Effect of *Nocardi a rubra* Cell Wall Skeleton on T-Cell-mediated Cytotoxicity in Mice Bearing Syngeneic Sarcoma

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**ABSTRACT**

Cell-mediated cytotoxicity against syngeneic MC104 fibrosarcoma cells was detected in C57BL/6N mice 7 days after tumor inoculation in the hind foot. This cytotoxicity was undetectable by Day 14 in the Winn test using spleen and draining popliteal lymph node (DPLN) cells. Similar results were obtained with the $^{51}$Cr release assay following *in vitro* activation of these lymphoid cells with mitomycin C-treated tumor cells. The antitumor cytotoxicity was shown to be mediated by T-cells. Spleen but not DPLN cells from 14-day tumor bearers enhanced tumor growth in the Winn test, suggesting the presence of immunosuppressor cells in the spleen.

Two intraleisional injections of 50 μg of cell wall skeleton (CWS) of *Nocardi a rubra* on Days 2 and 7 resulted in apparent tumor growth inhibition and prolongation of the survival period of tumor bearers. DPLN cells from tumor bearers treated with *N. rubra* CWS exhibited significant recovery in the cytotoxicity tested on Day 14, whereas the recovery in that of spleen cells was not apparent. The cytotoxicity augmented by *N. rubra* CWS was specific to MC104 tumor cells and was shown to be mediated by T-cells. These cytotoxic T-cells were shown to be able to localize not only in DPLN but also in the spleen and tumor in mice receiving the intraleSIONal immunotherapy with *N. rubra* CWS. These results suggest that T-cell-mediated cytotoxicity against syngeneic tumor can be augmented by *N. rubra* CWS and might play an important role in the systemic development of its antitumor effect, although the effector cell increase in the spleen might be suppressed by splenic suppressor cells during tumor growth.

**INTRODUCTION**

There have been many reports describing the effectiveness of cancer immunotherapy with bacterial immunoadjuvants administered to the growing tumor or the cancerous fluid in animals and humans (3, 7, 10, 11, 15, 22, 24). Inhibition of tumor growth that resulted from the i.l. injection of the immunoadjuvants has been considered to be mediated mainly by macrophages, from the findings that the granulomatous reaction is predominantly seen in the regressing tumor mass injected with the adjuvants (10) and that macrophages induced by the adjuvants in normal animals exhibit significant antitumor activity *in vivo* or *in vitro* (6, 12, 13, 15, 17).

On the other hand, T-cell-mediated cytotoxicity has been shown to be usually induced in animals at the early stage of tumor growth (14, 19). It is not well known how T-cell-mediated cytotoxicity induced in tumor-bearing hosts correlates to the systemic development of the antitumor effect of the i.l. immunotherapy, although T-cells appear to be not necessary for the immunoadjuvant-induced suppression of tumor growth observed at the injection site of the adjuvants (21).

We have reported that the i.l. injection of *Nocardi a rubra* CWS, mycobacteria-related organisms, is as effective as that of BCG-CWS for several experimental tumors (1, 2, 15). *N. rubra* CWS, as well as BCG CWS, not only activates macrophages to be nonspecifically cytotoxic against syngeneic or allogeneic tumor cells in mice and rats (12, 13, 15) but also acts as an adjuvant on the induction of T-cell-mediated cytotoxicity against allogeneic tumor cells in mice (1, 20). The latter strongly suggests that T-cell-mediated cytotoxicity against syngeneic tumor can be augmented by *N. rubra* CWS, and that, as well as macrophages, cytotoxic T-cells might play an important role in the development of the antitumor effect of the i.l. immunotherapy with *N. rubra* CWS.

The present study was undertaken to investigate the antitumor effect of the i.l. injection of *N. rubra* CWS in relation to its influence on T-cell-mediated cytotoxicity against syngeneic tumor and on the localization of the cytotoxic effector cells in mice bearing syngeneic sarcoma.

**MATERIALS AND METHODS**

**Animals.** Female 10-week-old C57BL/6N mice were obtained from Ohmura Animal Laboratories, Tokyo, Japan.

**Tumors.** 3-Methylcholanthrene-induced fibrosarcoma in C57BL/6N mice, designated as MC104, was maintained by serial trocar passages of a 1- to 2-mm fragment in C57BL/6N mice every other week for more than 2 years. Tumor cell suspensions were obtained by enzymatic digestion of tumor fragments with 0.25% trypsin (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). For use as target cells of cytotoxicity test or stimulator cells of *in vitro* generation of cytotoxic cells, MC104 tumor cells obtained by trypsinization were cultured in Roswell Park Memorial Institute Medium 1640 (Nissui Pharmaceutical Co., Tokyo, Japan) and supplemented with 100 units penicillin per ml (Meiji Seika Co., Tokyo, Japan), 100 μg streptomycin per ml (Meiji Seika Co.), 2 mm L-glutamine (Flow Laboratories, McLean, Va.), and 10% fetal calf serum (Microbiological Associates, Bethesda, Md.). This medium was designated as complete medium. Cultured MC104 tumor cells were collected with jet by pipetting. Viability of MC104 tumor cells obtained from tumor fragments or culture dishes was greater than 95%, as determined by trypan blue dye exclusion test.

**EL4, dimethylbenzanthracene-induced lymphoma in...**
C57BL/6 mice, was serially passaged in an ascites form by i.p. transplantation in C57BL/6N mice. 

**CWS of N. rubra.** N. rubra CWS, prepared by the method of Azuma et al. (1) was treated with squalene (Katayama Chemicals Co., Tokyo, Japan) in an oil-attached form as reported previously (23) and suspended in 0.85% NaCl solution at a concentration of 5 mg/ml just before use.

**Tumor Cell Inoculation.** MC104 tumor cells obtained by enzymatic digestion were suspended in Eagle's MEM (Nissui Pharmaceutical Co.) at a concentration of $10^6$ cells/ml, and $10^6$ cells were injected s.c. into the dorsum of the right hind feet of mice.

**Spleen and Lymph Node Cells.** Spleen and popliteal, inguinal, and cervical lymph nodes of normal mice and spleen and DPLN of tumor-bearing mice were aseptically removed, pooled, and washed, respectively. These spleens and lymph nodes were teased apart in MEM supplemented with 10% fetal calf serum and placed on ice for 5 min to settle the clumps. The cells were washed twice with MEM and suspended in an appropriate medium. Viability of both spleen and lymph node cells was greater than 80%.

**Winn Test.** The Winn-type cytotoxicity test was described previously (15). Ten thousand cultured MC104 tumor cells were mixed with $10^4$ cells of either spleen or lymph node in 0.1 ml of MEM and incubated for 30 min at 37°C. The mixture was injected i.c. into the backs of normal mice. Three weeks later, tumor incidence and tumor weight were examined. Mice that received 10^6 tumor cells alone served as controls.

**In Vitro Generation of Cytotoxic Cells.** Spleen and lymph node cells were activated in vitro with mitomycin C-treated tumor cells according to the method of Takei et al. (19). Ten million cultured MC104 tumor cells were incubated with 50 µg of mitomycin C per ml (Kyowa Hakko Industrial Co., Tokyo, Japan) in 1 ml of MEM at 37°C for 30 min and washed 3 times with MEM. Five million cells of either spleen or lymph node were mixed with $2 \times 10^7$ mitomycin C-treated MC104 tumor cells in 6 ml of complete medium supplemented with $5 \times 10^{-5}$ M 2-mercaptoethanol (Wako Jun'yaku Industrial Co., Tokyo, Japan) in 35-110-mm Falcon No. 2070 plastic tube (Falcon Plastics, Oxford, Calif.) and incubated for 5 days at 37°C in humidified 5% CO2 in air. After the 5-day incubation, the cells were collected by centrifugation, resuspended in complete medium, and used as effector cells for in vitro cytotoxicity test. Viability of the responder cells varied from 30 to 40%.

**In Vitro Cytotoxicity Test.** Cytolytic activities of spleen and lymph node cells activated in vitro with mitomycin C-treated tumor cells were determined by the method of Brunner et al. (5). Briefly, 5 million target tumor cells in 1 ml of complete medium were incubated with 100 µCi of $^{51}$Cr in sodium chromate form (Japan Atomic Energy Research Institute, Tokyo, Japan) for 1 hr at 37°C with occasional shaking. $^{51}$Cr-labeled tumor cells, after being washed 3 times with MEM, were resuspended in complete medium. One million viable effector cells were mixed with $10^4$ $^{51}$Cr-labeled target cells in 1 ml of complete medium in a 12- x 75-mm Falcon No. 2058 plastic tube, centrifuged at 200 x g for 1 min, and incubated for 4 hr at 37°C in humidified 5% CO2 in air. Each of the tubes was then centrifuged at 500 x g for 5 min, and the radioactivity in 0.5 ml of the resulting supernatant was counted by a well-type scintillation counter (Aloka Co.). Viability of both spleen and lymph node cells was determined by the method of Brunner et al. Maximum release was determined by freezing and thawing of $10^4$ target cells 3 times. Control release was lower than 20%, and maximum release was higher than 80% of the total radioactivity of $10^4$ target cells, respectively.

**Lymphocyte Trapping.** As reported previously (16), 100 million lymph node cells in 1 ml of MEM were incubated with 100 µCi of $^{51}$Cr for 1 hr at 37°C, washed 3 times with MEM, and resuspended in 1 ml of MEM. Twenty million $^{51}$Cr-labeled lymph node cells were injected into the tail vein of mice. Twenty hr later, these mice were sacrificed by bleeding, and the radioactivities of tumor, spleen, and popliteal, inguinal, and paraaortic lymph nodes of both the ipsilateral and contralateral sides were counted by a well-type scintillation counter (Aloka Co.). In the experiment for tumor and spleen trapping of $^{51}$Cr-labeled cells, results were expressed as a percentage of recovery of the total radioactivity per wet weight of respective organs. In the experiment for lymph node trapping, results were expressed as a ratio of the radioactivity of the right (draining ipsilateral) node to that of the left (contralateral) node.

**Anti-ß Antiserum and Complement Treatment.** Antiserum against brain-associated ß-antigen was raised in rabbits by injecting AKR mouse brain in complete Freund's adjuvant and extensively absorbed with AKR mouse liver cells, bone marrow cells, and RBC, as reported by Sugimura et al. (18). Guinea pig serum was absorbed with agarose (Nakarai Chemicals Co., Tokyo, Japan) and spleen cells from normal C57BL/6N mice and were used as a complement. Thirty million cells suspended in 0.3 ml of MEM and 0.3 ml of a 1:48 dilution of anti-ß antiserum were mixed, placed on ice for 15 min, and incubated at 37ºC for 30 min. The mixture was then added with 0.3 ml of a 1:3 dilution of complement and further incubated at 37ºC for 45 min with occasional shaking. After being washed 3 times with MEM, cells were resuspended in an appropriate medium. This treatment was cytotoxic to T-cells but not to B-cells, as indicated by the elimination of the response to concanavalin A and phytohemagglutinin without a concomitant reduction in lipopolysaccharide response (18).

**Histopathology.** Tumor and lymph nodes were surgically excised, fixed in 10% formalin, and routinely stained with hematoxylin and eosin.

**Experimental Procedure.** A preliminary experiment was undertaken to assess the change of T-cell-mediated cytotoxicity that generated in C57BL/6N mice bearing MC104 fibrosarcoma during the course of tumor growth. Mice were inoculated s.c. with $10^6$ MC104 tumor cells into the dorsum of the right hind foot. One or 2 weeks later, spleen and DPLN cells were obtained from these mice and applied to the Winn test and in vitro generation of cytotoxic cells. Age-matched normal mice were used as control.

To investigate the antitumor effect of the i.l. injection of N. rubra CWS of N. rubra CWS, prepared by the method of Azuma et al. (1) was treated with squalene (Katayama Chemicals Co., Tokyo, Japan) in an oil-attached form as reported previously (23) and suspended in 0.85% NaCl solution at a concentration of 5 mg/ml just before use.

**Experimental Procedure.** A preliminary experiment was undertaken to assess the change of T-cell-mediated cytotoxicity that generated in C57BL/6N mice bearing MC104 fibrosarcoma during the course of tumor growth. Mice were inoculated s.c. with $10^6$ MC104 tumor cells into the dorsum of the right hind foot. One or 2 weeks later, spleen and DPLN cells were obtained from these mice and applied to the Winn test and in vitro generation of cytotoxic cells. Age-matched normal mice were used as control.

To investigate the antitumor effect of the i.l. injection of N.
rubra CWS, the following experiments were carried out. Mice inoculated with MC104 tumor cells were divided into 3 groups. Mice in Group 1 were not treated after tumor inoculation (control group). Mice in Group 3 were twice given injections of 50 μg of N. rubra CWS into the growing tumor 2 and 7 days after tumor inoculation (N. rubra CWS group), and mice in Group 2 were given 50 μg of squalene in the same manner as mice in N. rubra CWS group (squalene group). Survival period, antitumor cytotoxicity of spleen and DPLN cells, and trapping of 51Cr-labeled DPLN cells in tumor, spleen, or lymph nodes in mice in each experimental group were examined. In the experiment for lymphocyte trapping, mice in each group were further divided into 2 subgroups, respectively, 2 weeks after tumor inoculation. DPLN cells were obtained from mice in one subgroup, labeled with 51Cr, and injected into the tail veins of mice in the other subgroup.

**Statistical Analysis.** The mean difference between experimental and control groups was evaluated statistically by Student’s t test. The χ² test was applied to evaluate the results of the Winn test. The survival rate of mice undergoing immunotherapy for solid tumor was examined by the Mann-Whitney U test.

**RESULTS**

**Cytotoxicity of Spleen and DPLN Cells from Mice Bearing MC104 Fibrosarcoma.** Spleen and DPLN cells from mice bearing MC104 tumor in the dorsum of the hind foot were tested for their cytotoxicity against MC104 tumor cells by a Winn-type cytotoxicity test. As shown in Table 1, complete inhibition of tumor growth was observed when tumor cells had been mixed with DPLN cells from 7-day tumor-bearing mice. Tumors developed in 4 of 10 mice inoculated with tumor cells mixed together with spleen cells from the same mice. However, tumors developed in all the mice when spleen and DPLN cells from 14-day tumor-bearing mice were used as effector cells. Tumor weights of mice that had received spleen cells from 14-day tumor-bearing mice were significantly greater than those from mice that received normal spleen cells.

To clarify the cytotoxic activity of DPLN cells from 7-day tumor-bearing mice shown in the Winn test, the DPLN cells were applied to an in vitro cytotoxicity test. When the DPLN cells were directly applied to 51Cr-release assay, no apparent cytolytic activity against MC104 tumor cells was detected at an effector:target cell ratio ranging from 100:1 to 200:1 for 8 hr, suggesting that the effector cells were not mature cytotoxic cells. Therefore, these cells were activated in vitro with mitomycin C-treated tumor cells before 51Cr release assay. The highest cytolytic activity was generated when the DPLN cells from 7-day tumor-bearing mice were cultured at a responder:stimulator cell ratio of 25:1 for 5 days; normal spleen or lymph node cells similarly exposed to the stimulator cells could not develop any cytotoxicity against MC104 tumor cells during this time course (data not shown). Chart 1 summarizes the cytolytic activities of spleen and DPLN cells from 7-day or 14-day tumor-bearing mice when these cells were activated in vitro under this condition. DPLN cells from 7-day tumor-bearing mice showed significant cytolytic activity against MC104 tumor cells. Spleen cells from these mice also exhibited apparent cytolytic activity, whereas it was lower than that of DPLN cells. The cytolytic activities of DPLN and spleen cells were significantly depressed in 14-day tumor-bearing mice. Particularly, spleen cells from 14-day tumor-bearing mice showed the considerably negative percentage of cytolysis, showing that the spleen cells from these mice became unresponsive rather than cytotoxic when cultured with mitomycin C-treated tumor cells in vitro.

Specificity and nature of effector cells of the cytotoxicity generated in vitro were analyzed. As shown in Table 2, DPLN cells from 7-day tumor-bearing mice, which became highly cytotoxic to MC104 tumor cells by in vitro activation with mitomycin C-treated MC104 tumor cells, did not show any cytotoxicity against unrelated EL4 tumor cells. The cytotoxicity against MC104 tumor cells was completely abolished by the treatment of the effector cells with anti-θ antisera plus complement before the 51Cr release assay.

**Effect of i.l. Injection of N. rubra CWS on Survival of Tumor-bearing Mice.** Chart 2 shows the survival rate of mice in control, squalene, and N. rubra CWS groups. Two i.l. injections of N. rubra CWS significantly improved the survival rate, whereas treatment with squalene had no effect on survival. Five of 17 mice treated with N. rubra CWS survived longer than 2 months, and 3 of these 5 survivors exhibited complete cure.

**Cytotoxicity of Spleen and DPLN Cells from Tumor-bearing Mice Treated with N. rubra CWS.** To investigate the effect of i.l. injection of N. rubra CWS on the antitumor cytotoxicity of spleen and DPLN cells, these lymphoid cells obtained from

| Table 1 | Winn test with spleen and draining lymph node cells from C57BL/6J mice bearing MC104 fibrosarcoma |
|------------------|---------------------------------|--------------------|----------------|
| **Effector cells obtained from** | **Tumor incidence (tumor take/total mice)** | **p** (χ² test) | **Tumor wt (g)** |
| **from** | | | **(Student’s t test)** |
| Medium alone | 15/15 | | 1.19 ± 0.18* |
| Normal mice | | | |
| Spleen cells | 9/9 | | 1.27 ± 0.24 |
| LN cells | 9/9 | | 1.08 ± 0.34 |
| 7-day tumor-bearing mice | | | |
| Spleen cells | 4/10 | <0.05 | 2.93 ± 0.69 |
| DPLN cells | 0/10 | <0.001 | <0.05 |
| 14-day tumor-bearing mice | | | |
| Spleen cells | 9/9 | | 1.74 ± 0.44 |
| DPLN cells | 9/9 | | NS* |

* Statistical analysis for tumor incidence between the experiment using spleen or LN cells from normal mice and that using spleen cells or DPLN cells from 7-day tumor-bearing mice, respectively.
* Statistical analysis for tumor weight between the experiment using spleen or LN cells from normal mice and that using spleen or DPLN cells from 14-day tumor-bearing mice, respectively.
* Mean ± S.E.
* LN, lymph node cells.
* NS, not significant.
and draining lymph nodes was the same as that seen in the control group. On the contrary, in the N. rubra CWS group, a significant increase in trapping of the DPLN cells in tumor, spleen, and DPLN was observed, compared with that in control or squalene groups. However, the trapping in the draining inguinal and paraaortic lymph nodes was not augmented by the i.l. injection of N. rubra CWS. The augmentation of the trapping of the DPLN cells in tumor, spleen, and DPLN resulted from the i.l. injection of N. rubra CWS and was completely abolished by the treatment of the DPLN cells with anti-θ serum plus complement before labeling.

**Histology.** Tumor and DPLN from mice in the N. rubra CWS group were examined histologically 2 weeks after tumor inoculation. Apparent mononuclear infiltration was observed around and in the tumor tissue (Fig. 1A). DPLN of these mice showed approximately a 5-fold increase in weight, compared with those of mice in the control or squalene group. A marked proliferation of cells was seen in the paracortical area of those lymph nodes (Fig. 1B). No apparent metastasis of tumor was detected in the DPLN of mice in control, squalene, and N. rubra CWS groups.

### DISCUSSION

The results of the Winn test showed that cell-mediated cytotoxicity against MC104 tumor cells was induced in spleen and DPLN cells of C57BL/6N mice bearing syngeneic MC104 fibrosarcoma at Day 7 of tumor growth and became undetectable by Day 14. Similar results were obtained in 51Cr release assay following in vitro activation of these lymphoid cells with mitomycin C-treated MC104 tumor cells. The antitumor cytotoxicity was shown to be specific to MC104 tumor cells and mediated by T-cells. Although the cytotoxicity was easily detected in the Winn test, the effector cells were thought to be immature cytotoxic T-cells (prekiller T-cells), because DPLN cells from 7-day tumor-bearing mice did not exhibit any cytotoxicity in 51Cr release assay before these cells were activated in vitro with stimulator cells. Barna et al. (4) have reported that tumor-specific cytotoxicity of draining lymph node cells becomes undetectable earlier than that of spleen cells during tumor growth in A/J mice bearing syngeneic Sarcoma 1 tumor. In the tumor system used in this study, however, the cytotoxicity of draining lymph node cells remained greater than that of spleen cells during tumor growth. These different results seem
to be based on the difference in immunological nature between both experimental tumor systems. In this study, spleen cells from 14-day tumor-bearing mice exhibited not only an enhanc-
ing effect on tumor growth in the Winn test but also a suppress-
ing effect on in vitro generation of nonspecific cytolytic activity of responder cells. These results suggest strongly that sup-
pressor cells increase in the spleen of these mice, although it remains to be determined which cells are responsible for this suppressor activity by using the methods described elsewhere (8, 13, 19).

Two injections of 50 μg of N. rubra CWS into the growing tumor resulted in significant inhibition of tumor growth and proliferation of the survival period of tumor-bearing mice. DPLN cells from 14-day tumor-bearing mice that were given this treatment exhibited significant recovery in the antitumor cytotoxicity. Squalene injection did not exhibit any recovery effect on the depressed cytotoxicity of DPLN cells in spite of its inhibition of tumor growth, suggesting that the augmentation of the cytotoxicity of DPLN cells with N. rubra CWS at the advanced stage is based not only on the inhibition of tumor growth but also on the immunopotentiation with N. rubra CWS. The cytotoxicity augmented with N. rubra CWS was also specific to MC104 tumor cells and was shown to be mediated by T-cells when tested by the in vitro cytotoxicity test, although this finding remains to be confirmed by the Winn test. Administration of N. rubra CWS into the hind feet of normal mice failed to result in the apparent augmentation of the T-cell-mediated cytotoxicity of spleen or DPLN cells against MC104 tumor cells in both direct Winn test and 51Cr release assay after in vitro activation with mitomycin C-treated tumor cells (data not shown). It seems possible that the T-cell-mediated cytotoxicity which had been induced in mice during tumor growth can be specifically augmented by i.i. immunotherapy with N. rubra CWS.

A trapping experiment with 51Cr-labeled DPLN cells revealed that the DPLN cells of which the cytotoxic activity had been augmented by N. rubra CWS could localize not only in DPLN but also in the spleen and tumor with a significant degree in the N. rubra CWS group, compared with those observed in the control or squalene groups. This enhancing effect of N. rubra CWS on the trapping of the DPLN cells in those organs was completely abolished by the treatment of the DPLN cells with anti-θ antiserum plus complement. Histologically, hyperplasia of the paracortical area (T-cell-dependent area) in the DPLN and infiltration of mononuclear cells into the tumor tissue were observed in these mice. These results suggest strongly that prekiller T-cells increase systemically in mice receiving i.i. immunotherapy with N. rubra CWS, and some of these cells can be mobilized in a tumor that has been injected with this adjuvant. Thus, it might be possible that, in addition to macrophage-mediated cytotoxicity, T-cell-mediated cytotoxicity plays an important role in the systemic development of antitu-

Table 3

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Specific cytosis (%)</th>
<th>Tumor diameter (mm)</th>
<th>No. of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Squalene</td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>N. rubra CWS</td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>p (Student's t test)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control vs. squalene</td>
<td>&lt;0.02</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Squalene vs. N. rubra CWS</td>
<td>&lt;0.001</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Control vs. N. rubra CWS</td>
<td>&lt;0.001</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

a Treatment for mice in each experimental groups was described in "Materials and Methods."

b Mean ± S.E.

c NS, not significant.

Table 4

<table>
<thead>
<tr>
<th>Treatment of effector cells</th>
<th>Target cells</th>
<th>Specific cytosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>MC104</td>
<td>32.6</td>
</tr>
<tr>
<td>None</td>
<td>EL4</td>
<td>3.7</td>
</tr>
<tr>
<td>Anti-θ antiserum</td>
<td>MC104</td>
<td>32.9</td>
</tr>
<tr>
<td>Complement</td>
<td>MC104</td>
<td>36.9</td>
</tr>
<tr>
<td>Anti-θ antiserum plus</td>
<td>MC104</td>
<td>-0.9</td>
</tr>
</tbody>
</table>

a C57BL/6N mice were given s.c. inoculations of 1 × 106 MC104 tumor cells into the dorsum of the hind foot on Day 0 and 2 intratumor injections of 50 μg of N. rubra CWS on Days 2 and 7. On Day 14, draining popliteal lymph node cells were activated in vitro with mitomycin C-treated MC104 tumor cells for 5 days. The responder cells were collected and treated with anti-θ antiserum and/or complement.
the immunotherapy with N. rubra CWS or other immunoadju-
vants for malignant diseases more effective.

The results presented in this paper also indicate that draining
lymph nodes constitute an important role in the development
of the antitumor effect of the i.l. immunotherapy with N. rubra
CWS. Fisher et al. (7) have described that the i.l. injection of
heat-killed Corynebacterium parvum results in the significant
augmentation of tumor-specific cytotoxicity of draining lymph
node cells in mice. Recently, Ogura et al. (16) have reported
the significant augmentation of lymphocyte trapping in draining
lymph nodes in rats that had received i.l. immunotherapy with
BCG-CWS. The results shown in this study are consistent with
those observations and suggest that cytotoxic lymphocytes
increased in draining lymph nodes might contribute to the
inhibition of metastases of tumor cells to these lymph nodes,
while Hanna et al. (10) have described that the inhibition or
rejection of tumor metastases to draining lymph nodes in
guinea pigs receiving the i.l. immunotherapy with living BCG
is mainly mediated by histiocytes accumulated in the granuloma-
tous reaction induced in the draining lymph nodes by BCG.

Further investigation of these possibilities is now under way.

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