Antitumor Effect of Nocardia rubra Cell Wall Skeleton on Syngeneically Transplanted P388 Tumors

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ABSTRACT

The antitumor activity of the immunomodulator, Nocardia rubra cell wall skeleton (N-CWS), was investigated using syngeneically transplanted P388 leukemia cells in a solid form. The s.c. growth of P388 tumors in DBA/2 mice was significantly suppressed by systemically administered N-CWS, and the effect was dose dependent. The antitumor effect of N-CWS was partially but significantly abrogated in splenectomized mice but not in T-cell- or natural killer cell-deficient mice. Although spleen cells from mice treated with 1600 μg N-CWS contained no cytolytic activity, they exerted a significant cytostatic effect on P388 cell growth both in vitro and in vivo. Splenic cytostatic activity did not reside in T- or natural killer cells, but in plastic adherent cell population, macrophages. The response to N-CWS immunotherapy appeared to be associated with the number of macrophages infiltrating into the tumors, and this was confirmed by histological analysis showing that P388 tumors from N-CWS-treated mice were intensively and dominantly infiltrated by macrophages. Furthermore, these were shown to be strongly tumor necrosis factor-positive by immunohistochemical analysis. These findings indicate that macrophages are the main effector cells playing a critical role in the suppression of P388 tumor growth in DBA/2 mice, and that tumor necrosis factor produced by these cells may be involved in the macrophage-mediated cytostatic effect induced by N-CWS. The fact that N-CWS suppressed the growth of weakly immunogenic P388 cells in syngeneic DBA/2 mice even when it was systemically injected would support the clinical potential of this agent.

INTRODUCTION

Over the past 10 years, evidence has accumulated to indicate that N-CWS has immunotherapeutic potential for the treatment of various tumors (1-3). Although the precise mechanism underlying its action in tumor cell elimination is not fully understood, it is becoming increasingly apparent that macrophage activation (4, 5) and induction of killer T-cells (6) are involved. In addition, it has also been shown that N-CWS is capable of inducing several cytokines which can activate monocytes/macrophages, including γ-interferon (7, 8), interleukin 1 (9), colony-stimulating factor (10), and TNF (11).

The objective of the present studies was to assess the tumor-suppressive properties of N-CWS when administered systemically against locally growing tumors lacking demonstrable immunogenic potential. For this purpose, we chose the P388-DBA/2 model, because it is known that P388 cells are weakly immunogenic. This trait makes it a difficult model of the host-mediated suppressive effects of biological response modifiers on tumor growth; but previous reports by Fleischmann et al. (12) and by this laboratory (13) demonstrated with this model the significant inhibitory effect of interferons and FK565, a macrophage modifier (14). These data strongly encouraged us in that when a proper immunotherapeutic protocol was designed, immune effector cells actually functioned in terms of in vivo tumor elimination even in the P388-DBA/2 model.

It was found that s.c. growth of P388 tumors in syngeneic DBA/2 mice was significantly suppressed by s.c. administration of N-CWS in a dose-dependent fashion. In this report, we describe the importance of macrophage-dependent antitumor immunity to the outcome of N-CWS therapy in the P388-DBA/2 model.

MATERIALS AND METHODS

Mice. Female DBA/2 mice 6 to 10 weeks old were purchased from Charles River Japan, Inc. (Atsugi, Japan). They were maintained under specific-pathogen-free conditions until use.

Tumor Cells. P388 leukemia cells were weekly passaged in DBA/2 hosts by i.p. inoculation of 106 leukemia cells suspended in serum-free RPMI 1640 (Flow Laboratories, Irvine, Scotland). N-CWS. Squalene-attached N-CWS was prepared by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan).

Evaluation of s.c. Tumor Growth. Tumor cells aspirated from the peritoneal cavities were washed with and resuspended in serum-free RPMI 1640. Only suspensions of single cells with more than 90% viability as determined by trypsin blue dye exclusion were used for injection. To induce solid tumors, 106 P388 cells/0.1 ml were injected s.c. into the left flank of DBA/2 mice. Tumors started to be palpable 5 days after tumor cell injection and grew progressively thereafter, leading to death of all the mice within 15 days. N-CWS or saline in 0.2-ml volumes was injected s.c. into the nuchal area of mice. Each tumor was removed and weighed 11 days after tumor inoculation. Because histological analysis of tumor sections showed that tumor nodules were predominantly composed of tumor cells and no edema was observed, it seemed quite reasonable to consider that tumor weights were representative of tumor cell content.

Cytostasis Assay. At varying intervals after tumor inoculation, mice were sacrificed, and spleen cell suspensions were prepared as described previously (7). Viable P388 cells (3 × 106 cells) with various numbers of spleen cells in 200 μl of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (GIBCO Laboratories, Grand Island, NY), 50 μM 2-mercaptoethanol, and antibiotics were cultured in 96-well round-bottomed microtiter plates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) at 37°C in a humidified CO2 incubator for 48 h. The end of culture, the cells were spun down, and the medium was replaced with fresh medium. Then 1850 Bq [3H]thymidine (New England Nuclear) were added to each well. After incubation for an additional 4 h, the labeled cells were harvested onto glass fiber strips with a microharvester (Bellco Glass, Inc., Vineland, NJ), and the amount of incorporated thymidine was determined by liquid scintillation counting. The data are presented as either mean ± SE (cpm) of triplicate determinations or as a percentage of cytostasis which was determined as

\[
\frac{\text{cpm of targets cultured with experimental splenocytes} \times 100}{\text{cpm of targets cultured with control splenocytes}}
\]

Spleen Cell Fractionation. T- or NK cells were eliminated as described in a previous paper (7). Mφ were removed from the cell suspension by glass adherence (culturing twice for 2 h at 37°C in glass flasks).

T-Cell-deficient Mice. T-Cell-deficient mice were prepared according to the methods of Fujiiura et al. (15). Briefly, thymectomy was performed on April 14, 2017. © 1991 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 1991 American Association for Cancer Research.
formed on 6-week-old DBA/2 mice, followed 2 days later by an i.p. injection of 0.25 ml rabbit anti-thymocyte serum. After resting for 3 weeks, they were X-irradiated with a dose of 750 rads and immediately reconstituted i.v. with $3 \times 10^6$ syngeneic adult bone marrow cells harvested from the femurs and tibiae. They were used for experiments 3 weeks after reconstitution. The effectiveness of T-cell depletion was verified by examining the cellular composition of the spleens by FACS IV (Becton Dickinson).

NK Cell-deficient Mice. NK cell-deficient mice were prepared according to the method of Habu et al. (16) with minor modifications. Lyophilized rabbit antiserum to the glycosphingolipid asialo-GM$_1$ (Wako Pure Chemical Industries, Ltd.) was reconstituted with water and diluted 1:3 with saline. DBA/2 mice received i.v. injections with 0.15 ml of the diluted antisera or normal rabbit serum 3 days before and 1, 4, and 7 days after the tumor inoculation. NK cell depletion in these mice was confirmed by conventional NK cell assay using $^{51}$Cr-labeled YAC-1 as a target.

Splenectomized Mice. Splenectomy was performed on 7-week-old DBA/2 mice. They were used for experiments 1 week later.

Passive Cell Transfer. Recipient DBA/2 mice were inoculated with $10^6$ P388 cells. They were divided into 2 groups, and each mouse was infused i.v. with the total spleen cell contents of one donor mouse that had been given saline or $1600 \mu g$ N-CWS on days 1 and 4 after tumor inoculation and sacrificed on day 8. SC used for transfer were resuspended in 0.5 ml of phosphate-buffered saline.

Determination of Macrophage Content in P388 Tumors. This determination was performed as described previously (17). The tumors were dissected free of the surrounding tissue and weighed 11 days after inoculation. They were finely minced with scissors and incubated for 20 min at 37°C with constant stirring in an enzyme cocktail containing 0.15% highly purified protease (Godo Shusei Co., Ltd., Tokyo, Japan), 0.1% collagenase (Type IV; Sigma, St. Louis, MO), and 0.002% DNase (Sigma). Subsequently, the cell suspension was filtered through a nylon mesh to remove cell debris, washed twice with RPMI 1640, and then resuspended in RPMI 1640 complete medium. Macrophage content was determined by morphology by Wright's stain using slides prepared with a Shandon centrifuge.

Histology. The s.c. tumors were removed 11 days after the inoculation of P388 tumor cells and fixed in 4% formaldehyde. After an adequate fixation, the tissues were processed in an automatic tissue processor for paraffin embedding. Sections, 5 \mu m thick, were cut and stained with hematoxylin and eosin.

Immunohistochemical Staining with Anti-TNF Antibody. Tumors and spleens were removed on day 11 after treatment of P388-bearing mice with saline or $1600 \mu g$ N-CWS on days 1, 4, and 7. Blocks of mouse tissue were snap-frozen in OCT compound (Miles Scientific, Naperville, IL) and stored at $-80^\circ$C. Frozen tissue sections, 7 \mu m thick, were fixed in a buffered picric acid-formaldehyde solution (18) at 4°C for 1 h and stained with antibodies by the biotin-streptavidin method. Sections were first incubated with a 1:1000 dilution of rabbit anti-mouse TNF-\alpha antiserum (Genzyme Corporation, Boston, MA) at 4°C for 18 h. Negative controls were run in parallel using the same dilution of normal rabbit serum in place of the priming antibody. After washing with 0.2 M TBS (pH 7.6), each section was incubated with 50 \mu l of biotinylated goat anti-rabbit immunoglobulins (BioGenex Laboratories, Dublin, CA) for 30 min at room temperature. The tissue sections were rinsed in TBS and incubated with 50 \mu l peroxidase-conjugated streptavidin (BioGenex Laboratories) per section for 30 min at room temperature. They were then washed in TBS again and incubated for 10 min at room temperature in 0.02%, 3,3' diaminobenzidine tetrahydrochloride (Nacalai Tesque, Kyoto, Japan), 0.02% hydrogen peroxide, and 0.065% sodium azide in TBS. Subsequently, the sections were washed in tap water, stained with hematoxylin and eosin, dehydrated in baths of increasing ethanol concentrations, cleared in xylene, and coverslipped.

Statistics. The results were evaluated for statistical significance by paired t tests.

RESULTS

Inhibitory Effect of N-CWS Treatment on the Growth of s.c. P388 Tumors in DBA/2 Mice. As an initial step toward investigating the effects of systemically administered N-CWS on the growth of P388 tumors in syngeneic DBA/2 mice, the drug was given s.c. remote from tumor with $10^7$ P388 cells at 1, 4, and 7 days after inoculation. As shown in Fig. 1, N-CWS exerted a significant and dose-dependent inhibition of tumor growth detected 11 days later. The mean tumor weight was reduced from 149 mg in the control group to 99, 78, and 60 mg in mice given 800, 1600, and $3200 \mu g$ N-CWS, respectively. The antitumor effect was not obtained when doses below $400 \mu g$ were used. N-CWS treatment produced a decrease in tumor weight; however, no significant differences were observed in the survival times between saline-treated and N-CWS-treated groups. Body weight did not decrease even in the group of mice treated with $3200 \mu g$ N-CWS (data not shown).

Antitumor Effect of N-CWS in T-Cell-deficient, NK Cell-deficient, or Splenectomized Mice. Since N-CWS showed no cytotoxicity to P388 cells in liquid suspension culture in the continuous presence of 50 \mu g/ml of the drug for 4 days (data not shown), it would be expected that the above-mentioned antitumor effects of N-CWS were elicited by a host-defense mechanism. To confirm this, a series of experiments was performed. Table 1 shows the combined data from three sets of independent experiments comparing the antitumor activity of N-CWS in T-cell-deficient, NK cell-deficient, or splenectomized mice to that in normal mice. In all experiments, $1600 \mu g$ of N-CWS was given on days 1, 4, and 7 after tumor inoculation. Removal of T- and NK cells did not affect the subsequent inhibition of tumor growth after treatment of P388-bearing mice with N-CWS (Table 1, Experiments 1 and 2), suggesting that neither T- nor NK cells were involved in the N-CWS-induced suppression of tumor growth in the P388-DBA/2

Fig. 1. Dose dependence of N-CWS-mediated inhibition of P388 tumor cell growth in DBA/2 mice. Mice inoculated with $10^7$ P388 cells were treated s.c. with the indicated doses of N-CWS on days 1, 4, and 7 after tumor inoculation. Tumors were removed and weighed on day 11. Points, mean of ten mice; bars, SE. **, statistically significant relative to the data for mice treated with saline. P < 0.01.
Table 1 Effect of N-CWS on local tumor growth of P388 cells in T-cell-deficient, NK cell-deficient, or splenectomized DBA/2 mice

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mice</th>
<th>Treatment</th>
<th>Tumor wt on day 11 P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal</td>
<td>Saline</td>
<td>167 ± 17*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-CWS</td>
<td>108 ± 18 (35)*</td>
</tr>
<tr>
<td></td>
<td>T-cell-deficient</td>
<td>Saline</td>
<td>147 ± 20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-CWS</td>
<td>97 ± 6 (34)</td>
</tr>
<tr>
<td>2</td>
<td>Normal</td>
<td>Saline</td>
<td>146 ± 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-CWS</td>
<td>74 ± 16 (49)</td>
</tr>
<tr>
<td></td>
<td>NK cell-deficient</td>
<td>Saline</td>
<td>173 ± 9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-CWS</td>
<td>90 ± 9 (47)</td>
</tr>
<tr>
<td>3</td>
<td>Normal</td>
<td>Saline</td>
<td>176 ± 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-CWS</td>
<td>92 ± 27 (48)</td>
</tr>
<tr>
<td>Splenectomized</td>
<td>Saline</td>
<td>194 ± 15</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-CWS</td>
<td>156 ± 28 (20)</td>
</tr>
</tbody>
</table>

* See "Materials and Methods."

Table 2 Ability of spleen cells from mice treated with N-CWS to suppress P388 cell proliferation in vitro

<table>
<thead>
<tr>
<th>Spleen</th>
<th>E:T</th>
<th>[3H]dThd uptake</th>
<th>Inhibition (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline-treated</td>
<td>100:1</td>
<td>35,794 ± 854a</td>
<td>33.1± 3.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>50:1</td>
<td>32,651 ± 2,319</td>
<td>33.1± 3.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>25:1</td>
<td>31,058 ± 1,682</td>
<td>33.1± 3.1</td>
<td>NS*</td>
</tr>
<tr>
<td>N-CWS-treated</td>
<td>100:1</td>
<td>8,816 ± 2,360</td>
<td>75.4 ± 6.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>50:1</td>
<td>20,165 ± 5,102</td>
<td>33.1± 3.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>25:1</td>
<td>26,867 ± 2,423</td>
<td>33.1± 3.1</td>
<td>NS*</td>
</tr>
</tbody>
</table>

* DBA/2 mice bearing P388 tumors were treated with saline or N-CWS (1600 µg/body) on days 1 and 4 after tumor inoculation. Spleens were harvested on day 7 for cytostasis assay. See "Materials and Methods."

Fig. 2. Dose response of N-CWS-induced splenocyte-mediated cytostasis. DBA/2 mice were treated s.c. with the indicated doses of N-CWS on days 1 and 4 after tumor inoculation. On day 7, their spleen cells were assayed for cytostatic activity as described in "Materials and Methods. The data shown were obtained at an E:T of 100:1 (O), 50:1 (Q), and 25:1 (Q). Bars, SE.}

Fig. 3. Kinetics of expression of splenocyte-mediated cytostasis in N-CWS-treated mice. Three groups of mice were treated s.c. with 1600 µg N-CWS on day 1, days 1 and 4, or days 1, 4, and 7 after tumor inoculation, respectively (left to right). Each spleen was obtained 3 days after the last injection of N-CWS, and cytostatic activity was measured as described in "Materials and Methods. Data were obtained at an E:T of 100:1 (O), 50:1 (A), and 25:1 (Q). Bars, SE; arrows, days of N-CWS injection.
with 1600 µg N-CWS on days 1 and 4 after tumor cell inoculation and were sacrificed on day 7. Spleen cells treated with anti-Thy-1.2 antibody plus C (A), those treated with anti-asialo-GMI antibody plus C (B), or plastic nonadherent spleen cells (C) were examined for cytostatic activity at an E:T ratio of 200:1 (■), 100:1 (■), 50:1 (■), and 25:1 (■). Columns, mean of triplicate determinations; bars, SE.

Experiments were then performed to determine whether these infiltrating Mφ play a pivotal role in suppressing P388 tumor growth in vivo. For this purpose, tumor nodules were obtained on day 11 after treatment of mice bearing P388 tumor with saline or 1600 µg N-CWS on days 1, 4, and 7. After the individual tumors were weighed, they were separately teased into small pieces and treated with an enzyme mixture. An aliquot of the resultant cell suspension was cyt centrifuged and Wright-Giemsa stained for differential count. Fig. 6 demonstrates that there was a strong correlation (r = 0.852) between the degree of suppression of P388 tumor growth and the number of Mφ infiltrating into the tumor. These data clearly indicate that in the N-CWS-treated tumor-bearing mice, Mφ was responsible for suppressing tumor growth in the P388-DBA/2 model.

Table 3  Ability of cytostatic spleen cells to inhibit tumor growth when adoptively transferred to P388-bearing mice

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor Treatment</th>
<th>Tumor wt on day 11 (mg)</th>
<th>Inhibition (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline</td>
<td>168 ± 29f</td>
<td>51.3 ± 5.7f</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>1</td>
<td>N-CWS</td>
<td>81 ± 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Saline</td>
<td>132 ± 11</td>
<td>31.2 ± 6.4</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>4</td>
<td>N-CWS</td>
<td>91 ± 8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* DBA/2 mice were inoculated with 10^7 P388 cells on day 0. The total spleen cell contents of one donor mouse treated with saline or 1600 µg N-CWS were transferred i.v. to each P388-bearing mouse on the indicated day. Each group consisted of 8 mice.

* Donor DBA/2 mice were treated with saline or 1600 µg N-CWS on days 1 and 4 after tumor inoculation. On day 8, mice were sacrificed, and their spleens were dissected for transfer.

* Mean ± SE.

DISCUSSION

We demonstrated in this paper that systemically administered N-CWS significantly suppressed the growth of P388 tumor cells in solid form, which are known to have extremely weak immunogenicity. That the P388 cells we used were poorly immunogenic was evident in the following experiment, in which DBA/2 mice were immunized with 2 s.c. injections of 10^7
mice immunized with MethA cells completely rejected the growth of the challenged tumor cells (data not shown).

Furthermore, we have reached a conclusion that cytostatic spleen cell activation by N-CWS was responsible for the subsequent growth inhibition of P388 tumors. This was based on the following findings: (a) neither growth inhibition of P388 tumors nor induction of splenic cytostatic activity was obtained when doses lower than 400 µg of N-CWS were used; (b) N-CWS-induced antitumor effect was significantly reduced in splenectomized mice compared to normal mice; and (c) adoptive transfer of SC from N-CWS-treated mice into P388-bearing mice resulted in the suppression of tumor growth. Moreover, the following findings strongly support the idea that Mφ are the main effector induced by N-CWS therapy: (a) cytostatic activity was induced in adherent fractions of SC from N-CWS-treated mice; and (b) a number of Mφ were infiltrating around the tumor nodules of P388-bearing mice treated with N-CWS, and there was a strong correlation between the number of Mφ and the inhibition rate of tumor growth.

For the first approach to clarify the N-CWS-induced effector mechanism in P388-DBA/2 model, DBA/2 mice were thymectomized or treated with anti-asialo-GM1 antibody as models for T-cell or NK cell deficiency, respectively (15, 16). The present studies using specific immune deficient mice showed that T-cell or NK cell deficiency did not abrogate the N-CWS-induced suppression of P388 growth but that splenectomy resulted in significant loss of the antitumor activity of N-CWS.

Fig. 5. Histological section of s.c. tumor nodules removed 11 days after P388 cell inoculation. a, saline-treated tumor-bearing mice; b, 1600 µg N-CWS-treated tumor-bearing mice. Prominent macrophage infiltration was observed around P388 tumors obtained from mice receiving N-CWS. Original magnification, × 62.5.

Fig. 6. Relation between tumor growth inhibition and the percentage of Mφ infiltrating into P388 tumors. DBA/2 mice bearing P388 tumors were treated with saline (○) or 1600 µg N-CWS (●) on days 1, 4, and 7 after tumor cell inoculation. Tumors were removed on day 11 and dissociated into single cell suspensions for the determination of macrophage content as described in "Materials and Methods."

irradiated (5000 rads) P388 cells at intervals of 14 days. Two weeks after the second injection, two groups of the immune or nonimmune mice were challenged s.c. with 10⁵ viable P388 cells. The life spans of the immune mice were comparable to those of nonimmune mice. In contrast, the data obtained using strongly immunogenic MethA fibrosarcoma and its syngeneic host BALB/c mice in the same procedure showed clearly that

Fig. 7. Immunohistochemical staining of P388 tumor nodules with anti-TNF antibody. Tumor specimens were prepared from mice treated with saline (a) or 1600 µg N-CWS (b). Most of the infiltrating Mφ were TNF-positive as indicated by diffuse "cytoplasmic" staining. Original magnification, × 62.5.
jection according to the method of Von Loveren et al. (20). The number of peritoneal M₀ in the silica-treated mice decreased to 25% of that of the untreated mice until day 33 after silica treatment, whereas that of the splenic M₀ was comparable in the two groups. Unexpectedly, the induced antitumor effect of N-CWS tended to be enhanced in the silica-treated mice (data not shown), suggesting that silica had activated rather than suppressed M₀. Although there is abundant evidence that in vitro treatment with silica, carrageenan, or 2-chloroadenosine (21) is effective in selectively eliminating M₀, a method selectively depleting these cells in vivo has not been developed.

In the spleens from the N-CWS-treated mice, significant cytostatic activity was induced in the plastic adherent cell populations but not in T-cells or NK cells. These adherent cells were identified as M₀ as assessed by morphology and uptake of neutral red (data not shown). The kinetic study demonstrated that cytostatic activity was maximal on day 7 after tumor inoculation and thereafter declined rapidly. A likely explanation for this would be that the N-CWS-induced cytostatic activity was down-regulated by splenic metastasizing P388 cells. Inoculation s.c. of 10⁷ P388 cells is followed by rapid metastatic spread from the primary tumor site to the liver and spleen (22), and host mice are killed within 15 days. No beneficial effect of N-CWS on the survival times of P388-bearing mice seemed to be attributable to the incapability of this agent to prevent metastasis. In some systems, the tumor cells themselves were directly responsible for the suppression of immune responses (23, 24). More recently, Yurochko et al. (25) demonstrated that the decrease in Igα antigen expression among the M₀ of a tumor-bearing host was associated with tumor progression in the EL4-C57BL/6 model (25). MacCubbin et al. (26) also reported that a variety of cell-mediated cytolytic effector functions were inhibited in animals bearing large tumors.

Our next approach to clarifying the N-CWS-induced effector mechanism in this model was to examine the capability of these splenic M₀ for suppressing tumor growth when adoptively transferred into P388-bearing mice. A basic technical problem in these experiments was, however, the difficulty of separating M₀ from SC without damaging their functions. Accordingly, whole SC from one donor mouse were used for injection into one tumor-bearing mouse. In this way, it was proved that cytostatic splenocytes were capable of suppressing tumor growth in vivo. In a study by Hampfries et al. (27), NK cell-stimulating activity of swainsonine was demonstrated when assessed per animal rather than per spleen cell, because swainsonine caused an increase in spleen cell numbers.

The cellular composition of the spleen was then examined from mice treated with saline or N-CWS on days 1 and 4 and harvested on day 7 after tumor inoculation. We found that the N-CWS-treated spleen comprised 50% lymphocytes, 13% M₀, and 26% granulocytes, whereas the proportions for the saline-treated spleen were 84%, 7%, and 5%, respectively. It has been reported that N-CWS-induced tumoricidal activity of polymorphonuclear leukocytes in vitro and that their release of hydrogen peroxide might have a part in tumor cell destruction as a direct cytotoxic mediator (28). Inflammatory neutrophils activated by i.p. injection of Corynebacterium parvum have also been reported to exert significant antitumor effects (29, 30). Accordingly, it is conceivable that neutrophils were involved in the cytostatic effects in our model, we exclude this for two reasons: (a) the effector cell population in the N-CWS-treated spleen cells was made up of plastic adherent mononuclear cells; (b) histology of s.c. P388 tumor dissected from the N-CWS-treated mice showed that M₀ were the dominant population in the cells accumulating around the tumor mass and that neutrophils were much less frequent (Fig. 5).

Of great importance is the fact that in the N-CWS-treated mice a number of M₀ were infiltrating around the growing tumor. This finding clearly indicates that M₀ are the most likely candidates for effector cells involved in the antitumor mechanisms of N-CWS in the P388-DBA/2 model. And more interestingly, these infiltrating M₀ were found to produce TNF as assessed by immunohistochemical analysis (Fig. 7). Our results provide the first evidence for N-CWS-induced TNF production by M₀ infiltrating into regressing tumor lesions. The results are consistent with those of our previous study (11), which demonstrated that N-CWS stimulated the release of TNF-like cytotoxic factor in vitro from murine and human M₀. It seems quite likely that the TNF produced within the microenvironment by infiltrating M₀ may exert cytostatic effects on P388 cells and lead to suppression of tumor growth. In support of this assumption is the previous observation that P388 cells were susceptible to TNF in vitro (11). TNF has been reported to be a possible mediator of cytokine- or lipopolysaccharide-activated monocyte/macrophage cytotoxicity in both human (31, 32) and murine (33) systems.

The precise mechanism by which the activated M₀ exerted their antitumor effect in the P388-bearing mice treated with N-CWS is unknown, but it should be noted that systemic administration of N-CWS caused a significant growth inhibition in the P388-DBA/2 model. Because of the very weak immunogenicity of P388 cells, this model is a clinically relevant and tough system for the evaluation of anticancer drugs with immunomodulating properties. We believe that the findings reported in this paper will strongly support existing clinical evidence that N-CWS is a promising immunomodulator for the treatment of cancer.

ACKNOWLEDGMENTS

We thank C. Ohkawa for processing the manuscript.

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