaccine in mice (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.3 ± 0.7c</td>
<td>7.63 ± 1.01c</td>
<td>Yes</td>
</tr>
<tr>
<td>CY (100 mg/kg)</td>
<td>2.0 ± 0.5c</td>
<td>8.62 ± 0.43</td>
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<tr>
<td>MMC (1.25 mg/kg)</td>
<td>3.8 ± 0.7c</td>
<td>8.28 ± 0.89</td>
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<tr>
<td>Daunomycin (0.625 mg/kg)</td>
<td>4.4 ± 1.2c</td>
<td>10.0 ± 0.91c</td>
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<tr>
<td>ACNU (20 mg/kg)</td>
<td>2.8 ± 0.2c</td>
<td>13.5 ± 0.84c</td>
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<tr>
<td>ara-C (200 mg/kg)</td>
<td>3.4 ± 0.1c</td>
<td>9.34 ± 0.49</td>
<td>No</td>
</tr>
<tr>
<td>6-Mercaptopurine (100 mg/kg)</td>
<td>8.7 ± 0.2c</td>
<td>8.05 ± 0.37</td>
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</tbody>
</table>

*a* Mean ± S.D. of more than 3 animals. The blastogenesis index of the control in the absence of peritoneal cells was 12.7 ± 0.22.

**Chart 3.** Kinetics of peritoneal suppressor cell production by Con A-bound L1210 vaccine. Mice were given i.p. injections of 10^7 Con A-bound L1210 vaccine once or twice (arrows). At the indicated times, their peritoneal cells were counted; the results are presented on a per-animal basis (C). Mixed cell populations (2.5 × 10^7/tube) (after the second vaccination) or cells adhering to plastic tubes after 2-hr incubation at 37°C in a CO2 incubator (after the first vaccination) were assayed for their potency in suppressing the blastogenesis of spleen cells by vaccine-bound Con A. The results are expressed as relative percentages of blastoid cell formation of the test (peritoneal cells added) and control (no peritoneal cells added) mixtures. For further details regarding the calculation, see "Materials and Methods." Bara, S.D.
other groups. Mean ± S.D. of 3 experiments. Delayed palpable s.c. tumor growth) were considered cured. Significant at p < 0.05 by the Fisher exact test as compared with any of the sensitized once or twice with Con A-bound tumor vaccine antitumor immunity by the vaccine.

Peritoneal cells Blastogenesis index* in recovered the presence of vaccine-bound Con A and peritoneal cells

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Group</th>
<th>Days</th>
<th>Exp.1</th>
<th>Exp.2</th>
<th>Exp.1</th>
<th>Exp.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>6.31 ± 1.2</td>
<td>5.4 ± 1.0</td>
<td>3.45 ± 1.0</td>
<td>6.33 ± 0.54</td>
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<td>2</td>
<td>-1</td>
<td>3.10 ± 0.60</td>
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<tr>
<td>3</td>
<td>-1</td>
<td>7.82 ± 0.03</td>
<td>9.20 ± 0.65</td>
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<tr>
<td>4</td>
<td>-1</td>
<td>5.4 ± 1.1</td>
<td>6.15 ± 0.17</td>
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<tr>
<td>5</td>
<td>-1</td>
<td>2.3 ± 0.8</td>
<td>7.73 ± 0.88</td>
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</table>

Chart 4. Selective inhibition of vaccine-associated suppressor cell production by vinblastine. Mice were given i.p. injections, at the indicated intervals, of 10^6 Con A-bound L1210 vaccine cells (V) and vinblastine (1 mg/kg) (O) or CY (100 mg/kg) (X). When the vaccine and drugs were administered on the same day, the vaccine preceded the drugs by 1.5 hr. Their peritoneal cells harvested 1 day after the second vaccination (arrows) were counted on a Coulter Counter (Experiment 1; see Table 6 for settings) or by a hemocytometer (Experiment 2), and 2.5 x 10^5 cells/ml were assayed in vitro for their suppressor cell activity of spleen cell blastogenesis.

- Means ± S.D. of more than 3 animals.
- Mean ± S.D. of quadruplicate determinations.
- The blastogenesis indices of the control in the absence of peritoneal cells were 10.7 ± 0.56 (Experiment 1) and 10.7 ± 0.38 (Experiment 2).
- Significant at p < 0.05 by the t test as compared with the corresponding Group 1.

No. of cured mice/total Survival days of tumor-bearer

<table>
<thead>
<tr>
<th>No. of cured mice/total</th>
<th>Survival days of tumor-bearer</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.3 ± 0.26</td>
</tr>
<tr>
<td>-1</td>
<td>15.4 ± 2.26</td>
</tr>
<tr>
<td>-1</td>
<td>10.5 ± 0.50</td>
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<tr>
<td>10^6/26</td>
<td>14.0 ± 1.05</td>
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</table>

Chart 5. Vaccine-induced antitumor immunity enhanced by vinblastine. Mice were given i.p. injections of 10^6 Con A-bound L1210 vaccine cells (V) and vinblastine (1 mg/kg) at the indicated times (E) and were inoculated i.p. with 10^6 live L1210 cells (arrows). When the vaccine and vinblastine was administered on the same day, the vaccine preceded the drug by 1.5 hr. The data are summarized results of 3 experiments, except where indicated differently.

- One-month survivors were further inoculated i.p. with 10^6 L1210 cells. Two-month survivors free of tumor signs (i.e., no increase in ascitic fluid and/or delayed palpable s.c. tumor growth) were considered cured.
- Significant at p < 0.05 by the Fisher exact test as compared with any of the other groups.
- * Means ± S.D. of 3 experiments.
- Data from a single experiment.

Although the present study identified the suppressor macrophages, it is not clear how they were associated with the eventual suppression of antitumor immunity induction by the vaccine. These macrophages may directly interfere with the production and/or function of immunocytes responsible for antitumor immunity. Alternatively, they may activate other cells, making them the ultimate suppressor cells. Our earlier finding that, although ara-C inhibited the numerical increase of peritoneal suppressor cells it was not effective in potentiating the Con A-bound vaccine (2), may point to the involvement of a second suppressor cell population. This matter requires further experiments.

The elimination of vaccine-associated suppressor cell activity leads to the production of enhanced antitumor immunity by the tumor vaccine. After the first or second vaccination, CY, MMC, daunomycin, and ACNU killed the suppressor cells and/or inhibited their production. Taking into account that these agents effectively potentiated the tumor vaccine (2), we posit that the decreased production of peritoneal suppressor cells (Table 6) may be associated with the eventual enhancement of antitumor immunity induction. The mechanism(s) underlying the inhibition of suppressor cell production remains to be clarified. However, our finding that vinblastine preferentially inhibited the increase of suppressor cells in the peritoneal cavity without affecting the general influx of host cells suggests that suppressor cells (and/or their progenitor cells) proliferate in the peritoneal cavity. Since the above antineoplastic agents were highly reactive with cellular DNA molecules, we hypothesize that they interfered with the proliferation of primed suppressor cells as well as the general influx of host cells in suppressed the in vitro spleen cell blastogenesis by vaccine-bound Con A. These peritoneal cells adhered to plastic vessels and were phagocytic, as determined by their uptake of latex beads. Their suppressor activity was abrogated by the antimalphage agent, silica, but not by anti-mouse brain antisem and complement. These findings led us to conclude that the vaccine-induced suppressor cells were macrophages.

The in vivo relevance of these suppressor macrophages to vaccine-induced antitumor immunity is substantiated by our previous finding that antimalphage agents, carrageenan and silica, eliminated the vaccine-induced, phagocytic, plastic-adherent peritoneal suppressor cells, resulting in the enhanced induction of antitumor immunity by the tumor vaccine (5).

Many previous studies have identified macrophages as suppressor cells under a variety of clinical and experimental situations and showed that the suppressor macrophages were induced by many agents. One of these agents, Con A, induced suppressor macrophages either by its direct binding to macrophages (11) or by its activation of resident macrophages by a soluble factor released from Con A-stimulated T-cells (8, 10). In the present study, the induction of suppressor macrophages was dependent on vaccine-bound Con A, since Con A-free vaccine induced much less suppressor activity (Table 1). However, free Con A in amounts equivalent to the amount bound to 10^6 L1210 vaccine cells (0.5 μg) did not induce the corresponding suppressor activity (Table 5). This indicates that both vaccine cells and vaccine-bound Con A molecules were involved in the production of these suppressor macrophages and that the present suppressor macrophages were at least partially different with respect to their induction mechanisms from those reported previously (8, 10, 11).

Although the present study identified the suppressor macrophages, it is not clear how they were associated with the eventual suppression of antitumor immunity induction by the vaccine. These macrophages may directly interfere with the production and/or function of immunocytes responsible for antitumor immunity. Alternatively, they may activate other cells, making them the ultimate suppressor cells. Our earlier finding that, although ara-C inhibited the numerical increase of peritoneal suppressor cells it was not effective in potentiating the Con A-bound vaccine (2), may point to the involvement of a second suppressor cell population. This matter requires further experiments.

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response to the second vaccination, although primed suppressor cells persisted and were functionally active until they died and were eliminated.

Among 7 antineoplastic agents tested for correlation of activities of inhibiting the suppressor cell production and enhancing the potency of tumor cell vaccines, ara-C was exceptional in that it inhibited the suppressor cell production although it did not enhance the vaccine-induced antitumor immunity. As pointed out above, it may be that a second suppressor cell population along with suppressor macrophages is involved in the vaccine-induced antitumor immunity and that ara-C is not effective in eliminating it whereas other potent antineoplastic agents are effective. Alternatively, ara-C may interfere with the process associated with the eventual production of antitumor immunity. Incidentally, both ara-C and 6-mercaptopurine, another agent incapable of potentiating the vaccine, are known for strongly suppressing the different aspects of host immunity. This is consistent with the second possibility. In this regard, further investigation is under way.

Although our present experiments were immunoprophylactic, their results are valuable in the therapy of tumor-bearing animals since they indicate the possibility of a novel type of combined immunotherapeutic and chemotherapeutic regimen. In this treatment system, chemotherapy can be expected to be dually effective by directly killing or inhibiting the proliferation of tumor cells and by eliminating or inhibiting the production of vaccine-associated suppressor cells, thereby leading to enhanced antitumor activity of host immunity. In fact, this feasibility was evidenced by our previous finding that Con A-bound tumor vaccine resulted in enhancement of the therapeutic effect when combined with antineoplastic agents capable of potentiating the vaccine immunoprophylactically (3). We hope that further experiments along this line, including the screening of more effective chemotherapeutic agents, may give rise to a novel combined modality of clinical applicability.

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Effect of Antineoplastic Agents on the Induction of Suppressor Macrophages by Concanavalin A-bound Tumor Vaccine

Tateshi Kataoka, Fujiko Oh-Hashi, Yoshio Sakurai, et al.


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