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Augmentative effect of *Nocardia rubra* cell-wall skeleton (N-CWS) on lymphokine-activated killer (LAK) cell induction

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Summary. The present study elucidated that N-CWS augments the cytolytic activity against 3LL tumor cells of LAK cells from N-CWS-immunized mice administered i.p. with rIL-2. This augmentative effect of N-CWS was not seen when the LAK cells were prepared from normal mice. The cytolytic activity was predominantly expressed in the NAPC prepared from the site of injection of rIL-2, and repeated administrations of rIL-2 were required to induce and maintain this potent cytolytic activity in vivo. Serological analysis revealed that the LAK cells were positive for Thy 1.2 and asialo GM1 antigens and that they were not classical CTL or NK cells. The administration of rIL-2 statistically prolonged the MST of mice bearing LAK-sensitive 3LL cells but not the MST of mice bearing LAK-resistant EL-4 leukemia. Furthermore, combination therapy with N-CWS and rIL-2 prolonged the MST of the mice more than the therapy with rIL-2 alone. These results suggest that LAK cells potentiated with N-CWS would be useful for immunotherapy of malignant neoplasms.

Introduction

Several investigators [4, 6, 9, 18, 21, 24] have reported that LAK cells are induced by brief incubation of murine lymphocytes or human peripheral blood lymphocytes with interleukin-2. These cells are capable of lysing a wide variety of freshly isolated autologous and NK cell-resistant tumor cells in vitro.

Rosenberg and his coworkers [17] have shown that combined immunotherapy consisting of LAK cells plus relatively low doses of rIL-2, but not rIL-2 alone, reduces the number of established pulmonary metastases in a weakly immunogeneic murine sarcoma. These results showed that both LAK cells and rIL-2 are essential for adoptive immunotherapy of cancer. In fact, Rosenberg et al. [19] have shown that in vivo administration of both LAK cells and rIL-2, but not rIL-2 alone up to the maximum dose, was therapeutically effective against human neoplasms. A problem with the in vivo use of rIL-2 is that the rIL-2 administered is rapidly cleared or inactivated in the host [3, 5]. Therefore, in order to improve the antitumor efficacy of rIL-2, it is necessary to administer high doses of rIL-2 frequently, which causes several adverse effects, for example, chilliness, water retention, and dyspnea. Therefore, it is important to reduce the dose of rIL-2.

On the other hand, N-CWS has been shown not only to activate macrophages [1, 14] but also stimulate T cells to produce a variety of cytokines [7, 11, 13, 14]. Under defined conditions N-CWS has shown therapeutic efficacy in clinical trials on various cancers, including small cell lung cancer [28], gastric cancer [22], acute nonlymphatic leukemia [27], and acute myelogenous leukemia [15].

We noted the multiple immunopotentiating effects of N-CWS and the present study was designed to analyze whether N-CWS could augment the cytotoxic activities of LAK cells induced with a small amount of rIL-2.

Materials and methods

Mice. Male C57BL/6 mice were purchased from Charles River Japan, Inc., Kanagawa, Japan. They were given food and water ad libitum and used at 8–10 weeks of age.

Cell lines. Lewis lung carcinoma, 3LL cells, syngeneic to C57BL/6 mice, P815 mastocytoma cells syngeneic to DBA/2 mice, and YAC-1 lymphoma cells syngeneic to A/SR mice were passed in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% FCS, 100 units/ml penicillin (Meiji Seika Kaisha, Ltd., Tokyo, Japan), 100 μg/ml streptomycin (Meiji Seika), and 2 m*M* L-glutamine (Flow Laboratories, Grand Island, NY) (TCM). EL-4 leukemic cells syngeneic to C57BL/6 mice were maintained by i.p. passage in C57BL/6 mice.

N-CWS. The N-CWS was supplied by Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan, and used as an aseptically lyophilized preparation formulated with N-CWS (2 mg), squalene (4 mg), polysorbate 80 (1 mg), and mannitol (28.2 mg). It was reconstituted in saline prior use.

Human rIL-2. Lyophilized human rIL-2, TGP-3 (3.6×10^4 Takeda units/mg and 1.2×10^7 units/mg as calculated ac-

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Abbreviations used: N-CWS, *Nocardia rubra* cell-wall skeleton; rIL-2, recombinant interleukin 2; LAK, lymphokine-activated killer; RPMI 1640, Roswell Park Memorial Institute 1640; FCS, fetal calf serum; TCM, tumor culture medium; PC, peritoneal cells; NAPC, nonadherent PC; APC adherent PC; MST, mean survival time; NK, natural killer; E:T ratios, effector to target ratios; Poly I:C, polyinosinic-polycytidylic acid; CTL, cytotoxic T lymphocytes; RLNC, regional lymphnode cells

cording to Biological Response Modifiers Program reference reagent human IL-2) was generously donated by Takeda Pharmaceutical Co., Ltd., Osaka, Japan, and dissolved in C57BL/6 mouse serum at a concentration of 200 units/ml. In the present study, the rIL-2 activity was assessed in Takeda units.

Immunization of mice with N-CWS. Mice were given weekly s.c. injections of 100 µg of N-CWS for 3 weeks. These mice showed significant increases in the blastogenic response of spleen cells in vitro and the foot pad reaction to N-CWS [8], and they were designated N-CWS-immunized mice. They were used for the experiment 1 week after the last s.c. injection of N-CWS.

Preparation of effector cells. Normal or N-CWS-immunized mice were injected i.p. with 50 µg of N-CWS on day 0 and/or 100 units of human rIL-2 on days 0, 1, 2, and 3. The PC were harvested by peritoneal lavage with cold Hanks' balanced salt solution containing 4 units/ml heparin using an 18-gauge needle on day 4. Then NAPC were prepared by incubation of PC in a 100-mm diameter plastic dish (Corning No. 25020, Corning Glass Works, Corning, NY) in a humidified 5% CO_2 atmosphere at 37° C for 2 h. On day 4, the spleens and the mesenteric lymph nodes were also removed from the mice and gently dissociated with the blunt end of a 2.5-ml syringe plunger. The spleen cells were treated in a buffered ammonium chloride solution for 1 min at room temperature to lyse red blood cells, washed 3 times, and resuspended in TCM. To prepare ADC monolayers, PC were suspended in TCM at a concentration of 2×10^6 cells/ml, 200 µl of the cell suspension was added to round-bottomed microtiter plates (Ms-3096U, Sumitomo Bakelites, Tokyo, Japan), and incubated for 2 h. The plates were washed to remove NAPC, and the resultant adherent cell monolayers were used as APC.

In the following all incubations were performed in a humidified 5% CO_2 atmosphere at 37° C unless otherwise stated.

Preparation of activated NK cells. Mice were given an i.p. injection of 100 µg of Poly I:C (Sigma Chemicals Co., St. Louis, Mo.), and 36 h after the injection, the spleen cells were removed from the mice and dissociated as described before.

Preparation of anti-P815 CTL. C57BL/6 mice were inoculated i.p. with 5×10^6 P815 mastocytoma cells, and 10 days after inoculation, the spleens were removed. Spleen cells were obtained as described before.

Cytotoxicity assay. Effector cells were tested for cytotoxicity as described elsewhere [18] with slight modifications. Briefly, tumor target cells were labeled with 100 µCi of $Na_2{}^{51}CrO_4$ for 60 min at 37° C. The cells were then washed 3 times and resuspended in TCM. ^{51}Cr-Labeled target cells (5,000) in 100 µl were placed in each well of a 96-well round-bottomed microtiter plate. Effector cells were added at the noted E:T ratios, generally 80:1. The microtiter plates were centrifuged and incubated for 4 h, and supernatants (100 µl) harvested for radioactivity counting. The percentage specific cytolysis was calculated using the following formula: % of specific cytolysis = $[(e-s)/(t-s)] \times 100$, where e represented the radioactivity in the supernatant from the culture containing both effector cells and target cells, s was the radioactivity in the supernatant from the culture containing only target cells, and t was one-half of the radioactivity of the total target cells added to each well. The spontaneous ^{51}Cr release was less than 10%. In some experiments, the cytotoxicity assay was performed in the presence of 25 or 50 µg/ml of carrageenan (Sigma Chemicals). The lytic units per mouse were determined using the following formula: lytic unit per mouse = a/b, where a represented the total number of NAPC harvested from the mice and b was the number of NAPC required to obtain 20% specific cytolysis against 5000 3LL target cells.

Treatment of NAPC with monoclonal antibodies and complement. Monoclonal anti-Thy 1.2, anti-Lyt 1.2, and anti-Lyt 2.2 antibodies were purchased from Cedarlane Laboratories, Ltd., Ontario, Canada, and used at dilutions of 1:50, 1:20, and 1:20, respectively. Monoclonal anti-asialo GM1 antibody was purchased from Wako Pure Chemicals Co., Ltd., Tokyo, Japan, and used at a dilution of 50:1. The NAPC were incubated with the indicated monoclonal antibody in RPMI-1640 medium containing 2% FCS on ice for 60 min, followed by incubation with Low-Tox-M rabbit complement (Cedarlane Laboratories) at a dilution of 1:8 for 60 min.

Therapeutic experiment. N-CWS-immunized C57BL/6 mice were inoculated i.p. with 1×10^6 3LL tumor cells which formed a solid tumor in the peritoneal cavity, or 2×10^5 EL-4 tumor cells on day 0 and then given an i.p. injection of 0.5 ml of saline or 50 µg of N-CWS on day 1. The mice were further given i.p. injections of 0.5 ml of syngeneic mouse serum or 100 units of rIL-2 on days 1, 2, 3, and 4. Mice were monitored daily for mortality.

Results

Augmentative effect of N-CWS on cytotoxic activity of LAK cells

We first examined whether an i.p. injection of N-CWS could augment the cytotoxic activity of LAK cells from normal and N-CWS-immunized mice which had received i.p. injections of rIL-2. The NAPC from the normal or the N-CWS-immunized mice given N-CWS alone or rIL-2 alone demonstrated only a slight increase in tumoricidal activity against 3LL tumor cells (Table 1). There was no difference in cytotoxic activity between NAPC from normal mice treated with rIL-2 alone and NAPC from normal mice treated with both rIL-2 and N-CWS. NAPC from N-CWS-immunized mice without an i.p. injection of N-CWS showed virtually no cytotoxic activity against 3LL and YAC-1 tumor cells (data not shown). On the other hand, the cytotoxic activity of NAPC from N-CWS-immunized mice was augmented by i.p. administration of both N-CWS and rIL-2. However, this synergistic effect was not seen when YAC-1 tumor cells were used as target cells. Furthermore, since the number of NAPC per mouse was increased, the lytic unit per mouse was significantly increased in the N-CWS-immunized mice injected i.p. with both N-CWS and rIL-2 (Fig. 1).

An i.p. injection of N-CWS and 1, 4, or 7 daily i.p. injections of rIL-2 were given to N-CWS-immunized mice, whose NAPC were tested for tumoricidal activity against

Table 1. Effect of N-CWS on the cytotoxic activity of NAPC from normal or N-CWS-immunized mice

NAPC from	i.p. with[b]	% Specific cytolysis[c]					
		Target cells					
		3LL			YAC-1		
		20 : 1	40 : 1	80 : 1	20 : 1	40 : 1	80 : 1
Normal mice	–	-0.3 ± 0.2[d]	1.1 ± 0.8	-1.0 ± 0.2	2.8 ± 0.5	2.1 ± 0.6	2.9 ± 0.8
	N-CWS	2.0 ± 0.1	3.7 ± 0.5	6.4 ± 0.2	7.9 ± 0.2	14.4 ± 0.6	21.4 ± 1.0
	rIL-2	8.9 ± 0.6	10.6 ± 0.8	16.9 ± 0.6	36.4 ± 1.7	55.2 ± 0.3	60.4 ± 3.3
	N-CWS+rIL-2	8.0 ± 0.6	10.8 ± 0.9	15.2 ± 0.9	40.4 ± 0.2	50.5 ± 2.3	64.7 ± 1.1
N-CWS-immunized mice[a]	N-CWS	3.5 ± 0.4	7.5 ± 0.6	14.6 ± 0.5	12.6 ± 0.8	25.5 ± 0.9	40.2 ± 1.3
	rIL-2	8.0 ± 0.4	10.1 ± 0.9	18.0 ± 1.2	43.7 ± 1.8	64.4 ± 1.1	73.9 ± 0.9
	N-CWS+rIL-2	10.6 ± 0.2	19.3 ± 0.3	32.2 ± 1.2	43.6 ± 1.1	60.0 ± 0.2	78.2 ± 0.8

[a] C57BL/6 mice received weekly s.c. injections of 100 µg of N-CWS 3 times
[b] Normal or N-CWS-immunized mice were injected i.p. with 50 µg of N-CWS on day 0 or 100 units of rIL-2 on days 0, 1, 2, and 3. NAPC were prepared on day 4
[c] NAPC were assayed for cytotoxic activity against 3LL or YAC-1 cells at E : T ratios as indicated
[d] Mean ± SE

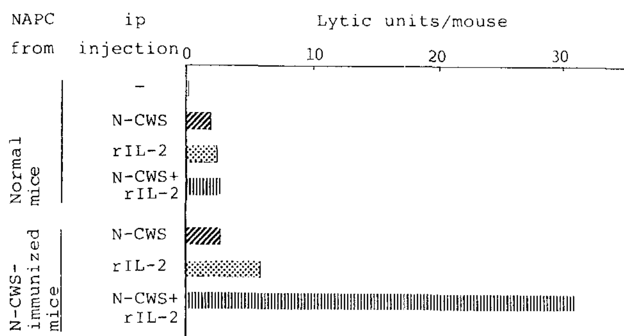

Fig. 1. Lytic units/mouse of the mice treated i.p. with N-CWS and/or rIL-2. Normal mice or N-CWS-immunized mice were injected i.p. with 50 µg of N-CWS on day 0 and 100 units of rIL-2 on days 0, 1, 2, and 3. NAPC were assayed for cytotoxic activity against 3LL cells at various E:T ratios. The lytic unit/mouse was determined by dividing the total number of NAPC harvested from the mice by the number of NAPC required to obtain 20% specific cytolysis against 5000 3LL target cells

3LL tumor cells 1 day after the last rIL-2 injection (Fig. 2). Cytotoxic activity was not induced by a single injection of rIL-2 with N-CWS. However, 4 and 7 i.p. injections of rIL-2 induced potent tumoricidal activity in NAPC from the mice treated i.p. with N-CWS.

Furthermore, various doses of rIL-2 or N-CWS were injected i.p. in N-CWS-immunized mice, and their NAPC were tested for cytotoxic activity. The tumoricidal activity showed a dose-dependent increase in terms of rIL-2 (Fig. 3) and N-CWS (Fig. 4).

Temporal changes in LAK activity. NAPC were tested for antitumor activity from day 1 to day 6 after the last rIL-2 injection (Fig. 5). The most potent cytotoxic activity was elicited by the NAPC harvested 1 day after the last injection, and this activity then diminished progressively to the control level by 4 days.

The effect of i.p. injections of N-CWS and rIL-2 on tumoricidal activities of spleen and mesenteric lymph node cells

NAPC, spleen cells, and mesenteric lymph node cells were obtained from N-CWS-immunized mice which had been injected i.p. with N-CWS and rIL-2, and they were tested for cytotoxic activity. As shown in Table 2, neither the lymph node cells nor the spleen cells demonstrated cytotoxic activity against 3LL tumor cells. On the other hand, potent tumoricidal activity was shown by the NAPC.

Characterization of effector cells in NAPC. In order to clarify the characteristics of the effector cells in NAPC, PC from mice injected i.p. with N-CWS and rIL-2 were fractionated into APC and NAPC. Although both the whole PC and the NAPC demonstrated a potent tumoricidal activity, the APC demonstrated no cytotoxic activity (Fig. 6). Furthermore, addition of carrageenan to the cytotoxic assay system did not abolish the antitumor activity of the NAPC (Table 3). These results suggest that the effector cells in the NAPC were not contaminating peritoneal macrophages.

NAPC induced by N-CWS and rIL-2 were tested for their phenotype by various antibodies and complement (Table 4). It was found that NAPC were susceptible to anti-Thy 1.2 or anti-asialo GM1 antibodies plus complement but completely resistant to anti-Lyt 1.2 or anti-Lyt 2.2 antibodies and complement. On the other hand, anti-P815 CTL lost their cytotoxic activity by anti-Thy 1.2 or anti-Lyt 2.2 antibodies and complement but not by anti-asialo GM1 antibodies and complement. In contrast, the NK activity was eliminated by anti-asialo GM1 antibodies and complement but not by anti-Thy 1.2 or anti-Lyt 2.2 antibodies and complement. These results show that the effector cells induced with i.p. injection of rIL-2 and N-CWS were not classical CTL or classical NK cells.

Therapeutic effect of N-CWS and rIL-2 administered i.p. on survival time of tumor-bearing mice. NAPC induced with N-CWS and rIL-2 showed potent cytolytic activity against

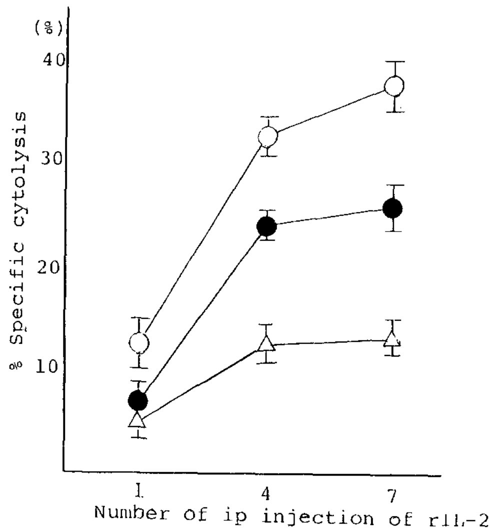

Fig. 2. Effect of repeated injections of rIL-2 on the cytotoxic activity of NAPC. N-CWS-immunized mice received an i.p. injection of 50 µg of N-CWS and 1–7 i.p. injections of 100 units of rIL-2. NAPC prepared 1 day after the last rIL-2 injection were assayed for cytotoxic activity against 3LL cells at E:T ratios of 20:1 (△), 40:1 (●), and 80:1 (○)

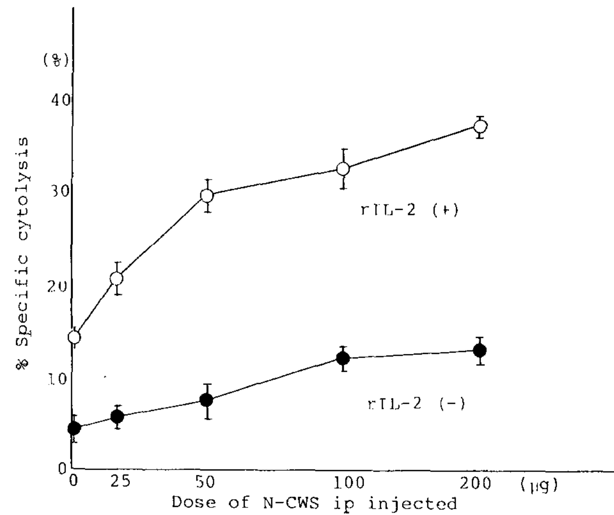

Fig. 4. Dose-dependent augmentation of the cytotoxic activity of NAPC relative to the doses of N-CWS. N-CWS-immunized mice were injected i.p. with various doses of N-CWS on day 0, and injected i.p. with (○) or without (●) 100 units of rIL-2 on days 0, 1, 2, and 3. On day 4, NAPC were assayed for cytotoxic activity against 3LL cells at an E:T ratio of 80:1. Each *vertical bar* represents mean ± SE

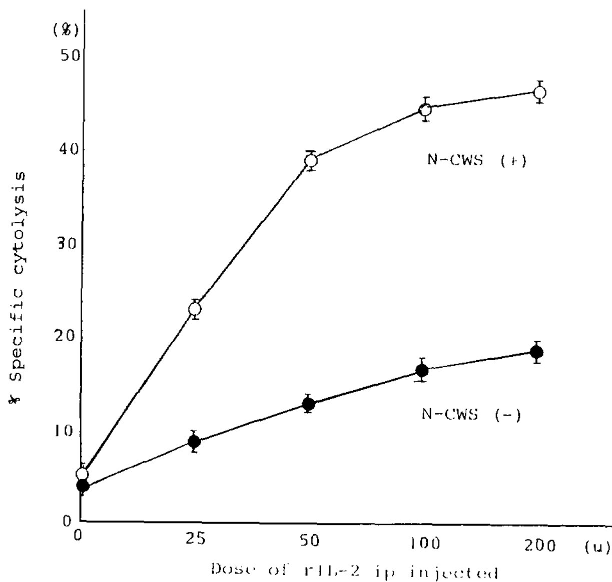

Fig. 3. Dose-dependent augmentation of the cytotoxic activity of NAPC relative to the doses of rIL-2. N-CWS-immunized mice were injected i.p. with (○) or without (●) 50 µg of N-CWS on day 0, and various doses of rIL-2 on days 0, 1, 2, and 3. On day 4, NAPC were assayed for cytotoxic activity against 3LL cells at an E:T ratio of 80:1. Each *vertical bar* represents mean ± SE

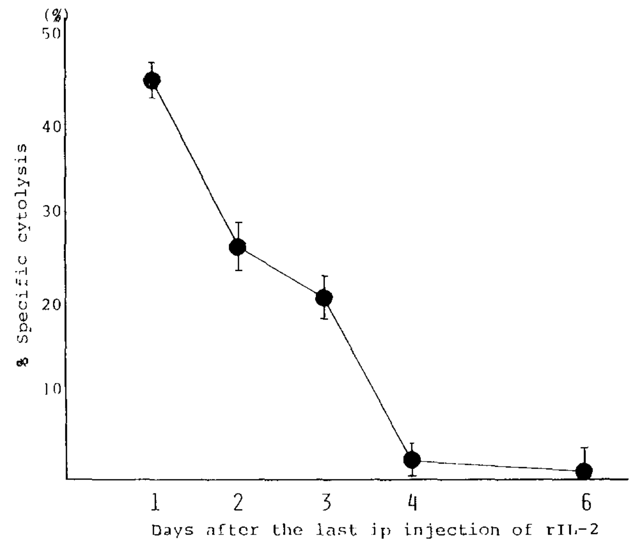

Fig. 5. Temporal changes of the cytotoxic activity of NAPC after the last i.p. injection with rIL-2. N-CWS-immunized mice were injected i.p. with 50 µg of N-CWS on day 0 and 100 units of rIL-2 on days 0, 1, 2, and 3. NAPC were prepared on day 4 – day 9. The cytolytic activity against 3LL cells was determined at an E:T ratio of 80:1. Each *vertical bar* represents mean ± SE

3LL and YAC-1 tumor cells, but not against EL-4 leukemic cells (Table 5). We next examined the effect of i.p. injections of N-CWS and rIL-2 on the survival time of 3LL- or EL-4-bearing mice. A single administration of N-CWS alone to 3LL-bearing N-CWS-immunized mice resulted in a slight prolongation of the MST compared to the control mice (Fig. 7B). Multiple administrations of rIL-2 alone significantly prolonged the MST. Furthermore, administration of both N-CWS and rIL-2 significantly prolonged the MST compared not only to the control mice but also to the mice given rIL-2 alone. However, combination therapy with N-CWS and rIL-2 did not prolong the MST of N-CWS-immunized mice bearing LAK-resistant EL-4 tumor (Fig. 7A).

Discussion

Previous studies have shown that combined immunotherapy with LAK cells and rIL-2 is therapeutically effective not only against experimental models but also against hu-

Table 2. The cytoxic activity of NAPC, spleen cells, or regional lymph node cells from N-CWS-immunized mice treated with N-CWS and rIL-2

Effector cells[a]	% Specific cytolysis		
	E : T ratios		
	20 : 1	40 : 1	80 : 1
NAPC	17.1 ± 1.4[b]	27.3 ± 0.7	38.7 ± 2.3
Spleen cells	0.0 ± 0.9	1.5 ± 0.9	2.4 ± 0.3
Regional lymph node cells	− 1.1 ± 2.3	2.2 ± 0.5	1.5 ± 0.7

[a] N-CWS-immunized mice were injected i.p. with 50 μg of N-CWS on day 0 and 100 units of rIL-2 on days 0, 1, 2, and 3. On day 4, NAPC, spleen cells, and regional lymph node cells were assayed for cytotoxic activity against 3LL cells at E : T ratios as indicated
[b] Mean ± SE

Table 3. Effect of the treatment with carrageenan on the cytotoxic activity of NAPC[a]

Concentration of carrageenan (μg/ml)[b]	% Specific cytolysis[c]
0	44.6 ± 4.9[d]
25	43.9 ± 2.7
50	36.9 ± 0.8

[a] N-CWS-immunized mice were injected i.p. with 50 μg of N-CWS on day 0 and 100 units of rIL-2 on days 0, 1, 2, and 3. NAPC were 4 prepared on day 4
[b] The treatment of NAPC with carrageenan was performed during the cytotoxic assay
[c] Cytotoxic activity against 3LL cells was determined at an E : T ratio of 80 : 1
[d] Mean ± SE

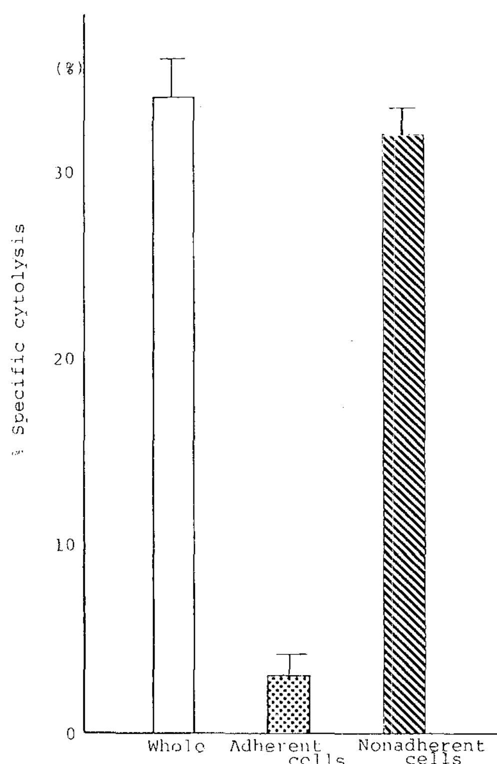

Fig. 6. Fractionation of PC induced by i.p. injections of N-CWS and rIL-2. PC from the N-CWS-immunized mice treated i.p. with N-CWS and rIL-2 were fractionated into APC and NAPC. The cytolytic activity against 3LL cells was determined at an E:T ratio of 80:1. Each *vertical bar* represents mean ± SE

man neoplasms [4, 6, 12, 19, 21, 23]. To induce tumoricidal activity, it is necessary to administer high doses of rIL-2 with LAK cells because rIL-2 administered in vivo is rapidly cleared or inactivated in the host [2, 5]. Therefore, many investigators have attempted to maintain a high level of rIL-2 activity in the serum by using a sustained-release vehicle such as gelatin or a miniosmotic pump [2, 16].

In this report, we have demonstrated that N-CWS augments the cytolytic activity of NAPC from N-CWS-immunized mice given i.p. rIL-2 against NK-resistant 3LL tumor cells. But NAPC from N-CWS-immunized mice without an i.p. injection of N-CWS showed virtually no cytotoxic activity against 3LL and YAC-1 tumor cells. There was no difference in the cytolytic activity between NAPC induced with rIL-2 and NAPC induced with both N-CWS and rIL-2 when NAPC were prepared from normal mice (Table 1).

Furthermore, since the number of NAPC per mouse was increased, the lytic units per mouse was significantly increased in the N-CWS-immunized mice injected i.p. with both N-CWS and rIL-2 (Fig. 1).

The cytotoxic activity was predominantly expressed at the local site of administration of rIL-2 (Table 2), suggesting that N-CWS and rIL-2 should be administered at the tumor site. One wonders why this enhancement is not demonstrated in regional lymphnode cells (RLNC). One possibility is that we administered rIL-2 by i.p. injection, therefore the levels of rIL-2 at RLNC may be too low to induce tumoricidal activity. We have demonstrated that i.p. injection with N-CWS activates peritoneal macrophages more effectively than i.v. or s.c. injection [10].

The tumoricidal activity of NAPC was not induced by an i.p. injection of N-CWS or a single injection of rIL-2. However, repeated i.p. injections of rIL-2 induced potent tumoricidal activity in NAPC from N-CWS-immunized mice given N-CWS i.p. (Fig. 2).

The NAPC prepared from N-CWS-immunized mice showed cytotoxic activity, but the APC did not. Moreover, treatment with carrageenan did not affect the cytotoxic activity of NAPC (Table 3, Fig. 2). These results suggest that the effector cells are nonadherent cells and that they are not contaminating peritoneal macrophages.

Rosenberg and coworkers [17] have reported that combined immunotherapy with LAK cells plus rIL-2, but not with rIL-2 alone, reduces the number of established pulmonary metastases of weakly immunogeneic murine sarcomas. We examined the effect of i.p. injections with N-CWS and rIL-2 on the survival time of LAK-resistant EL-4 tumor-bearing or LAK-sensitive 3LL tumor-bearing N-CWS-immunized mice. As shown in Fig. 7B, multiple administration of rIL-2 to 3LL-bearing mice statistically prolonged the MST compared with the control mice

Table 4. Phenotypic analysis of peritoneal LAK cells induced by injections of N-CWS and rIL-2

Treatment of effector cells	% Specific cytolysis		
	Effector cells		
	LAK[a] (3LL)	Poly I : C-induced[b] NK (YAC-1)	Anti-P815 CTL[c] (P815)
Medium alone	24.1 ± 1.2[d]	30.9 ± 1.6	88.8 ± 1.2
Complement (C′) alone	27.9 ± 0.3	32.7 ± 0.4	85.3 ± 0.7
Anti-Thy1.2 + C′	11.1 ± 0.9	14.4 ± 2.0	3.8 ± 0.1
Anti-Lyt1.2 + C′	25.8 ± 1.0	20.7 ± 1.5	69.9 ± 0.3
Anti-Lyt2.2 + C′	35.6 ± 0.4	29.7 ± 0.9	1.0 ± 0.2
Anti-asialo GM1 + C′	9.6 ± 0.9	−0.4 ± 0.5	76.1 ± 2.0

[a] N-CWS-immunized mice were injected i.p. with 50 μg of N-CWS on day 0 and 100 units of rIL-2 on days 0, 1, 2, and 3. NAPC were prepared on day 4, and assayed for cytotoxic activity against 3LL cells at an E : T ratio of 80 : 1
[b] C57BL/6 mice were injected i.p. with Poly I : C, and 36 h later, spleen cells were assayed for cytotoxic activity against YAC-1 cells at an E : T ratio of 80 : 1
[c] C57BL/6 mice were injected i.p. with P815 cells, and 10 days later, spleen cells were assayed for cytotoxic activity against P815 cells at an E : T ratio of 80 : 1
[d] Mean ± SE

Table 5. The cytotoxic activity of NAPC against various tumor cells

Target cells	% Specific cytolysis[a]		
	E : T ratios		
	20 : 1	40 : 1	80 : 1
3LL	17.1 ± 1.4[b]	27.3 ± 0.7	38.7 ± 2.3
YAC-1	46.9 ± 2.3	53.2 ± 3.1	62.0 ± 1.7
EL-4	0.2 ± 0.2	7.0 ± 0.3	9.7 ± 0.6

[a] N-CWS-immunized mice were injected i.p. with 50 μg of N-CWS on day 0 and 100 units of rIL-2 on days 0, 1, 2, and 3. On day 4, NAPC were assayed for cytotoxic activity against various tumor cells at E : T ratios as indicated
[b] Mean ± SE

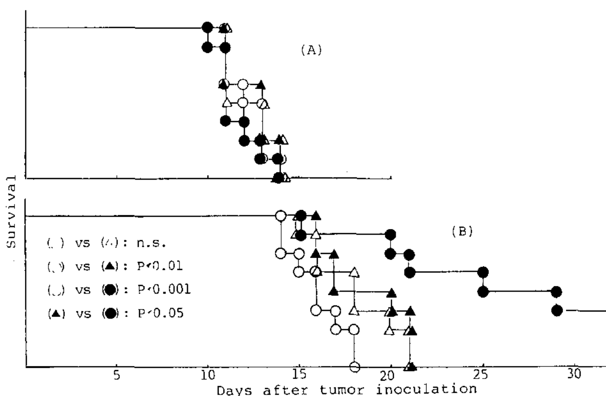

Fig. 7. Effect of the treatment with N-CWS and rIL-2 on the survival period of mice bearing EL-4 (**A**) or 3LL (**B**) tumor. N-CWS-immunized C57BL/6 mice were inoculated i.p. with tumor cells on day 0 followed by: no treatment (○), i.p. injection of N-CWS on day 1 (△), i.p. injections of rIL-2 on days 1, 2, 3, and 4 (▲), or i.p. injections of N-CWS on day 1 and rIL-2 on days 1, 2, 3, and 4 (●)

($P<0.01$). Furthermore, combined administration of N-CWS with rIL-2 statistically prolonged the MST compared with not only the control mice ($P<0.001$) but also mice given rIL-2 alone ($P<0.05$). This synergistic effect of N-CWS and rIL-2 was observed in LAK-sensitive 3LL-bearing mice but not in LAK-resistant EL-4-bearing mice (Fig. 7 A).

The mechanism by which N-CWS augments the tumoricidal activity of LAK cells remains unclear. In preliminary experiments, however, it was shown that N-CWS stimulated N-CWS-immunized spleen cells to produce some cytokines that cooperated with rIL-2 to augment the tumoricidal activity (data not shown). Furthermore, N-CWS augmented the capacity of N-CWS-immunized spleen cells to bind radioiodinated rIL-2 (data not shown). The peritoneal effector cells were susceptible to anti-Thy 1.2 antibody and anti-asialo GM1 antibody plus complement, but were completely resistant to anti-Lyt 1.2 antibody and anti-Lyt 2.2 antibody but the killing ability of anti-P815 CTL was completely eliminated by treatment with anti-Lyt 2.2 antibody plus complement, which does not suggest that the effector cells are Lyt 2.2^+ CTL induced by immunization of N-CWS. It suggests that there are no cross-reacting antigens on the N-CWS that are antigenically similar to those found on 3LL cells. The killing ability of the peritoneal effector cells induced by N-CWS and rIL-2 was partly eliminated by treatment with anti-asialo GM1 plus complement but the killing ability of Poly I:C-induced NK cells was completely eliminated by the same treatment.

Therefore, the peritoneal effector cells might belong to the NK lineage and they were not classical CTL or NK cells.

On the other hand, several investigators [21, 24, 25] have reported that murine LAK cells induced in vitro express mature T cell antigens on their surfaces (Thy 1 and Lyt 2). Treatment with anti-Lyt 2.2 antibodies plus complement under our experimental conditions failed to eliminate the cytotoxic activity of NAPC cells induced with N-CWS and rIL-2. Recently, Hinuma et al. [9] reported that

nonspecific killer cells induced by rIL-2 in vitro and in vivo express Thy 1.2 and asialo GM1 antigens on their surface; this is in agreement with our findings. Therefore NAPC induced by N-CWS and rIL-2 are likely to be so-called in vivo LAK cells. Rosenberg et al. [18, 26] have demonstrated that LAK cells induced in vitro act as effector cells to prevent tumor growth in vivo in mice. In addition, adequate administration of rIL-2 induced LAK activity in mice without transfer of in vitro cultured LAK cells [20]. But our results showed that combination therapy with N-CWS and rIL-2 was more effective in preventing tumor growth compared to administration of rIL-2 alone. Immunotherapy with LAK cells plus rIL-2 is expensive, and administration of high doses of rIL-2 causes severe adverse effects in some patients. The use of N-CWS might save money and allow reduction of the dose of rIL-2 which must be administered. Therefore, combination immunotherapy with N-CWS and rIL-2 is beneficial. Studies are in progress in our laboratory to elucidate the mechanism of N-CWS-induced augmentation of LAK activity.

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