Antitumor Effect of *Nocardi a rubra* Cell Wall Skeleton on Syngeneically Transplanted P388 Tumors

Shizue Izumi, Toshikazu Ogawa, Michiyo Miyachi, Keiko Fujie, Masakuni Ohkura, and Masanobu Kohsaka


**ABSTRACT**

The antitumor activity of the immunomodulator, *Nocardi a 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 naturally occurring low-molecular-weight biological response 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 10³ 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 trypan blue dye exclusion were used for injection. To induce solid tumors, 10³ 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 the 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 dominantly 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 96 h. At the end of culture, the cells were spun down, and the medium was replaced with fresh medium. Then 150 μl per well [3H]Thd (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

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

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 Fujiwara et al. (15). Briefly, thymectomy was per-

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1 To whom requests for reprints should be addressed.

2 The abbreviations used are: N-CWS, *Nocardi a rubra* cell wall skeleton; dThd, thymidine; NK cell, natural killer cell; SC, spleen cells; Mφ, macrophages; E:T ratio, effector:target cell ratio; asialo-GM₁, ganglio-N-tetraosylceramide; TNF, tumor necrosis factor; TBS, Tris-buffered saline.
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 x 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 analysis.

NK Cell-deficient Mice. NK cell-deficient mice were prepared according to the method of Habu et al. (16) with minor modifications. Lympholized rabbit antiserum to the glycosphingolipid asialo-GM1 (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 ^51Cr-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 ^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 V; 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 with 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 ^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 ^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°C. Frozen tissue sections, 7 ^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 1:1000 dilution of rabbit anti-mouse TNF- 0 antiserum (Genzyme Corporation, Boston, MA) at 4°C for 3 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 ^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 ^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 systematically administered N-CWS on the growth of P388 tumors in syngeneic DBA/2 mice, the drug was given s.c. remote from tumor with 10^5 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 ^g N-CWS, respectively. The antitumor effect was not obtained when doses below 400 ^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 ^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 ^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 ^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
ANTITUMOR EFFECT OF NOCARDIA RUBRA CELL WALL SKELETON

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</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal</td>
<td>Saline</td>
<td>167 ± 17</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-CWS</td>
<td>108 ± 18 (35)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Normal</td>
<td>Saline</td>
<td>147 ± 20</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-CWS</td>
<td>97 ± 6 (34)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Normal</td>
<td>Saline</td>
<td>146 ± 14</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-CWS</td>
<td>74 ± 16 (49)</td>
<td></td>
</tr>
</tbody>
</table>

*See “Materials and Methods.”

Saline or 1600 ng N-CWS were administered s.c. on days 1 and 4 after tumor inoculation.

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 (cpm)</th>
<th>Inhibition (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline-treated</td>
<td>100:1</td>
<td>35,794 ± 854</td>
<td>75.4 ± 6.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>50:1</td>
<td>32,651 ± 2,319</td>
<td>73.1 ± 11.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>25:1</td>
<td>31,058 ± 1,682</td>
<td>73.9 ± 7.8</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>73.1 ± 11.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>25:1</td>
<td>26,867 ± 2,423</td>
<td>73.9 ± 7.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

* DBA/2 mice bearing P388 tumor 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 cytosis assay. See "Materials and Methods."

Fig. 2. Dose response of N-CWS-induced splenocyte-mediated cytosis. 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 spleens were assayed for cytosytic 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 (D). Bars, SE.

Fig. 3. Kinetics of expression of splenocyte-mediated cytosis 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 cytotic activity was measured as described in "Materials and Methods." Data were obtained at an E:T of 100:1 (O), 50:1 (Q), and 25:1 (D). Bars, SE; arrows, days of N-CWS injection.

The data presented thus far give rise to the question of whether SC from mice treated with N-CWS were capable of exerting an antitumor effect was, however, significantly reduced in splenectomized mice (Table 1, Experiment 3), suggesting that some cell type(s) in the spleen, other than T- or NK cells, had an important part in the N-CWS-induced antitumor effect in this model.

Induction of Cytostatic Activity in SC from Mice Given N-CWS. The above findings led us to test the SC from N-CWS-treated mice for their effect on [3H]dThd uptake and 51Cr release by P388 cells in vitro. Incubation of the N-CWS-treated SC with P388 cells, at an E:T ratio of 100:1 and 50:1 for 96 h, suppressed the [3H]dThd uptake of P388 cells by 75.4% and 33.1%, respectively, when compared with that of P388 cells cocultured with saline-treated SC (Table 2). The degree of suppression was dependent on the doses of N-CWS injected (Fig. 2) and the E:T ratio utilized. In contrast to the generation of significant cytostatic activity in the N-CWS-treated SC, cytology could not be detected using 51Cr-labeled P388 cells as a target even when the 51Cr release assay was extended to 24 h (data not shown). Since the culture medium was replaced with fresh medium prior to the addition of [3H]dThd, these findings exclude the possibility that the depression of thymidine uptake was due to excess cellular pools of cold thymidine (19).

In the next experiments, we examined the kinetics of the generation of cytostatic SC in N-CWS-treated mice. The results of a representative experiment are given in Fig. 3. The cytostatic effect of N-CWS-treated SC was evident at a higher E:T ratio of 100:1 on day 4 following a single injection of 1600 µg N-CWS and was maximal by day 7 after 2 injections of N-CWS on days 1 and 4 after tumor inoculation. Cytostatic activity then declined rapidly to low levels on day 10.

Fractions of N-CWS-treated SC were then tested for cytostatic activity. As shown in Fig. 4, while depletion of Mφ almost completely abrogated the cytostatic activity of the SC, depletion of Thy-1.2+ cells and asialo-GM1+ cells failed to reduce it. These results suggest that the ability of SC from N-CWS-treated tumor bearers to suppress P388 cell growth in vitro resides in Mφ but not in T- and NK cells.

Suppression of P388 Tumor Growth in Vivo by Adoptive Transfer of SC from N-CWS-treated P388-bearing Mice. The data presented thus far give rise to the question of whether SC from mice treated with N-CWS were capable of exerting an
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-GM₁ 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.

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*</th>
<th>Tumor wt on day 11 (mg)</th>
<th>Inhibition (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day of injection</td>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Saline</td>
<td>168 ± 29</td>
<td>51.3 ± 5.7</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td></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></td>
<td>N-CWS</td>
<td>91 ± 8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Donor DBA/2 mice were inoculated with 10⁶ 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.

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 cytocentrifuged 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.

TNF Production by Infiltrated Mφ in P388-bearing Mice Treated with N-CWS. The strong evidence for macrophage-mediated suppression of P388 tumor growth and our earlier studies on the induction of a TNF-like activity by N-CWS-treated Mφ in vitro (11) prompted us to examine whether infiltrated Mφ surrounding the P388 tumor cells from N-CWS-treated mice produce TNF. The tumors were removed on day 11 after treatment of P388-bearing mice with saline or 1600 μg N-CWS on days 1, 4, and 7. They were snap-frozen in dry ice/acetone, and 10-μm frozen sections were examined for cellular localization of TNF by serial staining with anti-murine TNF antibody and peroxidase-conjugated anti-mouse IgG antibody. As shown in Fig. 7, the majority of infiltrating Mφ from the N-CWS-treated mice were strongly stained with the anti-TNF antibody, whereas no detectable TNF was present in the saline-treated mice.

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 10⁷
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 thymec-tomized 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.

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 10^5 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
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 was 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 monocellulars; (b) histology of s.c. P388 tumor dissected from the N-CWS-treated mice showed that MΦ 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 MΦ were infiltrating around the growing tumor. This finding clearly indicates that MΦ 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 MΦ 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 MΦ 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 MΦ. It seems quite likely that the TNF produced within the microenvironment by infiltrating MΦ 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 MΦ 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.

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