Killing of Tumor Cells in Vitro by Macrophages from Mice Given Injections of Squalene-treated Cell Wall Skeleton of Nocardia rubra 1

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ABSTRACT

Peritoneal exudate cells (PEC) harvested from mice after i.p. injection of squalene-treated cell wall skeleton of Nocardia rubra (N. rubra-CWS) demonstrated vigorous cytolytic activity in vitro toward tumor target cells. Fractionation of these PEC by adherence to plastic dishes showed that the cytolytic activity in PEC was associated with an adherent phagocytic cell. Induction of the cytolytic adherent PEC required an optimal dose of 50 μg N. rubra-CWS and i.p. injection. Cytolytic activity of N. rubra-CWS-induced adherent PEC was maximal after 5 days and fell steadily thereafter. Susceptible tumor targets included cells syngeneic, allogeneic, and xenogeneic to the effector cell source. In contrast, nonneoplastic xenogeneic cells were not affected by N. rubra-CWS-induced adherent PEC. The effector cells were not found in the spleen or peripheral lymph nodes.

In addition, the cytolytic activity of N. rubra-CWS-induced adherent PEC was completely inhibited by treatment with antimacrophage serum and complement or carrageenan. Treatment with monoclonal anti-Thy 1.2 antibody and complement, however, did not affect the cytolytic activity of the adherent PEC. These features make it likely that N. rubra-CWS-induced cytolytic effector cells are macrophages.

INTRODUCTION

In studies from our laboratory, mineral oil-attached N. rubra-CWS 2 was shown to possess a potent antitumor activity (2, 3, 23). We have also described that PEC harvested from rats (23) or mice (18, 19) given injections of mineral oil-attached N. rubra-CWS i.p. are cytotoxic toward syngeneic tumor cells in vitro. Fractionation of these PEC by adherence to plastic dishes or tubes suggested that the cytotoxicity of PEC induced by N. rubra-CWS is associated with an adherent phagocytic cell. A Winn-type assay showed that adherent PEC obtained from mineral oil-attached N. rubra-CWS-injected rats suppress the growth of a syngeneic fibrosarcoma in vivo (23). These results lead us to speculate that N. rubra-CWS may exert cytotoxicity against a variety of tumors through activation of macrophages. However, further characterization of N. rubra-CWS-induced cytotoxic adherent PEC remained to be done.

We have recently treated N. rubra-CWS with squalene, a cholesterol metabolite, to replace mineral oil used as a vehicle for preparations since mineral oil causes inflammatory changes at the injected site (32). In addition, mineral oil-attached N. rubra-CWS is unstable and its immunostimulatory activity decays with storage. The squalene-treated N. rubra-CWS showed the same degree of adjuvant and antitumor protection activity as mineral oil-attached N. rubra-CWS (32). The cellular mechanism of immunity to syngeneic tumors was not determined.

The present study was undertaken to further characterize the cytotoxic cell induced by N. rubra-CWS, to compare the sensitivity of lymphoma targets to fibrosarcoma targets, and to use the newly prepared squalene-treated N. rubra-CWS to reduce inflammation at the injection site.

MATERIALS AND METHODS

Animals. Seven- to 9-week-old male C57BL/6 and CBA mice were obtained from The Shizuoka Jikken-Dobutsu Nokyo, Shizuoka, Japan, and The Omura Jikken-Dobutsu, Kanagawa, Japan, respectively. They were kept on a standard diet and water throughout the experiments.

Effector Cells. PEC were harvested by massage of the peritoneal cavity after injection of 5 ml cold HBSS containing heparin (5 units/ml) and aspiration of the exudate with a syringe and 26-gauge needle. This procedure was repeated with two 5-ml injections of HBSS-heparin, and the exudates were pooled. The PEC were washed twice in cold HBSS and then placed into cold RPMI/HI-FCS. For preparation of adherent PEC monolayer, the appropriate number of PEC suspended in RPMI/HI-FCS was seeded into 100-mm-diameter tissue culture dishes (Falcon Plastics, Oxnard, Calif.) and incubated for 2 hr at 37° in a humidified atmosphere of 5% CO2:95% air. Nonadherent cells were removed, each dish was then washed by vigorous pipetting, and the remaining adherent cells were recovered by scraping with a rubber policeman (70% viable by trypsin blue exclusion). Of the viable adherent PEC, over 95% of the cells had a typical macrophage morphology and phagocytosed latex beads. Spleens and mesenteric lymph nodes were excised, minced, and teased mechanically.

All of the resulting cells were washed twice in cold HBSS and resuspended in RPMI/HI-FCS at a required concentration before use as effector cells for cytolytic assays.

Target Cells. Tissue culture tumor cell lines, ascites tumor cells, and nonneoplastic human fibroblasts were used as target cells in the cytolytic assays. The tissue culture tumor cell lines used were FBL-3, a Friend virus-induced leukemia of C57BL/6 mouse origin (12), and CEM, a human T-cell lymphoma (24).
The human fibroblasts designated HF-1 were obtained from Dr. Tatsuo Yamashita, Institute of Fujisawa Pharmaceutical Co., Osaka, Japan. Cultures of HF-1 fibroblasts were prepared by trypsinization of a 4-month aborted fetus after removal of head and viscera. They were cytologically normal and contact inhibited. Tissue culture tumor cell lines and HF-1 fibroblasts were grown in RPMI/HI-FCS at 37° in a humidified atmosphere of 5% CO₂:95% air. The HF-1 fibroblasts used in the cytolytic assays were from the 9th and 15th passage in vitro, and assays were done with cultures in the exponential growth phase. Ascites tumor cells were Meth A cells from a methylcholanthrene-induced fibrosarcoma of BALB/c mouse origin (32). They were maintained by weekly transfer in syngeneic mice. The tumor target cells were prelabeled in vitro with [125I]ldUrd (The Radiochemical Centre, Amersham, United Kingdom) as described previously (23). Single-cell suspensions of HF-1 target cells were prepared by trypsinization of confluent monolayer cultures. HF-1 fibroblasts were then labeled with [125I]ldUrd at 1 µCi/ml for 8 hr. All target cells labeled with [125I]ldUrd were washed 3 times with RPMI/HI-FCS and adjusted to 1 x 10⁵ cells/ml.

Cytolytic Assay. Various effector cells and 1 x 10⁴ [125I]ldUrd-labeled target cells were combined in a total volume of 1 ml in the wells of the microplate (Falcon No. 3008; Falcon Plastics). The plates were incubated for 24 hr at 37° in a humidified atmosphere of 5% CO₂:95% air. Cells were centrifuged, and 0.5-ml supernatant aliquots were removed for assay of the percentage of released radioactivity. The specific cytolysis was expressed as net percentage of cytolysis as calculated by the following formula.

\[
\text{Net } \% \text{ of cytolysis} = \frac{\text{cpm in supernatant} - \text{cpm in pellet + cpm in supernatant}}{\text{cpm in pellet + cpm in supernatant}} \times 100
\]

The total percentage of cytolysis obtained with medium control (the spontaneous release observed in wells containing target cells alone) was usually between 15 and 25% for all the targets. Assays were performed in triplicate.

Treatment of Adherent PEC with Anti-Macrophage Serum and Complement. A rabbit anti-macrophage serum was kindly provided by Dr. Toshiyuki Hamaoka, Institute for Cancer Research, Osaka University Medical School, Osaka, Japan. These antisera were produced by multiple i.v. injections of a murine macrophage cell line, J774.1 cells (25), into rabbits (29). The resulting sera used in the experiments reacted strongly with mouse peritoneal macrophages as well as J774.1 cells at a 1:64 dilution, as judged by indirect immunofluorescence with fluorescein-labeled goat anti-rabbit globulin antibodies but not with over 90% of spleen cells or lymph node lymphocytes. The antisera at a 32-fold dilution killed both more than 90% of mouse peritoneal macrophages and J774.1 cells in the presence of guinea pig complement as tested by trypan blue exclusion, whereas lymph node lymphocytes remained unaffected. Adherent PEC (1 x 10⁵) in 0.2 ml RPMI/HI-FCS were mixed with 0.2 ml of 1:10 diluted anti-macrophage serum. After 30 min on ice, the supernatant of the culture was decanted, and 0.2 ml of 1:4 diluted guinea pig complement was added. After a further 30 min incubation at 37°, the reaction was terminated by washing the cultures. Complement had to be preabsorbed on ice by FBL-3 cells in order to abolish the background cytotoxicity for FBL-3 cells.

Cytolytic Activity of PEC from N. rubra-CWS-injected Mice. PEC obtained from C57BL/6 mice given i.p. injections of 50 µg of squalene-treated or mineral oil-attached N. rubra-CWS 5 days previously showed marked cytolytic activity against [125I]ldUrd-labeled FBL-3 target cells at effector cell:target cell ratios of 100:1 and 50:1 (Chart 1). The cytolytic activity of squalene-treated N. rubra-CWS-induced PEC did not differ significantly from that of mineral oil-attached N. rubra-CWS-induced PEC. On the other hand, cytolytic activity of PEC recovered from squalene alone- or 0.9% NaCl solution-injected mice remained low at any effector cell:target cell ratios.

Separation by adherence to plastic dishes showed that most of the cytolytic activity was associated with the adherent PEC fraction. Likewise, PEC and adherent PEC harvested from squalene-treated or mineral oil-attached N. rubra-CWS i.p.-injected CBA mice at Day 5 demonstrated almost the same degree of strong cytolytic activity toward the allogeneic tumor target cell, FBL-3.

Effect of N. rubra-CWS Dose, Route, and Timing on Cytolytic Activity of Adherent PEC. Chart 2 shows that 50 µg...
squalene-treated *N. rubra*-CWS induced high cytolytic activity in adherent PEC at Day 5, and 10 μg squalene-treated *N. rubra*-CWS caused a low but significant cytotoxicity. These results were observed in both C57BL/6 and CBA mice.

C57BL/6 mice were given injections of 50 μg squalene-treated *N. rubra*-CWS i.p., s.c., or i.v. Five days later, adherent PEC were assayed for their cytolytic activity toward FBL-3 target cells. The results demonstrated that the i.p. route was more efficacious than the s.c. route. In contrast to the i.p. and the s.c. routes, the i.v. route did not affect the cytolytic activity of adherent PEC.

Chart 3 shows that maximal cytolytic activity occurred 5 days after squalene-treated *N. rubra*-CWS injection and declined steadily thereafter.

**Target Cells Lysed by *N. rubra*-CWS-induced Adherent PEC.** The susceptibility of a variety of [125I]IdUrd-labeled target cells to be lysed by squalene-treated *N. rubra*-CWS-induced PEC was measured. As shown in Chart 4, adherent PEC obtained from C57BL/6 mice given injections of 50 μg squalene-treated *N. rubra*-CWS i.p. at Day 5 had marked cytolytic activity against syngeneic FBL-3, allogeneic Meth A, and xenogeneic CEM tumor target cells. In contrast, HF-1 fibroblasts were not affected by *N. rubra*-CWS-induced adherent PEC. A small degree of toxicity was also seen with the 0.9% NaCl solution and squalene control PEC for Meth A, CEM, and HF-1 target cells.

**Effect of Anti-Macrophage Serum on Cytolytic Activity of Adherent PEC.** Adherent PEC were obtained from CBA mice 5 days after squalene-treated *N. rubra*-CWS injection. C57BL/6 mice were given i.p. injections on Day 0 of 50 μg squalene-treated *N. rubra*-CWS (A). The adherent PEC were harvested from 1 to 21 days later and assayed as in Chart 2. The adherent PEC taken from the mice given i.p. injections of squalene alone (B), corresponding to that attaching to 50 μg *N. rubra*-CWS, or 0.9% NaCl solution (C) only (C) were used as controls. Each point represents the mean of 3 experiments; bars, S.D.
given 50 μg squalene-treated N. rubra-CWS i.p. 5 days before harvesting. The adherent PEC were treated with anti-macrophage serum and guinea pig complement and then assayed for cytolytic activity toward FBL-3 target cells. Whereas the cells treated with normal mouse serum, with complement alone, or with normal mouse serum plus complement led to normal lysis, the cultures treated with the anti-macrophage serum plus complement lost their cytolytic activity. Thus, adherent PEC used here possess macrophage antigens on their surface and appear to be macrophages.

**Sensitivity of Cytolytic Adherent PEC to Hybridoma Anti-Thy 1.2 Antibody.** Anti-Thy 1.2 antibody was used in the presence of guinea pig complement to deplete T-lymphocytes from adherent PEC. The PEC were harvested from CBA mice given injections of 50 μg squalene-treated N. rubra-CWS i.p. 5 days previously. The cytolytic activity of adherent PEC treated with anti-Thy 1.2 antibody and complement did not differ from that of control populations.

**Abrogation of Cytolytic Activity of Adherent PEC by Carrageenan.** To determine the effect of the anti-macrophage agent carrageenan on production of cytolytic PEC, C57BL/6 mice were given injections of 5 or 1 mg carrageenan and 50 μg squalene-treated N. rubra-CWS i.p. simultaneously. The cytolytic activity of adherent PEC against FBL-3 target cells tested at Day 5 was decreased significantly in response to the injected doses of carrageenan (Table 1).

**Cytolytic Activity of Spleen Cells and Lymph Node Cells from N. rubra-CWS-Injected Mice.** Spleen cells and lymph node cells obtained from 50 μg squalene-treated N. rubra-CWS i.p.-injected C57BL/6 or CBA mice at Day 5 were tested for cytolytic activity against FBL-3 target cells. Neither spleen cells nor lymph node cells, in contrast to adherent PEC, showed cytolytic activity toward the tumor target at any effector cell:target cell ratios from 10:1 to 100:1.

**DISCUSSION**

This study shows that the cytolytic activity of PEC taken 5 days after the i.p. injection of squalene-treated N. rubra-CWS is associated with an adherent phagocytic cell. Furthermore, the cytolytic activity of squalene-treated N. rubra-CWS-induced adherent PEC was markedly inhibited by treatment with anti-macrophage serum and complement or carrageenan. The treatment with anti-Thy 1.2 antibody and complement, however, did not affect the cytolytic activity of squalene-treated N. rubra-CWS-induced adherent PEC toward tumor target cells. From these results, it is strongly suggested that squalene-treated N. rubra-CWS-induced cytolytic effector cells are macrophages.

In addition to morphological studies and phagocytic activity, we have used in the present study 2 criteria for identification of the cytolytic adherent cell as macrophages. (a) Susceptibility to treatment with anti-macrophage serum and complement was examined in the cytolytic adherent PEC population. The treatment with anti-macrophage serum and complement, which does not cross-react with lymphocytes (29), provides evidence that the effector cell is a macrophage. Similarly, anti-macrophage serum was used by Lohmann-Matthes et al. (16) in order to identify a cytotoxic effector cell from bone marrow cultures against antibody-coated tumor targets to be the promonocyte.

(b) The effect of carrageenan on induction of cytolytic adherent PEC by squalene-treated N. rubra-CWS was treated. Carrageenan has been well known to inhibit selectively macrophage function (15). The administration of carrageenan has been reported to enhance tumor growth, which is probably due to inhibition of macrophage function (14, 17). The data presented in Table 1 suggest that squalene-treated N. rubra-CWS-induced effector cells impaired by carrageenan may be macrophages.

To determine optimal dose, time, and route of N. rubra-CWS injection is indispensable to establish the protocol of N. rubra-CWS immunotherapy. The strong adherent PEC response after the i.p. injection of squalene-treated N. rubra-CWS was apparent with 50 to 100 μg, although 10 μg squalene-treated N. rubra-CWS produced a response which was low but significantly above control levels. Usually, we have used a dose of 50 μg of squalene-treated N. rubra-CWS per mouse to elicit cytolytic adherent PEC. This dose did not cause any unusual physiological reactions in mice. We have also observed that the i.p. injection of 100 to 500 μg mineral oil-attached N. rubra-CWS per rat is appropriate for in vivo induction of cytolytic adherent PEC (presumably activated macrophages) in rats (23).

The time course of the appearance of effector cells after i.p. squalene-treated N. rubra-CWS injection showed that the reactivity peaks at Day 5 and falls steadily thereafter. By about 12 days, the activity of the N. rubra-CWS-induced adherent PEC has fallen nearly to control levels. Likewise, maximum cytotoxic activity of peritoneal macrophages was reported to be observed 4 to 7 days after Corynebacterium parvum (8) or pyran administration (13). However, these findings are in contrast to the effector cell activities of viable Bacillus Calmette-Guérin-induced PEC where activity does not appear until about 14 days and is maximal at 20 to 25 days (7, 11). Differences in the time required for in vivo macrophage activation are described with different adjuvants. In the present study, cytotoxicity was observed at effector cell:target cell ratios of 100:1 and 50:1 but seldom at 10:1. The absolute number of macrophages per unit area of culture may be more important than this ratio, as shown by Doe and Henson (5).

A noteworthy aspect of the cytolytic adherent PEC response to N. rubra-CWS is the route of injection. The adherent PEC of mice given injections of squalene-treated N. rubra-CWS i.p. showed a marked cytolytic activity. When squalene-treated N. rubra-CWS was injected s.c., a small response was detected.

<table>
<thead>
<tr>
<th>Treatment (i.p.)</th>
<th>% of cytolytic a</th>
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<tbody>
<tr>
<td>N. rubra-CWS (50 μg)</td>
<td>23.5 ± 2.3 a</td>
</tr>
<tr>
<td>No addition</td>
<td>14.0 ± 1.3 a</td>
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<tr>
<td>+ carrageenan (1 mg)</td>
<td>9.5 ± 1.0 a</td>
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<tr>
<td>+ carrageenan (5 mg)</td>
<td>5.7 ± 1.5</td>
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<tr>
<td>Squalene alone</td>
<td>1.0 ± 0.3</td>
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a Cytolysis of FBL-3 cells was measured in a 24-hr [3H]TdR release assay at an effector cell:target cell ratio of 100:1.

b Mean ± S.D. of 4 experiments.

p < 0.05 compared to N. rubra-CWS controls.

<ref>Table 1</ref>
in the adherent PEC. In contrast, cytolytic adherent PEC was not produced at all by i.v. administration. Thus, the increase in cytolytic activity of adherent PEC appears to be due to a local activation within the peritoneal cavity or a migration of cells from the blood similar to the exudation of macrophages and granulocytes following an inflammatory stimulus. It was observed previously that the therapeutic effect of mineral oil-attached *N. rubra*-CWS was dependent on the route of administration in the experimental immunotheraphy of rat syngeneic ascites and solid tumors (23). These studies demonstrated that repeated i.p. injection of mineral oil-attached *N. rubra*-CWS completely prevented the accumulation of cancerous ascites fluid and prolonged the survival period. Similarly, in the case of the solid tumor inoculated i.m., treatment with repeated i.t. injections inhibited the growth of primary tumor and also prevented metastasis. In both of the cases, the PEC harvested from the survived rats exhibited a potent cytolytic activity for the tumor cells *in vitro*.

In the present study, we have treated *N. rubra*-CWS with squalene, a metabolizable oil and an intermediate in the biosynthesis of cholesterol, to replace mineral oil used as a vehicle for preparations. As reported elsewhere (32), we have observed that mineral oil-attached *N. rubra*-CWS is unstable when stored for more than 1 day. Furthermore, the i.p. injection of mineral oil alone caused inflammation including increased numbers of PEC and often cytotoxic PEC production (19). However, the squalene i.p.-injected control showed no antigenic effects (32) and rarely induced cytolytic PEC and little increase in PEC numbers in the present experiments. In contrast to the squalene control, the i.p. injection of squalene-treated *N. rubra*-CWS induced markedly cytolytic PEC (Chart 1) and strong antigenic effects *in vivo* (32), similar to mineral oil-attached *N. rubra*-CWS. Therefore, squalene-treated *N. rubra*-CWS provides a good experimental system for macrophage activation and possible immunotherapy of cancer. Since *N. rubra*-CWS alone without squalene or mineral oil is a poor stimulant (18), these treatments probably maintain a depot of *N. rubra*-CWS in the peritoneal cavity allowing long-term stimulation.

There is increasing evidence that the immunologically nonspecific conversion of the resting macrophage to an activated state by a variety of biological and synthetic agents constitutes an effective host defense mechanism against malignant tumors (9, 15). Activation of macrophages *in vitro* and *in vivo* can lead to increased lysis of target tumor cells (1, 4–8, 10, 13, 19–22, 26, 27, 30, 31, 34). This ‘activated’ macrophage killing is described as nonspecific because it occurs in the absence of known immunity or antibody to the target. The squalene-treated *N. rubra*-CWS-induced adherent PEC were capable of lysing a broad range of target tumor cells. In addition, they were selectively cytotoxic to the neoplastic cells but not to the nonneoplastic cells tested. It has been postulated that nonneoplastic cells, irrespective of histocompatibility differences, are less affected by activated macrophages than are nonneoplastic cells (4, 9, 15, 21).

The mechanism of activation of macrophages by *N. rubra*-CWS remains to be elucidated. Macrophages either may be directly activated by various immunostimulants or may require activating factors from sensitized T-lymphocytes in the presence of appropriate antigen (10, 16). It has recently been shown that rat alveolar macrophages can respond to direct stimulation by squalene-treated *N. rubra*-CWS to become tumoricidal *in vitro* (28). In addition, partial regression of tumors growing in nude mice as a consequence of mineral oil-attached *N. rubra*-CWS administration has been reported, suggesting that this agent may act independently of an active T-lymphocyte system (33). It is therefore likely that activation of macrophages with *N. rubra*-CWS can occur by direct interaction and may not require lymphocyte participation.

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