Inhibition of Pulmonary Metastasis by Nocardia rubra Cell Wall Skeleton, with Special Reference to Macrophage Activation

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ABSTRACT

The antimetastatic activity of Nocardia rubra cell wall skeleton (N-CWS) with or without cyclophosphamide was examined in an experimental model of pulmonary metastasis induced by Lewis lung carcinoma in C57BL/6 mice. Lewis lung carcinoma cells were implanted into the footpads of mice, and the implanted tumors were removed 9 to 10 days later. Pulmonary metastatic nodules began to develop a few days after the implanted tumor was removed. The inhibitory effect of N-CWS was evaluated from the number of pulmonary surface nodules about 3 weeks after tumor implantation. The antimetastatic activity of N-CWS depended upon the dose, time, and route of its injection. Injection of N-CWS i.v. after removal of the implanted tumor caused the greatest inhibition of development of pulmonary metastases. Therapy with N-CWS plus cyclophosphamide prolonged significantly the survival of mice with metastases.

The cytotoxic activities of peritoneal macrophages and macrophages in the lung against Lewis lung carcinoma cells were enhanced in mice treated with N-CWS. Injection i.v. of peritoneal macrophages activated with N-CWS inhibited pulmonary metastases. The role of macrophages in inhibition of micrometastasis in the lung is discussed.

INTRODUCTION

The pathogenesis of metastasis involves many complicated biological processes, and its outcome depends on the interaction of tumor cells with their host and is influenced by various factors in each metastatic step (30, 39). The role of host defense in control of metastasis is not well understood. The increased metastasis observed after generalized immunosuppression (3, 23, 33) or suppression of T-cell activity (4, 5) and macrophages (14) and in dysfunction of natural killer cell activity (37) indicates the roles of these components of the immune system in control of metastases.

Recently, immunotherapy of animal and human cancers has been studied extensively. However, immunotherapy is of little therapeutic value in patients with clinically detectable primary tumors or overt visceral metastases. Thus, much attention has been directed to the possible value of immunotherapy in patients with minimal residual malignant disease after surgical resection. It might be possible to eradicate or prevent micrometastases by immunological modulation of the host defense mechanism. Indeed, so-called BRM (17, 21), Corynebacterium parvum (9, 25), bacterial cellular components (27, 28), polysaccharides extracted from plants (31, 40), and liposomes containing muramyl dipeptide (8) or lymphokines (7) that activate the reticuloendothelial system of the host have been found to inhibit development of metastases.

The present study was undertaken to investigate the antimetastatic effect of N-CWS on spontaneous pulmonary metastases in relation to its influence on macrophage-mediated cytotoxicity against a syngeneic tumor.

MATERIALS AND METHODS

Animals. Six- to 8-week-old male C57BL/6N mice, weighing 20 to 25 g, were obtained from Shizuoka Jikken-Dohbustu Nokyo, Shizuoka, Japan. They were maintained on standard diet and water throughout experiments.

Tumor. 3LL is an undifferentiated squamous cell carcinoma that arose spontaneously in the lung of a C57BL/10 mouse and has been maintained by serial biweekly s.c. passage in the same strain of mice. A local tumor grown in the thigh was removed aseptically and minced. The tumor fragments were stirred in RPMI 1640 (Microbiological Associates, Bethesda, Md.) containing 0.2% trypsin (1:250; Difco Laboratories, Inc., Detroit, Mich.) at 37° for 30 min. The isolated tumor cells were washed twice with RPMI 1640 containing 10% FCS (Grand Island Biological Co., Grand Island, N. Y.), resuspended in fresh RPMI 1640, and counted in a hemocytometer. The viability of tumor cells was estimated as more than 90% by the trypan blue dye exclusion method. A suspension of 10⁶ viable cells in 0.05 ml of RPMI 1640 was implanted s.c. into a footpad of each mouse.

N-CWS. N-CWS prepared by the method of Azuma et al. (1) was treated with squalene in an oil-attached form as reported elsewhere (41) and suspended in 0.9% NaCl solution at a concentration of 0.5 mg/ml just before use. N-CWS preparations (Fujisawa Pharmaceutical Co., Osaka, Japan) were provided from Dr. Yuichi Yamamura.

Anticancer Agent. CY was dissolved at a concentration of 50 mg/kg in 0.9% NaCl solution for i.p. injection into mice.

Assay of Pulmonary Metastases. Implantation s.c. of 10⁶ 3LL cells into the footpads of mice led to progressive tumor growth and subsequent pulmonary metastases. When the implanted tumor in the footpad had become 5 to 6 mm in diameter 9 to 10 days after implantation of tumor cells, the mice were anesthetized by i.p. injection of 50 mg of sodium pentobarbital (Nembutal) per kg, and the tumor-bearing leg was amputated by the cautery clamp technique. The edge of the wound was then closed with Michel clips under aseptic conditions. Mice were autopsied 14 days after removal of the implanted tumor, and their organs were examined grossly. Pulmonary metastases were estimated grossly by counting the numbers of metastatic nodules on the pulmonary surface after fixation in 10% formaldehyde solution.

Preparation of Effector Cells. PEC were collected by washing the peritoneal cavity with 10 ml of RPMI 1640 5 days after i.p. injection of N-CWS. PEC were washed 3 times with medium and suspended at a fixed concentration in RPMI 1640 containing 10% FCS. For separation of adherent cells, suspensions of PEC were put into plastic flasks coated with serum and incubated at 37° in a 5% CO₂ incubator to allow macrophages to become attached (19). After incubation for 20 min, nonadherent cells were decanted, and flasks were washed 3 times with
medium in an ice bath. Adherent cells were then removed from the flasks by incubation at 4°C in medium containing 0.2% EDTA and 5% FCS, washed, and resuspended in RPMI 1640.

Macrophages in the lung were isolated by the method of Howard et al. (12). The lungs were finely minced in ice-cold RPMI 1640. The fragments of lungs were vigorously pipetted with a Pasteur pipet in the same medium and removed by passing the suspension through fine gauze, and the cells were separated by centrifugation at 450 x g for 5 min. Lung cells were resuspended in fresh medium, and nucleated cells were counted. Adherent cells were then separated from lung cells by adherence to glass and collected by rubber policeman.

Assay of In Vitro Cytolytic Activities of Peritoneal Macrophages and Macrophages in the Lung. For assay of cytolytic activity, peritoneal macrophages or macrophages in the lung, as effector cells, were suspended at 10^6 cells/ml in RPMI 1640 containing 10% FCS, supplemented with 15 x 10^-3 M N-2-hydroxymethylpiperazine-N-2-ethanesulfonic acid, streptomycin (100 µg/ml), and penicillin (100 IU/ml). Target cells, 3LL cells, were labeled with 0.5 µCi of [3H]thymidine per ml (methyl-3H)-thymidine; specific activity, 2 Ci/10^-3 M; Radiochemical Centre, Amer- sham, England) in 5 ml of RPMI 1640 containing 10% FCS in 25-sq cm plastic tissue culture flasks (Nunc, Roskilde, Denmark) for 24 hr. The labeled tumor cells were washed, and 1 x 10^6 cells/0.1 ml were introduced into the wells of a tissue culture plate (No. 3042; Falcon Plastics, Oxnard, Calif.) with effector cells and incubated for 48 hr in a 5% CO2 incubator. Then, the target cells labeled with [3H]thymidine were sucked out of the wells with glass fiber paper (GF/B; Whatman, Maidstone, England), the paper was washed with 20 ml of 10% cold trichloroacetic acid, and the paper was dried and transferred to glass vials containing toluene-based scintillation fluid (32). The radioactivity was counted in a scintillation counter. All experiments were done in triplicate. The percentage of cytolyis was calculated from the following formula:

\[
\text{Cytolysis (\%)} = \frac{cpm \text{ in control group} - cpm \text{ in test group}}{cpm \text{ in control group}} \times 100
\]

Statistical Analysis. Experimental results were analyzed for their statistical significance by the Student t test (2-tailed) and Mann-Whitney U test.

RESULTS

Inhibition of Growth of Pulmonary Metastases by N-CWS. The effect of N-CWS on the growth of pulmonary metastases of 3LL was examined by injecting N-CWS into mice after removal of the implanted primary tumor. As shown in Table 1, a single dose of 5.0 or 10.0 mg/kg was injected i.v. into mice on the day when the implanted tumor was removed. Significant inhibition of pulmonary metastasis was noted in mice given injections of 10.0 mg of N-CWS per kg. Subsequently, a dose of 0.5, 2.5, or 10.0 mg of N-CWS per kg was injected i.v. 3 times, once every 2 days, after excision of the tumor. Its inhibition of pulmonary metastasis was greatest at a dose of 2.5 mg/kg. These results indicate that there is an optimal dose of N-CWS for inhibition of pulmonary metastases. However, N-CWS had no inhibitory effect against primary tumors in the leg under these conditions.

Effects of the Time and Route of N-CWS Administration on Its Inhibition of Pulmonary Metastases. The effects of the time and route of N-CWS administration on its inhibition of pulmonary metastases were examined. As shown in Table 2, in Experiment 1, a dose of 2.5 mg of N-CWS per kg was injected i.v. 3 times, once every 2 days, before and after removal of the implanted tumor. When N-CWS was injected i.v. on Days 3, 5, and 7 during growth of the implanted tumor, it caused only slight inhibition of metastases, but when it was injected i.v. on Days 10, 12, and 14 during growth of metastases in the lung, it caused significant inhibition of pulmonary metastases. On the other hand, it caused no significant reduction of metastases when injected i.p. into mice. These results indicate that its inhibition of pulmonary metastases depends on its time and route of injection.

Additive Inhibitory Effects of N-CWS and CY on Pulmonary Metastases. The effect of combined therapy with N-CWS and CY on pulmonary metastases was examined. A dose of 2.5 mg of N-CWS per kg was injected i.v. 3 times on Days 10, 12, and 14 after tumor implantation, and a single dose of 50 mg of CY per kg was injected i.p. on the day after removal of the implanted tumor. As shown in Table 3, development of pulmonary metastases was inhibited by each of these treatments alone, and the effects of the 2 treatments were additive: no metastatic nodules could be seen in 4 of 10 mice treated with N-CWS plus CY. The survival of mice was also prolonged significantly by this combined therapy (Chart 1). No visible metastases were seen in any organs of surviving mice killed on Day 60.

In Vitro Cytolytic Activities of PEC. In vitro cytolytic activities were measured to determine whether N-CWS activated peritoneal cells to be cytotoxic against 3LL cells. N-CWS caused significantly increased cytolytic activity of PEC when injected i.p. at 10.0 mg/kg into normal mice 5 days before harvesting PEC.
Table 2

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>N-CWS</th>
<th>Pulmonary metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose (mg/kg)</td>
<td>Route</td>
</tr>
<tr>
<td>1 Untreated</td>
<td>2.5 i.v.</td>
<td>3, 5, 7</td>
</tr>
<tr>
<td>N-CWS</td>
<td>2.5 i.v.</td>
<td>10, 12, 14</td>
</tr>
<tr>
<td>2 Untreated</td>
<td>2.5 i.p.</td>
<td>10, 12, 14</td>
</tr>
<tr>
<td>N-CWS</td>
<td>2.5</td>
<td>10, 12, 14</td>
</tr>
</tbody>
</table>

* Inocula of 10<sup>6</sup> tumor cells were injected s.c. into a footpad. The implanted tumor was removed on Day 10 after tumor implantation. In Experiment 1, a dose of 2.5 mg of N-CWS per kg was injected i.v. 3 times before or after removal of the implanted tumor. In Experiment 2, N-CWS was injected i.p. after removal of the implanted tumor.

<sup>a</sup> Number of mice with pulmonary metastases of the number of mice tested.

<sup>b</sup> Mean ± S.E.

<sup>c</sup> Numbers in parentheses, median number of pulmonary metastases.

<sup>d</sup> Range of the number of surface nodules.

<sup>e</sup> p < 0.01 (Student's t test); p < 0.025 (Mann-Whitney's U test) compared with untreated group.

Table 3

<table>
<thead>
<tr>
<th>Experimental group&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CY (i.p.)</th>
<th>N-CWS (i.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose (mg/kg)</td>
<td>Day of injection</td>
</tr>
<tr>
<td>Untreated</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>N-CWS</td>
<td>2.5</td>
<td>10</td>
</tr>
<tr>
<td>CY</td>
<td>10</td>
<td>2.5</td>
</tr>
<tr>
<td>CY + N-CWS</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>CY</td>
<td>10</td>
<td>2.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Inocula of 10<sup>6</sup> tumor cells were injected s.c. into a footpad. The implanted tumor was removed on Day 10 after tumor implantation.

<sup>b</sup> Number of mice with pulmonary metastases of the number of mice tested.

<sup>c</sup> Survival time of treated group as a percentage of that of the control.

<sup>d</sup> Mean ± S.E.

<sup>e</sup> Numbers in parentheses, median number of pulmonary metastases.

<sup>f</sup> Range of the number of surface nodules.

<sup>g</sup> p < 0.05 compared with untreated group.

<sup>h</sup> p < 0.01 compared with untreated group.

<sup>i</sup> p < 0.001 compared with untreated group.

<sup>j</sup> p < 0.05 compared with CY.

The cytolytic activity of PEC from mice given injections of N-CWS was 37.5%, whereas that from untreated mice was 12.5% (p < 0.01).

Next, the cytolytic activities of adherent or nonadherent cell fractions of PEC against 3LL cells were examined. Whole PEC and adherent cells were strongly cytolytic, whereas nonadherent cells did not cause cytolyis (Chart 2). These results indicate that peritoneal macrophages activated by N-CWS play a role in the in vitro cytolyis of 3LL cells.

Inhibition of Pulmonary Metastases by i.v. Injection of Peritoneal Macrophages Activated with N-CWS. Peritoneal adherent cells from mice activated with N-CWS as described above were injected i.v. on Days 10, 12, and 14 into mice with pulmonary metastases after removal of the implanted primary tumor.
on Day 10. As shown in Table 4, significant reduction in the number of pulmonary metastatic nodules was observed in mice given injections of peritoneal adherent cells activated with N-CWS but not in mice given injections of untreated adherent cells or nonadherent cells treated with N-CWS. Furthermore, peritoneal adherent cells from mice bearing tumors at different stages were injected into mice with pulmonary metastases. Significant inhibition of metastases was noted only in mice that had received peritoneal adherent cells from normal mice or mice with tumors excised on Day 10 activated with N-CWS; no inhibition was seen in mice given injections of peritoneal adherent cells from mice bearing primary tumors on Day 15 or 16 (Table 5). These results indicate that transfer of peritoneal macrophages activated with N-CWS has an inhibitory effect on pulmonary metastases.

**Cytolytic Activities of Macrophages in Lung Activated with N-CWS.** Little is known about tumoricidal effector cells in the lung in animals with blood-borne pulmonary metastases. The *in vitro* cytolytic activity on 3LL cells of adherent cells in lungs from mice treated with N-CWS was examined. When doses of 2.5, 5.0, and 10.0 mg of N-CWS per kg were injected i.v. into mice, the numbers of total cells and macrophages in the lung increased with the dose of N-CWS (Chart 3). Macrophages from the lung from normal mice and mice with excised tumors activated with N-CWS were much more tumoricidal than those from untreated mice (Chart 4). These results indicate that macrophages in the lung may act as effector cells for eradication of pulmonary metastases.

**DISCUSSION**

Inhibition or prevention of metastases is a major problem in cancer research. It may be possible to inhibit micrometastasis, although it is difficult to inhibit overt metastasis or primary tumor growth. We used 3LL as an experimental model of spontaneous pulmonary micrometastasis. In our model, the influence of the primary tumor on metastasis was excluded by removing the implanted tumor. Thus, this system seemed suitable for investigating the effects of host modulation on micrometastasis (39). There are a few reports of eradication of micrometastasis by immunological modulation of the host by means of factors in BRM, such as BCG (2, 17, 21), its cellular component (28), *C. parvum* (9, 18, 25), or plant polysaccharides (31). We have...
reported that the β(1-3)glucan, Schizophyllan, inhibits the development of pulmonary metastases in this experimental model (40).

It has been found that the cell wall skeleton of Nocardia rubra has many advantages over BCG cell wall skeleton as an immunotherapeutic agent in cancer therapy (42). Immunological antitumor effects of N-CWS have been reported in various experimental tumor-host systems, including suppressive effects on syngeneic rat tumors (27), suppressive effects on syngeneic (41) and autochthonous murine tumors (38), regressive effects on syngeneic rat tumors (27), and suppressive effects on carcinogene

In this work, N-CWS was effective in preventing pulmonary micrometastases in syngeneic mice after removal of an implanted 3LL tumor in the footpad. A single injection of 10 mg/kg or 3 N-CWS injections of 2.5 mg/kg inhibited the development of pulmonary micrometastases only when given i.v. against a minimal tumor burden in the lung. We also found that N-CWS and CY had additive inhibitory effects on pulmonary metastases of 3LL. This combined therapy not only reduced the number of pulmonary metastatic nodules but also prolonged the survival of mice bearing pulmonary metastases. These results are consistent with observations on the effects of CY and a glucan in mice bearing 3LL (40). It was supposed that augmentation of the antitumor cytotoxicity, as a result of elimination of suppressor elements in the spleen by CY, might be induced in addition to a direct antitumor effect of CY. There is a report that effectiveness of CY therapy resulted in the elimination of suppressor elements in the spleen after timely treatments of CY (11).

The antitumor effect of N-CWS on 3LL is thought to be mediated by activation of cytostatic and cytolytic properties of macrophages (24, 27, 36). Using in vitro cytotoxic tests, we demonstrated that adherent PEC were cytolytic to 3LL cells, whereas nonadherent PEC were not. We also demonstrated that i.v. injections of peritoneal macrophages activated with N-CWS inhibited the development of pulmonary micrometastases. Ito et al. (13) reported that adherent PEC harvested from mice after i.p. injections of squalene-treated N-CWS showed cytolytic activity in vitro on tumor target cells and that this activity was inhibited markedly by treatment with antimacrophage serum and complement or carageenan. Flyder (6) demonstrated that, when macrophages that had been specifically activated in vitro were injected i.v. into mice, they significantly reduced the incidence of pulmonary metastatic nodules formed after i.v. injection of B16 melanoma cells. Moreover, Liotta et al. (22) reported that i.v. transfer of BCG-activated PEC inhibited pulmonary metastases in mice with implanted T241 fibrosarcomas. Furthermore, significant inhibition of pulmonary metastases was noted in mice after injection of adherent PEC activated with N-CWS from normal mice or mice after excision of a tumor; however, i.v. injection of adherent PEC from mice bearing primary tumor on Day 15 or 16 did not inhibit pulmonary metastases. It was suggested that the inhibitory effect of adherent PEC might be suppressed by an immunosuppressive substance or cells (20). Klykken and Munson (16) reported that 3LL-bearing mice showed reduced ability to elicit an antibody response, develop a delayed hypersensitivity reaction, and phagocytize sheep erythrocytes and that surgical removal of the primary tumor resulted in a rebound increase in antibody-forming cells to the control level.

Little is known about tumoricidal effector cells in the lung, the target organ for hematogenous pulmonary metastasis. We demonstrated that macrophages in the lungs of mice treated with N-CWS showed enhanced in vitro cytotoxic activity against 3LL cells. Gorman (10) and Olivotto and Bomford (29) reported that alveolar or lung macrophages from animals treated with BCG or C. parvum showed nonspecific cytotoxicity against tumor cells. We have also demonstrated that these cells from mice treated with a glucan, Schizophyllan, showed tumoricidal activity against 3LL cells. Sone et al. (36) demonstrated that, after direct stimulation with N-CWS, alveolar macrophages became tumoricidal in vitro against syngeneic, allogeneic, and xenogeneic tumor cells. Furthermore, Sone and Flyder (35) found that the number of rat alveolar macrophages and their ability to respond to activation stimuli, such as N-CWS in vitro and in vivo, to become tumoricidal were not diminished in the presence of progressively growing pulmonary metastases of a syngeneic mammary adenocarcinoma. Thus, these alveolar macrophages or macrophages in the lung may be important in destruction of tumor cells in the lung.

On the other hand, it has been reported that cytotoxic T-cells could be detected not only in regional lymph nodes but also in the spleen and tumor of C57BL/6 mice receiving intraleSIONal immunotherapy with N-CWS, suggesting augmentation of T-cell-mediated cytotoxicity by N-CWS (15). Therefore, it is necessary to consider activation of the tumoricidal activity of cytotoxic T-cells as well as macrophages by N-CWS.

We conclude that N-CWS has potent antitumor activity at the stage of micrometastasis in the lung by causing nonspecific activation of macrophages in the target organ and that its effect depends on the time, dose, and route of its injection.

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