POTENTIATION OF TUMORICIDAL PROPERTIES OF MURINE MACROPHAGES BY *NOCARDIA RUBRA* CELL WALL SKELETON (N-CWS)

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Intraperitoneal injection of squalene-treated cell wall skeleton of *Nocardia rubra* (N-CWS) caused increase in number of peritoneal exudate cells (PEC). Adherent macrophages obtained from N-CWS-treated PEC suppressed growth of methylcholanthrene-induced fibrosarcoma (Meth-A), when injected intradermally with the tumor cells into BALB/c mice. The macrophages showed strong cytotoxicity against Meth-A cells *in vitro*.

When treated with 10 μg/ml of N-CWS *in vitro*, proteose peptone-induced macrophages acquired tumoricidal property but resident macrophages showed no cytotoxicity after the treatment. In the supernatant of spleen cells cultured for 72 hours in the presence of N-CWS (10 μg/ml), the presence of (a) factor(s) with macrophage activating effect was observed. This factor, shown to be identical to macrophage activating factor (MAF) in molecular weight, showed synergy with N-CWS in potentiating macrophage cytotoxicity against tumor cells.

Cells, cell walls or cell wall skeletons of several microbial species, such as *Mycobacterium*[1~5] or *Corynebacterium*[6~9], have been reported to have the immunopotentiating activities in experimental animals and man. Recently, cell wall skeleton of *Nocardia rubra* (N-CWS) has been shown to enhance host resistance against tumor cell transplantation[10~13]. The suppressive effect of N-CWS was investigated in mice intradermally or subcutaneously implanted with a mixture of N-CWS and a variety of syngeneic tumor cells[13,14]. The injection of N-CWS in tumor site results in inhibition of tumor growth and sometimes in regression of the established tumors[15], and showed prolongation of survival time in mice bearing syngeneic tumor[16,17].

The exact mechanisms of the antitumor effect of N-CWS are not yet fully elucidated, but are thought to be augmentation of general immune responses, induction of specific cell-mediated cytotoxicity mediated by T lymphocytes[11,15] and/or macrophages[18~20]. Intraperitoneal or intravenous injection of N-CWS activates the cytotoxicity of peritoneal[1,18,20], or alveolar macrophages[19]. Alveolar macrophages of rat were rendered tumoricidal after direct interaction with N-CWS *in vitro*[21].

In this paper, we report that tumoricidal activity of peritoneal macrophages can be induced not only by direct contact with N-CWS but indirectly by a factor produced by spleen cells under the influence of N-CWS. Cultivation of splenic lymphocytes with N-CWS yielded a factor which, when added to the macrophage culture, activates macrophage tumoricidal.

Materials and Methods

Drug

N-CWS was used for experiments as a lyophilized preparation (Fujisawa Pharmaceutical Co., Ltd., Lot 106712 K). It contained 2 mg N-CWS, 4 mg squalene, 1 mg polysorbate 80 and 28.2 mg mannitol. A placebo was used as a control. The placebo preparation (Lot 112913 K) was comprised

of the same formulation except N-CWS. Prior to use, both preparations were reconstituted and suspended in physiological saline at the same ratio. Then, N-CWS was compared with placebo with the equivalent volume of the suspensions. To simplify the explanations, placebo amounts were expressed as equivalent doses of N-CWS, for instance, "100" μg placebo corresponding to 100 μg N-CWS. These preparations did not contain endotoxins as detected by the Limulus amebocyte lysate assay.

Animals

Specific-pathogen-free BALB/c female mice, 8-week old were obtained from Shizuoka Agriculture Cooperative Association for Laboratory Animals.

Cell Line

Meth-A is 3-methylcholanthrene-induced fibrosarcoma and passed serially in BALB/c mice as ascites. The cells were harvested from the ascites 6 or 7 days after the inoculation of the host animals, suspended and washed in HANKS' balanced salts solution (HBSS) by low speed centrifugation, and finally suspended in RPMI 1640 medium with 10% fetal calf serum (FCS). When used in Winn test, cells were suspended in phosphate buffered saline without Ca^{2+} (PBS (−)).

Peritoneal Exudate Cells

Peritoneal exudate cells (PEC) were harvested by lavage with HBSS containing 10 U/ml of heparin, washed by low speed centrifugation and suspended in EAGLE's minimum essential medium (E-MEM) without FCS. For Winn test, PEC were suspended in PBS (−).

Winn Test

Expt 1. PEC were harvested after intraperitoneal injection of 100 μg of N-CWS at day-4, -8 and -12, pooled, washed and suspended in PBS (−) at a cell density of 4×10^7/ml. Meth-A cells (8×10^5/ml) suspended in PBS (−) were mixed with the same volume of PEC suspension and 50 μl of the mixture was inoculated intradermally into the flank of normal mice. Two weeks later, tumor taken was checked grossly.

Expt 2. PEC (2×10^7) were harvested from mice 4 days after intraperitoneal injection of 100 μg N-CWS, pooled, washed with PBS (−) and suspended in 10 ml of E-MEM with 10% FCS, and cultured in plastic dishes at 37°C in 5% CO_2 and moist air. After 2-hour incubation, adherent cells, scraped with a rubber policeman, were suspended in PBS (−) at a cell density of 4×10^7 viable cells/ml and mixed with the same volume of tumor cells (8×10^5 Meth-A cells/ml). The mixture was injected intradermally and tumor taken was checked 14 days after the inoculation.

Induction of Cytotoxic Macrophage *In Vivo*

PEC was harvested by lavage with HBSS with heparin (10 U/ml) after injection of 100 μg N-CWS 4 and 6 days before the experiment. Cells were washed, suspended at an appropriate cell density and 200 μl of the cell suspension was plated into each well of a microtitration plate (Nunc, Denmark). Non-adherent cells were removed by washing with E-MEM 3 hours after initial plating. This procedure provided uniform monolayer of macrophages (>95%) identified with Giemsa staining and phagocytosis of latex beads. Macrophages were suspended in RPMI 1640 with 10% FCS and 5×10^{-5} M 2-mercapto-ethanol (2-ME) and used for cytotoxicity assay.

Preparation of Lymphokines with Macrophage Activating Effect

Spleens were aseptically removed from normal BALB/c mice and minced by gently teasing each spleen between frosted ends of microscope slides in RPMI 1640 with 10% FCS. Cellular debris was removed by passing through nylon mesh and red cells were removed by treatment with red cell lysing buffer comprised 8.3 mg/ml $NHCl_4$, 1 mg/ml $KHCO_3$ and 3.7 mg/ml EDTA·3Na, for 3 minutes at 4°C. The collected cells were washed and suspended in RPMI 1640 with 10% FCS and 5×10^{-5} M 2-ME at a cell density of 5×10^6/ml. Solution of N-CWS in physiological saline (10 μg/ml) was added to the cell suspension (1 ml) and the mixture was plated into microtitration plates. After 72-hour incubation at 37°C, N-CWS was filtered off with a Millipore filter and the resultant supernatant was used for macrophage activation.

Sephadex G-75 Chromatography

Sephadex G-75 chromatography was performed essentially as described by NANCY *et al.*[22]. Columns

of freshly prepared Sephadex were usually made for each run. The gel slurry (328 ml) was poured into a 26-mm diameter-glass column (Pharmacia, C 26/70). Height of the gel bed after setting was approximately 62 cm. The eluting fluid was 0.15 M NaCl containing 50 μg/ml of cytochrome *C*. In total 500 ml of eluting fluid was run through the column before sample application. Forty ml of spleen cell culture supernatant was lyophilized, reconstituted with 4 ml of RPMI 1640, and passed through the Millipore filter in order to remove undissolved residue. An aliquot was retained for bioassay. Elution rate was approximately 25 ml/hour, and fraction volume was 3.3 ml. Fractions were stored at -80°C until assay.

Induction of Cytotoxic Macrophages *In Vitro*

One ml of 10% Proteose peptone solution was injected intraperitoneally into BALB/c mice. Four days after the injection, PEC were harvested in E-MEM and plated in microtitration plates at a density of 2×10^5/well for 3 hours. PEC were washed to remove nonadherent cells with E-MEM and 100 μl of RPMI 1640 with 10% FCS was added. At this time, greater than 95% were macrophages as identified with Giemsa staining and phagocytosis of latex beads. The adherent cells were incubated for 24 hours in the presence of supernatant of splenocyte culture and/or N-CWS solution, washed with RPMI 1640 and added with 100 μl of RPMI 1640 with 10% FCS and 5×10^{-5} M 2-ME.

Macrophage-mediated Cytotoxicity

Target cells (3×10^6/3 ml RPMI 1640) in the exponential growth phase were labeled with 2 μCi [^{3}H]-uridine (specific activity, 23 Ci/mmol), washed with RPMI 1640 and resuspended in RPMI 1640 with 10% FCS and 5×10^{-5} M 2-ME at a cell density of 1×10^5/ml. Radiolabeled target cells (100 μliter) were plated into each well containing activated macrophages and incubated at 37°C for 24 hours. At the end of incubation period, target cells were harvested on the glass filter paper with Titerteck Cell Harvester (Flow Laboratories, U.S.A.), dried and residual radioactivity was counted by a liquid scintillation counter.

Cytotoxicity (%) was calculated as follows:

$$\text{Cytotoxicity (\%)}=\left(1-\frac{\text{cpm in target cells cultured with activated macrophages}}{\text{cpm in target cells cultured with normal macrophages}}\right)\times100$$

Results

Winn Type Transfer Assay

In the first experiment, the Winn type transfer assay was carried out to identify the effector cells to be potentiated by N-CWS administration. Meth-A cells (2×10^4 cells) suspended in 25 μl PBS($-$) were mixed with 25 μl of 1×10^6 PEC and inoculated intradermally into the flank of BALB/c mice. Two weeks later, tumor taken was checked grossly. As shown in Table 1, complete growth inhibition of tumor cells inoculated with N-CWS-activated PEC was observed in 9 of 10 mice. However, tumor developped in 8 of 10 mice when inoculated with PEC from placebo-treated mice. Complete inhibition of tumor growth was attained also by adherent cells from N-CWS-treated mice. It seems, therefore, that macrophage activated with N-CWS were the main effector cells in the suppression of the tumor growth *in vivo*.

In Vivo Activation of Murine Peritoneal Macrophages by N-CWS

BALB/c mice were injected intraperitoneally with 100 μg of N-CWS or 100 μg of placebo at 4 and 6 days before the experiment. The lavaged cells from peritoneal cavity were plated for 3 hours and washed throughly to yield a monolayer of macrophages and their tumoricidal capacity was measured against [^{3}H]uridine-labeled Meth-A cells (1×10^4 cells) in a 24-hour cytotoxicity assay. The significant cytolytic activity was shown in the N-CWS-treated group, 49.6% and 54.7% in 7.5 and 15.0 ratio of effector to target, respectively. In contrast, intraperitoneal injection of placebo did not render macrophages tumoricidal (Table 2).

Table 1. Winn type transfer assay with PEC and macrophage activated by N-CWS.

Expt No.	Treatment	Tumor cell inoculation[a]	Tumor taken / Total mice[c]
I	None	Alone	8 / 10
	None	With PEC[b]	8 / 9
	100 μg N-CWS 3 injections	With PEC	1 / 10
	100 μg Placebo 3 injections	With PEC	8 / 10
II	None	Alone	5 / 5
	100 μg N-CWS 1 injection	With adherent cell	0 / 5
	100 μg Placebo 1 injection	With adherent cell	4 / 5

a) Tests were performed with mixed inoculation of 2×10^4 tumor cells with PEC or adherent cells at an effector: target cell ratio of 50: 1.
b) Peritoneal exudate cells.
c) Examined 14 days after inoculation of tumor cells.

Table 2. Cytolytic effect of adherent PEC stimulated by N-CWS.

Treatment	PEC yield ($\times10^{-6}$)	Cytolytic activity (%)[a] (mean±S.E.)[c]	
		E/T 7.5	15
N-CWS 100 μg 2 injections[b]	6.6±1.0[d]	49.6±11.6[d]	54.7±5.7[d]
Placebo 100 μg 2 injections	2.4±0.4	8.8±8.9	10.5±12.6

a) Cytolytic activity was calculated as follows:

$$\text{Cytolytic activity (\%)} = \left(1 - \frac{\text{cpm of Meth-A incubated with adherent PEC treated with drugs}}{\text{cpm of Meth-A incubated with adherent PEC treated with saline}}\right) \times 100$$

The cpm of Meth-A alone was 6,426, and cpm of Meth-A incubated with adherent PEC from saline treatment was 5,243 at E/T 7.5, and 5,007 at 15.
b) BALB/c mice were injected intraperitoneally with 100 μg of N-CWS or 100 μg of placebo at −4 and −6 days.
c) Mean of five mice per group±S.E.
d) Significant difference from control (placebo) at $P<0.05$ (Student's t test).

In Vitro Activation of Peritoneal Macrophages by N-CWS

Murine peritoneal exudate macrophages were treated *in vitro* with graded concentration of N-CWS or placebo and examined for their cytolytic activity against Meth-A cells. As shown in Table 3, N-CWS at a concentration of 10 μg/ml showed significant enhancement of cytotoxicity of peritoneal exudate macrophages. However, when the resident macrophages were used as effector cells, no significant cytolytic activity was seen at any concentration of N-CWS.

To exclude the effect of contaminating T-lymphocytes and ascertain the reproducibility, nu/nu mice were employed. Macrophage induced from BALB/c nu/nu mice could also be activated to become tumoricidal at concentration of 20 and 50 μg/ml of N-CWS. In contrast to the cytotoxicity with normal mice, at 10 μg/ml, only a trend to an augmented cytolytic activity over placebo was shown (Table 4).

Thus it was shown that peritoneal exudate macrophages could be rendered tumoricidal following their direct interaction with N-CWS in the culture medium. But, we could not exclude that the activa-

Table 3. Effect of N-CWS on cytotoxicity of resident and exudate macrophages[a].

Drug	Concentration (μg/ml)	Cytolytic activity (%)[b] (mean±S.E.)[e]	
		PEC[c]	PRC[c]
N-CWS	0.1	4.6±3.2	−3.2±3.3
	1.0	−3.1±4.3	−1.6±2.4
	10.0	22.8±4.2[d]	8.5±2.7
Placebo	0.1	2.5±2.9	3.0±5.7
	1.0	−1.3±1.8	−2.8±3.8
	10.0	−1.2±3.0	2.0±1.8

[a] Adherent macrophages (2×10^5/well) from PEC or PRC were incubated with N-CWS or placebo for 24 hours. [^{3}H]Uridine-labeled Meth-A cells (1×10^4/well, E/T=20) were added onto the macrophage monolayers. After incubation for 24 hours, the radioactivity of viable Meth-A cells was counted by a liquid scintillation counter.

[b] Cytolytic activity was calculated as follows:

$$\text{Cytolytic activity (\%)} = \left(1 - \frac{\text{cpm of Meth-A incubated with macrophage treated with N-CWS or placebo}}{\text{cpm of Meth-A incubated with macrophage treated with the medium}}\right)\times 100$$

[c] Five days after intraperitoneal injection of Proteose peptone, peritoneal exudate cells (PEC) were harvested from BALB/c mice. Peritoneal resident cells (PRC) were collected from non-treated BALB/c mice.

[d] Significant difference from control (placebo) at $P<0.05$ (Student's t test).

[e] Mean±S.E. of triplicate cultures.

Table 4. Effect of N-CWS on cytotoxicity of adherent peritoneal exudate cells of BALB/c nu/nu mice.

Drug	Conc (μg/ml)	Cytolysis (%)[a] mean±S.E.[b]
N-CWS	10	15.8±4.2
	20	22.0±4.8[c]
	50	18.5±2.7[c]
Placebo	10	9.0±0.9
	20	5.5±5.8
	50	11.6±1.6

[a] See Table 3.

[b] Mean±S.E. of triplicate cultures.

[c] Significantly different from control (placebo) at $P<0.10$ (Student's t test).

tion of macrophages might be associated with the indirect mechanism such as the release of lymphokines from lymphocytes.

Production of Macrophage Activating Factor by Spleen Cells

Spleen cells were incubated with 10 μg/ml of N-CWS for 72 hours and the cell-free supernatant, ranging from 2.5 to 20% (v/v) was added onto macrophage monolayers. As shown in Table 5, significant enhancement of cytolytic activity was observed when macrophages were treated with spleen cell supernatant prepared with N-CWS; 60.7% and 82.7% of cytolysis at 5% and 20% (v/v) of added supernatant were shown. However, at 2.5% (v/v) spleen cell supernatant showed no enhancement. The results of the Limulus amebocyte lysate assays verified that macrophage activation by the culture supernatant of N-CWS-treated splenocytes was not caused by contamination with LPS.

When 10 μg/ml of N-CWS was added to the macrophage culture in combination with culture supernatant of N-CWS-treated splenocyte, synergistic enhancement effect was observed (Fig. 1). Even low concentration of the supernatant (2.5% v/v) was sufficient to achieve full activation of macrophage cytotoxicity in the presence of 10 μg/ml of N-CWS.

Sephadex G-75 Chromatography of Macrophage Activating Factor

To characterize the macrophage activating factor produced by the N-CWS-treated spleen cells, we

Table 5. Effect of supernatant prepared by incubating splenocytes with N-CWS on macrophage-mediated cytotoxicity[a].

Drug	Conc (μg/ml)	Cytotoxicity (%)[b] of macrophage incubated with splenocyte supernatant at a concentration (%) of			
		20	10	5	2.5
N-CWS	10	82.7±1.1[c]	80.3±2.3[c]	60.7±0.8[c]	4.1±2.2
Placebo	10	−1.0±1.2	−3.2±8.3	−5.5±8.4	−9.3±3.8

a) BALB/c splenocytes (5×10^6 cells/ml) were incubated with N-CWS (10 μg/ml) or placebo (10 μg/ml) for 72 hours and supernatants were collected through Millipore filters. Adherent macrophages (2.5×10^5/well) were incubated in medium containing graded conc of supernatant for 24 hours. After through washing, [^{3}H]uridine-labeled Meth-A cells (1.25×10^4 cells/well) were added onto the macrophage monolayer. After 24 hours of incubation, label retained in viable Meth-A cells was counted by a liquid scintillation counter.

b) Cytotoxicity (%) was calculated as follows (Mϕ=macrophage):

$$\text{Cytolytic activity}=\left(1-\frac{\text{cpm of Meth-A incubated with M}\phi\text{ stimulated with splenocyte sup}}{\text{cpm of Meth-A incubated with M}\phi\text{ treated with medium}}\right)\times100$$

c) Significantly different from control (placebo) at $P<0.01$. (Student's t test).

Fig. 1. Synergistic effect of N-CWS-treated splenocyte supernatant with N-CWS on macrophage-mediated cytotoxicity.

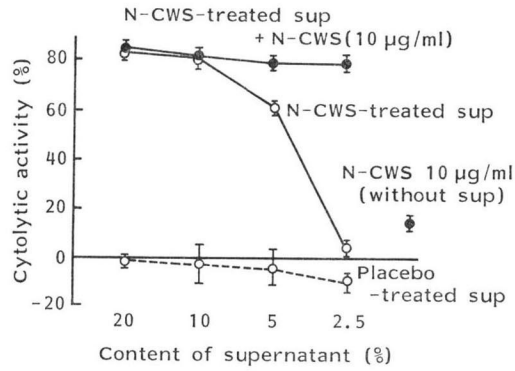
Splenocytes obtained from BALB/c mice (5×10^6 cells/ml) were incubated with 10 μg/ml of N-CWS or 10 μg/ml of placebo for 72 hours and supernatants were collected through Millipore filters. Adherent macrophages prepared from BALB/c mice (2.5×10^5 cells/well) were incubated in medium containing graded concentration of the supernatant for 24 hours. After through washing, [^{3}H]uridine-labeled Meth-A cells (1.25×10^4 cells/well, E/T=20) were added onto the macrophage monolayer. After 24 hours of incubation, label retained in viable Meth-A cells was counted by a liquid scintillation counter.

Cytotoxicity was calculated as follows:

$$\text{Cytotoxicity (\%)}=\left(1-\frac{\text{cpm of Meth-A incubated with macrophage stimulated with splenocyte}}{\text{cpm of Meth-A incubated with macrophage treated with medium}}\right)\times100$$

○—○, cytotoxicity of macrophage stimulated with N-CWS-treated splenocyte sup; ●—●, cytotoxicity of macrophage stimulated with N-CWS-treated splenocyte sup together with 10 μg/ml of N-CWS; ○---○, cytotoxicity of macrophage stimulated with placebo-treated splenocyte sup.

Mean±S.E. of triplicate cultures was plotted.



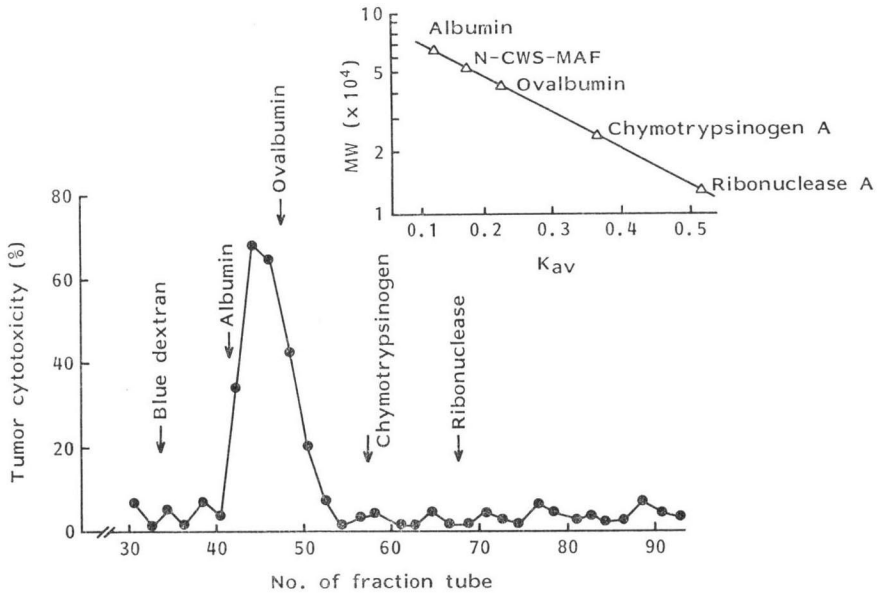
examined its molecular weight in Sephadex column chromatography according to the procedure mentioned in the Materials and Methods.

Macrophage activating activity of the supernatant was obtained at a position corresponding to a molecular weight of 55,000 daltons, which was confirmed in two independent experiments (Fig. 2).

Fig. 2. Gel filtration on Sephadex G-75 of MAF prepared by incubating spleen cells with N-CWS.

The apparent molecular weight of N-CWS-MAF was determined on Sephadex G-75. The following proteins were utilized as standards: Albumin (MW 67,000); ovalbumin (MW 43,000); chymotrypsinogen A (MW 25,000); and ribonuclease A (MW 13,700) (Pharmacia Fine Chemicals). Blue dextran (Pharmacia Fine Chemicals) was used for the determination of the column void volume.

Arrows indicate protein markers used to calibrate column.



The MAF prepared by Con A stimulated splenocytes was also eluted in a fraction of the same molecular weight (data not shown). Thus the macrophage activating factor produced by the N-CWS-treated splenocytes seems similar to so-called MAF produced by lectin-stimulated splenocytes.

Discussion

In the present study, we examined *in vitro* activation of tumor cell cytotoxicity of macrophages to undertake and evaluation of the immunotherapeutic efficacy of N-CWS.

After intraperitoneal injection of N-CWS in BALB/c mice, PEC yield increased and the macrophages showed significant tumoricidal activity. This finding is in agreement with the experimental results of other investigators[18~20], who have shown that the *in situ* administration of N-CWS can activate macrophages for tumor cell killing. When these macrophages were mixed with Meth-A cells and inoculated subcutaneously into normal mice, complete growth suppression of tumor was observed in majority of mice. A similar therapeutic effect has been reported by OGURA *et al.*[20], using the Winn type transfer test in rats. YAMASHITA *et al.*[28] has shown that intraperitoneal injection of macrophages activated with N-CWS produced significant eradication of Lewis lung carcinoma metastasis. We interpret these results to indicate that the main therapeutic efficacy of N-CWS treatment is a potentiation of macrophage tumoricidal activity.

In this report, we also intended to determine how N-CWS augmented the tumoricidal activity of macrophages. At first, as shown in Tables 3 and 4, we showed murine macrophages could respond to direct stimulation by N-CWS to become tumoricidal *in vitro*. These results are consistent with the data of SONE *et al.*[21], which show that alveolar macrophage lavage from lung of F344 rats are rendered tumoricidal following their direct interaction with N-CWS. However, such effectiveness was recognized only in peritoneal exudate macrophages and not in resident macrophages.

Macrophages were rendered tumor cell cytotoxic by MAF prepared by incubation of spleen cells

with Con A[24]. In our hand, we found that the supernatant prepared by incubating spleen cells with N-CWS rendered macrophage tumoricidal, and the minimal volume of the supernatant necessary to activate macrophage *in vitro* is 5% (v/v) (Table 5). MELTZER[24] has reported that high concentration of lymphokine, which is the supernatant from antigen- or lectin-stimulated spleen cell cultures, potentiated tumoricidal activity of exudate macrophages, but that low concentration of lymphokine to potentiate tumoricidal activity needed additional signal; MAF achieving priming (first signal) and LPS needed as second signal (triggering). Considering this two signal hypothesis, we added N-CWS (10 μg/ml) into macrophage cultures in addition to the splenic supernatant to expect the synergistic augmentation of tumoricidal activity. As shown in Fig. 1, N-CWS and supernatant from N-CWS-treated splenic lymphocytes showed synergistic effect in activation of tumoricidal activity of macrophages.

Molecular weight of macrophage activating factor from N-CWS-treated spleen cells was examined by Sephadex G-75 column chromatography and found to be about 55,000 daltons, which is comparable to that of MAF produced by Con A-stimulated splenocytes. Recently, MAF has been partially purified[22,25~27] and the molecular weight was reported to be 45,000~60,000 daltons. It seems probable that the factor from N-CWS-treated spleen cells is similar to or identical with MAF so far reported. Interferon activity in the fractions of Sephadex G-75 chromatography was also examined. The peak of interferon activity coincided with that of macrophage activation (data not shown), which gives an additional support for the similarity of our factor with MAF, as several investigators reported the close association of MAF and interferon activities[28~30].

In conclusion, the results we report here indicate that the immunotherapeutic activity of N-CWS is achieved not only by direct activation of macrophage cytotoxicity but also by enhancement of the release of lymphokine(s) with character of MAF from spleen cells and moreover by synergistic effect of the factor and N-CWS itself.

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