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Synergy of *Nocardia rubra* Cell Wall Skeleton and Interleukin 2 in the *in vivo* Induction of Murine Lymphokine-activated Killer Cell Activity

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Combination of an ip injection of *Nocardia rubra* cell wall skeleton (N-CWS) and 3 daily sc injections of human recombinant interleukin 2 (rIL 2) into C3H/HeN mice resulted not only in a significant increase in the number of peritoneal cells (PC) but also in a potent induction of their lymphokine-activated killer (LAK) activity, compared with results obtained with N-CWS or rIL 2 alone. The augmented LAK activity of PC was mediated by nonadherent, nonphagocytic, Thy-1.2$^{+\sim-}$ and asialo GM$_1^+$ cells. Nonadherent PC induced by an ip injection of N-CWS bound more ^{125}I-labeled rIL 2 than did normal, nonadherent PC, and generated high LAK activity when cultured overnight with rIL 2. In contrast, normal, nonadherent PC responded only weakly to the overnight stimulation with rIL 2. The phenotype of N-CWS-induced PC with an elevated IL 2 responsiveness was Thy-1.2$^{+\sim-}$, Lyt-1.1$^-$, Lyt-2.1$^-$ and asialo GM$_1^+$, suggesting that the N-CWS-stimulated LAK precursors were derived mainly from the NK cell lineage. However, mature T cells may also be involved in this mechanism, because N-CWS failed to augment the IL 2 responsiveness of nonadherent PC in BALB/c *nu/nu* mice. Treatment of C57BL/6N mice bearing solid Lewis lung carcinoma (3LL) tumors with an intratumoral injection of N-CWS followed by 6 daily sc injections of rIL 2 resulted in the apparent suppression of tumor growth, while N-CWS or rIL 2 alone produced no such suppression. These results suggest that N-CWS augments the antitumor effect of rIL 2 by accumulating LAK precursors and elevating their responsiveness to rIL 2 at the injection site.

Key words: N-CWS — Human rIL 2 — LAK cells

Cancer immunotherapy with adoptive transfer of autologous lymphokine-activated killer (LAK)[2] cells followed by systemic administration of human recombinant interleukin 2 (rIL 2) has been reported to be effective against not only animal but also human cancers.[1,2] In humans, however, this treatment produced good results only against tumors of selected histological types including melanoma, renal carcinoma and lymphoma, whereas other common malignancies were hardly affected. This limitation of adoptive immunotherapy seems to be caused mainly by the low sensitivity of tumor cells to LAK cells and, at least in part, by the poor accumulation of the cytotoxic effectors at the tumor sites. Furthermore, current LAK therapy is hampered by other problems: it requires a large number of LAK cells prepared *ex vivo*, and the subsequent administration of a large amount of rIL 2 often results in severe side effects such as pulmonary edema and hypotension. Therefore, alternative procedures capable of inducing high LAK activity *in vivo* with a relatively low dose of rIL 2 and of accumulating LAK cells at the tumor sites are needed.

The cell wall skeleton of *Nocardia rubra* (N-CWS), a bacterial immunoadjuvant with a low toxicity, has been found to exhibit potent antitumor effects against both animal and human cancers.[3,4] Recent studies have revealed that N-CWS stimulates T cells and macrophages to produce a variety of cytokines including the colony-stimulating factor (CSF), the macrophage-activating factor (MAF), interferons (IFN), and the tumor necrosis factor (TNF).[5–8] This study was undertaken to investigate whether the combined use of N-CWS and rIL 2 could augment the antitumor effects of rIL 2. In this paper, we report that N-CWS not only accumulates LAK precursors but also elevates their responsiveness to rIL 2 at the injection site of N-CWS, and that an intralesional injection of N-CWS significantly augments the antitumor effect of rIL 2 administered systemically to a LAK-sensitive tumor.

MATERIALS AND METHODS

Animals and tumors Male C3H/HeN and C57BL/6N mice were purchased from Charles River Japan, Inc.,

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[2] Abbreviations used are: LAK, lymphokine-activated killer; CSF, colony-stimulating factor; FBS, fetal bovine serum; IFN, interferon; IL 2, interleukin 2; MAF, macrophage-activating factor; N-CWS, *Nocardia rubra* cell wall skeleton; NK, natural killer; PC, peritoneal cells; rIL 2, recombinant interleukin 2; TNF, tumor necrosis factor.

Kanagawa. Male BALB/c *nu/nu* and *nu/+* mice were purchased from Shizuoka Agricultural Cooperative for Experimental Animals, Shizuoka. Male C57BL/6 *bg/bg* and *bg/+* mice were purchased from The Jackson Laboratory, Bar Harbor, ME. All mice were used when they were 8 weeks old. Lewis lung carcinoma (3LL), a LAK-sensitive murine tumor of C57BL/6 mouse origin, was maintained by serial passage *in vitro* using RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (General Scientific Laboratories, Los Angeles, CA), 2 m*M* L-glutamine (Flow Laboratories, North Lyde, Australia), 100 units/ml of penicillin (Meiji Seika Co., Tokyo), and 100 μg/ml of streptomycin (Meiji Seika). This medium was designated the complete medium.

rIL 2 rIL 2 was kindly provided by Takeda Pharmaceutical Industries, Ltd., Osaka. The production of rIL 2 in *Escherichia coli* and its purification have been described in detail previously.[9] The specific activity of the purified rIL 2 used in this study was 3.5×10^4 units/mg. This corresponds to 1.2×10^7 units/mg, calculated on the basis of the Biological Response Modifiers Program reference reagent human IL 2 (Jurkat).[10] This material was dissolved in the complete medium for use *in vitro* or in the RPMI 1640 medium supplemented with 5% heat-inactivated mouse serum for use *in vivo*. Mouse serum was obtained from mice syngeneic to those used as recipients for rIL 2 administration.

N-CWS Squalene-attached N-CWS was generously provided by Fujisawa Pharmaceutical Co., Osaka, and dissolved in a 0.85% NaCl solution before use.

Preparation of spleen cells and peritoneal cells (PC) Spleen cells were prepared by teasing spleens in the RPMI 1640 medium supplemented with 2% heat-inactivated FBS (2% FBS-RPMI 1640 medium), washing the cells twice, and suspending them in the complete medium. PC were obtained by lavage of the peritoneal cavities with Hanks' medium (Nissui) supplemented with 5 units/ml heparin (Novo Industries, Bagsvaerd, Denmark), washing the cells twice, and suspending them in the complete medium. PC were further fractionated into nonadherent and adherent cells by using 60-mm plastic dishes (No. 25010, Corning, New York, NY) or 96-well microculture plates (No. 76-013-05, Linbro, McLean, VA) as described previously.[11]

Treatment of cells with antibodies and complement Seven million nonadherent PC were suspended in a 2% FBS-RPMI 1640 medium containing anti-Thy-1.2 (Cedarlane, Ontario, Canada; 1/10 dilution), anti-Lyt-1.1 (Cedarlane; 1/10 dilution), anti-Lyt-2.1 (Cedarlane; 1/10 dilution) or anti-asialo GM_1 antibody (Wako Pure Chemicals Co., Osaka; 1/50 dilution), kept at 4°C for 30 min, and washed once. The cells were resuspended in the 2% FBS-RPMI 1640 medium containing rabbit complement (Cedarlane; 1/8 dilution), incubated at 37°C for 45 min, and washed twice. After a viable cell number count, the cells were suspended in the complete medium at 1.25–2.5×10^6 cells/ml.

Incubation of cells with rIL 2 Two million five hundred thousand nonadherent PC were suspended in 2 ml of the complete medium supplemented with 5×10^{-5} *M* 2-mercaptoethanol (Wako) and rIL 2 at a concentration of 0.1–5.0 units/ml in a well of a 24-well culture plate (Corning), and incubated overnight at 37°C in a humidified 5% CO_2 atmosphere. After incubation, cells were collected by centrifugation, washed once, and then resuspended in the complete medium.

Cytotoxicity assay Five thousand ^{51}Cr-labeled 3LL tumor cells were mixed with $0.625–5\times10^5$ spleen cells or PC in 200 μl of the complete medium in a well of a microculture plate (No. 76-013-05, Linbro). In some experiments, carrageenan (Sigma, St. Louis, MO) was added to the well at a final concentration of 50 μg/ml. The plate was centrifuged at 800 rpm for 1 min, and then incubated at 37°C for 4 h in a humidified 5% CO_2 atmosphere. After incubation, 100 μl of the supernatant was harvested and its radioactivity was counted with a gamma counter. Cytolytic activity in triplicate cultures was calculated as follows:

$$\%\ \text{Specific cytolysis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}}\times 100.$$

Spontaneous cpm, measured by incubating labeled target cells alone, was less than 10% of total cpm.

IL 2 binding assay For the IL 2 binding assay, ^{125}I-labeled rIL 2 (specific activity: 2.3×10^5 cpm/pmol) was used as described elsewhere.[12] Briefly, 7×10^5 nonadherent PC in 100 μl of complete medium were incubated in duplicate with increasing concentrations of ^{125}I-labeled rIL 2 at 4°C for 1 h, and washed once, after which the radioactivity bound to the cells was measured with a gamma counter. Nonspecific binding was assessed by incubating, in parallel, aliquots with an excess of unlabeled rIL 2. Specific binding was calculated by subtracting the cpm of nonspecific binding from that shown in the absence of unlabeled rIL 2.

Experimental procedure Three or four mice were given an ip injection of 25–100 μg of N-CWS, followed by sc injection of 100 units of rIL 2 into the back once a day for 3 days from the day following N-CWS injection. One day after the last rIL 2 injection, spleen cells and PC were obtained and tested for cytolytic activity against 3LL tumor cells. In other experiments, nonadherent PC obtained 3 days after an ip injection of 100 μg of N-CWS were subjected to an IL 2 binding assay, or to overnight culture with rIL 2 followed by a cytotoxicity assay using

3LL tumor cells as a target. In these experiments, control mice were given injections of a 0.85% NaCl solution or the RPMI 1640 medium supplemented with 5% heat-inactivated syngeneic mouse serum in the same manner, respectively, as used for the administration of N-CWS or rIL 2.

Therapeutic experiment C57BL/6N mice were inoculated sc with 10^6 3LL tumor cells into the right side of the back. Five days after tumor inoculation, when the tumors had grown to approximately 5 mm in diameter, 100 μg of N-CWS dissolved in 50 μl of 0.85% NaCl solution was injected once into the growing tumors. One hundred units of rIL 2 dissolved in 100 μl of the RPMI 1640 medium supplemented with 5% heat-inactivated C57BL/6N mouse serum was injected sc into the contralateral side of the back once a day for 6 days from 6 to 11 days after tumor inoculation. Mice given injections of the medium used for preparing N-CWS or rIL 2 solution served as controls. The mean of the maximum and the perpendicular minimum diameters of the tumors was monitored every other day. Each group consisted of 6 mice.

Statistical analysis The mean difference between control and experimental groups was evaluated statistically by Student's t test, and P less than 0.05 was defined as statistically significant.

RESULTS

Augmentative effect of N-CWS on *in vivo* LAK cell induction When C3H/HeN mice were given 3 daily sc injections of rIL 2 alone, both spleen cells and nonadherent PC exhibited a weak but distinct cytotoxicity against 3LL tumor cells, compared with the results shown by cells of control mice. By combining the rIL 2 treatment with an ip injection of N-CWS, the peritoneal LAK cell induction was further augmented in an N-CWS dose-dependent manner, while splenic LAK activity was no more elevated than that induced with rIL 2 alone (Fig. 1). The combined effect of N-CWS and rIL 2 on peritoneal LAK cell induction was further analyzed, and the results are summarized in Table I. Three daily sc injections of rIL 2 weakly induced LAK activity of the nonadherent PC without affecting the number of PC. An ip injection of N-CWS increased the number of PC, while the nonadherent fraction of the cells also exhibited LAK activity. The combination of these two treatments resulted not only in a greatest increase in the number of PC but also in the more potent induction of their LAK activity than could be obtained with rIL 2 or N-CWS alone. These findings were similar for both BALB/c and C57BL/6N mice, and the cytotoxic effectors induced by the combined use of N-CWS and rIL 2 aggressively lysed

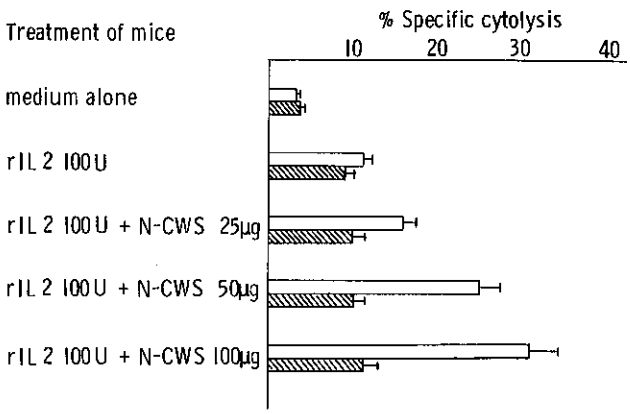

Fig. 1. Induction of splenic and peritoneal LAK cells by rIL 2 and N-CWS. C3H/HeN mice were given 3 daily sc injections of 100 units of rIL 2 on days 1, 2 and 3, or both an ip injection of N-CWS at the dose indicated on day 0 and 3 daily sc injections of rIL 2 as described above. On day 4, the cytolytic activity of spleen (▨) and nonadherent PC (□) against 3LL tumor cells was determined with a 4-h ^{51}Cr-release assay at respective E/T ratios of 100/1 and 50/1. Bars represent the mean±SD for triplicate cultures.

Table I. Combined Effect of N-CWS and rIL 2 on the Induction of LAK Activity of Nonadherent PC

Treatment of mice[a]	No. of PC per mouse	% Non-adherent cells	% Specific cytolysis[b]
Medium	4.4×10^6	32.0	1.7 ± 0.1[c]
rIL 2	3.8×10^6	41.0	10.6 ± 0.9[d]
N-CWS	7.3×10^6	34.5	22.5 ± 2.1[e]
N-CWS+rIL 2	11.0×10^6	31.3	41.0 ± 3.6[f]

a) C3H/HeN mice were given an ip injection of 100 μg of N-CWS on day 0, 3 daily sc injections of 100 units of rIL 2 on days 1, 2 and 3, or both N-CWS (day 0) and rIL 2 injections (days 1, 2 and 3). Control mice were given the medium used for preparing the N-CWS or rIL 2 solution in the same manner. PC were obtained from these mice on day 4.

b) Cytotoxicity against 3LL tumor cells was determined by 4-h ^{51}Cr-release assay at an E/T ratio of 50/1. Data represent the mean±SD for triplicate cultures. Statistical significance: *c*) vs. *d*), $P<0.001$; *c*) vs. *e*), $P<0.001$; *d*) vs. *f*), $P<0.001$; *e*) vs. *f*), $P<0.01$.

NK-sensitive YAC-1 but killed only a small number of LAK-resistant MH134 tumor cells (data not shown). As shown in Table II, the LAK activity of PC induced by the combined treatment was shown to be mediated mainly by nonadherent and nonphagocytic cells whose cell surface phenotype was Thy-1.2$^{+\sim-}$ and asialo GM$_1^+$.

Table II. Characteristics of Peritoneal LAK Cells Induced by N-CWS and rIL 2

Experiments	Treatment of mice[a]	Fractionation of PC[b]	Treatment of PC[c]	Addition of carrageenan[d]	% Specific cytolysis[e]
I	medium	unfractionated PC	−	−	3.1±0.2
	N-CWS+rIL 2	unfractionated PC	−	−	19.1±1.4
		adherent PC	−	−	−1.1±0.1
		nonadherent PC	−	−	31.1±2.5
II	medium	nonadherent PC	−	−	3.0±0.5
	N-CWS+rIL 2	nonadherent PC	−	−	35.4±3.8[f]
				+	29.0±3.0[g]
III	medium	nonadherent PC	medium	−	4.4±0.2
	N-CWS+rIL 2	nonadherent PC	medium	−	34.3±2.5[h]
			C	−	33.0±2.6
			anti-Thy-1.2+C	−	24.5±1.9[i]
			anti-asialo GM_1+C	−	2.8±0.1[j]

a) C3H/HeN mice were given an ip injection of 100 μg of N-CWS one day 0, and given sc injections of 100 units of rIL 2 on days 1, 2 and 3. Control mice were given injections of the medium used for preparing the N-CWS or rIL 2 solution in the same manner. PC were obtained from these mice on day 4.
b) PC were fractionated into nonadherent and adherent cells by using plastic dishes and 96-well microculture plates.
c) Nonadherent PC were treated with antibodies and complement. The number of cells was then counted, and the cell density adjusted to 2.5×10^6/ml. Cell depletion data showed that Thy-1.2$^+$ accounted for 1.3% and asialo GM_1^+ cells for 16.9%.
d) Carrageenan was added at a final concentration of 50 μg/ml.
e) See footnote *b*) in Table I. Statistical significance: *f*) vs. *g*), not significant; *h*) vs. *i*), $P<0.01$; *h*) vs. *j*), $P<0.001$.

Elevated responsiveness of N-CWS-induced PC to rIL 2 Nonadherent PC induced by N-CWS generated high LAK activity in response to the overnight stimulation with rIL 2, while only a low LAK activity was induced in control, nonadherent PC by this treatment (Fig. 2). The IL 2 binding assay using ^{125}I-labeled rIL 2 showed that N-CWS-induced, nonadherent PC bound rIL 2 more than did control, nonadherent PC (Table III).

Requirement of both NK and T cells for augmented LAK cell induction by N-CWS and RIL 2 N-CWS-induced, nonadherent PC were treated with antibodies and complement, and then cultured overnight with rIL 2. As shown in Table IV, peritoneal LAK precursors induced by N-CWS were Thy-1.2$^{+\sim-}$, Lyt-1.1$^-$, Lyt-2.1$^-$, and asialo GM_1^+. Similar experiments were performed on C57BL/6 *bg/bg* and BALB/c *nu/nu* mice. LAK cell induction from nonadherent PC by rIL 2 alone was lower in C57BL/6 *bg/bg* than that shown in *bg/+* mice. When N-CWS-induced, nonadherent PC were used as responder cells, their responsiveness to rIL 2 was still weaker in *bg/bg* than in *bg/+* mice, while an increase in the number of PC induced by N-CWS was generally seen in *bg/bg* mice (Table V). On the other hand, nonadherent PC of BALB/c *nu/nu* mice generated a higher LAK activity in response to the overnight stimulation with rIL 2 than those of *nu/+* mice when control, nonadherent PC were used as responders. However, the responsiveness of BALB/c *nu/nu* nonadherent PC to rIL 2 was not further augmented by the preceding stimulation with N-CWS, while the response of BALB/c *nu/+* cells was apparently augmented by N-CWS (Table VI).

Antitumor effect of the combined therapy with rIL 2 and N-CWS C57BL/6N mice bearing 3LL tumors were given an intratumoral injection of N-CWS, or 6 daily sc injections of rIL 2 into the back at the contralateral side of the tumor inoculation, or a combination the two treatments. As shown in Fig. 3, the combined treatment with N-CWS and rIL 2 resulted in a significant retardation of the tumor growth, while the treatment with N-CWS or rIL 2 alone produced no apparent effect. However, the combined effect lasted only a few days, and tumors started to grow again after rIL 2 injections were stopped.

DISCUSSION

Because of the short half-life of IL 2 in the blood flow, several protocols, including continuous infusion of IL 2, repeated sc injections of high doses of IL 2 and inoculation of slow-release IL 2-containing substances, have been tried to obtain the continuous presence of IL 2 at low concentrations in the blood stream.[13–15] The present study showed that 3 daily sc injections of 100 units of human rIL 2, corresponding to 34,000 Jurkat units, into

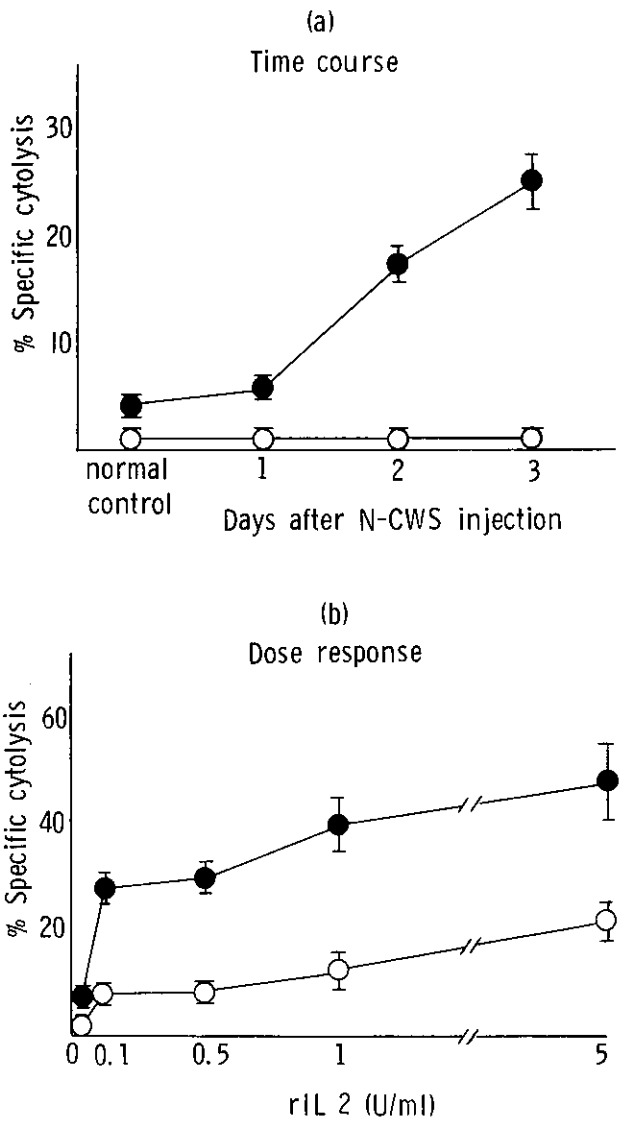

Fig. 2. Augmented LAK cell induction from N-CWS-induced, nonadherent PC. (a) Nonadherent PC were obtained from C3H/HeN mice 1–3 days after an ip injection of 100 μg of N-CWS, and incubated overnight with medium alone (○) or in the presence of 1 unit of rIL 2/ml (●). (b) Nonadherent PC were obtained 3 days after an ip injection of 1 ml of a 0.85% NaCl solution (○) or 100 μg of N-CWS (●), and cultured overnight in the presence of rIL 2 at the concentration indicated. After incubation, cytotoxicity against 3LL tumor cells was determined with a 4-h ^{51}Cr-release assay at an E/T raito of 50/1. Bars represent the mean±SD for triplicate cultures.

C3H/HeN mice resulted in systemic LAK cell induction, elevating the LAK activity of both spleen cells and nonadherent PC. Preliminary experiments confirmed that no further elevation of the LAK activity of these

Table III. Augmentation of IL 2 Binding Capacity of Non-adherent PC by N-CWS

Cells[a]	Specific rIL 2 binding (cpm) at ^{125}I-rIL 2 concentration of[b]		
	0.2 n*M*	0.8 n*M*	3.2 n*M*
Medium-induced nonadherent PC	80	191	967
N-CWS-induced nonadherent PC	449	865	1825

a) Nonadherent PC were obtained 3 days after an ip injection of 100 μg of N-CWS or 1 ml of 0.85% NaCl solution.
b) Specific binding of ^{125}I-labeled rIL 2 by 7.5×10^5 non-adherent PC was assayed at the concentrations indicated. Data represent the mean of duplicate cultures.

Table IV. Phenotype of Peritoneal LAK Precursors Induced by N-CWS in C3H/HeN Mice

Cells[a]	Treatment[b]	Addition of rIL 2[c]	% Specific cytolysis[d]
Medium-induced nonadherent PC	−	−	1.9±0.2
	−	+	9.2±0.5
N-CWS-induced nonadherent PC	medium	−	7.8±0.8
	medium	+	30.8±2.5[e]
	C	+	28.2±2.0
	anti-Thy-1.2+C	+	15.9±1.0[f]
	anti-Lyt-1.1+C	+	29.6±3.0[g]
	anti-Lyt-2.1+C	+	26.4±2.0[h]
	anti-asialo GM_1+C	+	−3.9±0.1[i]

a) See footnote *a*) in Table III.
b) Seven million nonadherent PC were treated with antibodies and complement. The number of cells was counted, and the cell density adjusted to 1.25×10^6/ml. Cell depletion data showed that Thy-1.2$^+$ accounted for 3.1%, Lyt-1.1$^+$ for 4.7%, Lyt-2.1$^+$ for 3.1% and asialo GM_1^+ cells for 25.1%.
c) Two million five hundred thousand nonadherent PC were cultured overnight in the presence of 4 units of rIL 2/ml.
d) See footnote *b*) in Table I. Statistical significance: *e*) vs. *f*), $P<0.001$; *e*) vs. *g*), not significant; *e*) vs. *h*), not significant; *e*) vs. *i*), $P<0.001$.

lymphoid cells was achieved by repeating sc injection of rIL 2 more than 3 times (data not shown). However, the cytotoxicity induced by repeated sc injections of rIL 2 alone was not as high as expected, and so it was investigated whether a combination of rIL 2 and other cytokines or cytokine-inducing stimulators could augment LAK cell induction.

Table V. Loss of the Augmented LAK Cell Induction from N-CWS-induced Nonadherent PC in C57BL/6 *bg*/*bg* Mice

Mice	Treatment of mice[a]	No. of PC/mouse	Addition of rIL 2[b]	% Specific cytolysis[c]
C57BL/6 *bg*/+	medium	2.9×10^6	−	0.2±0.1
			+	15.7±1.0[d]
	N-CWS	8.3×10^6	−	0.6±0.1
			+	43.4±3.3[e]
C57BL/6 *bg*/*bg*	medium	3.6×10^6	−	−1.0±0.1
			+	7.0±0.2[f]
	N-CWS	7.0×10^6	−	1.4±0.1
			+	10.7±1.0[g]

a) See footnote *a*) in Table III.
b) See footnote *c*) in Table IV.
c) See footnote *b*) in Table I. Statistical significance: *d*) vs. *e*), $P<0.001$; *f*) vs. *g*), $P<0.01$; *d*) vs. *f*), $P<0.001$; *e*) vs. *g*), $P<0.001$.

Table VI. Loss of the Augmented LAK Cell Induction from N-CWS-induced Nonadherent PC in BALB/c *nu*/*nu* Mice

Mice	Treatment of mice[a]	No. of PC/mouse	Addition of rIL 2[b]	% Specific cytolysis[c]
BALB/c *nu*/+	medium	2.7×10^6	−	2.7±0.2
			+	13.5±1.0[d]
	N-CWS	9.8×10^6	−	1.6±0.1
			+	36.4±3.3[e]
BALB/c *nu*/*nu*	medium	2.1×10^6	−	1.6±0.1
			+	25.6±2.4[f]
	N-CWS	12.0×10^6	−	1.6±0.1
			+	23.9±2.0[g]

a) See footnote *a*) in Table III.
b) See footnote *c*) in Table IV.
c) See footnote *b*) in Table I. Statistical significance: *d*) vs. *e*), $P<0.001$; *f*) vs. *g*), not significant; *d*) vs. *f*), $P<0.01$; *e*) vs. *g*), $P<0.01$.

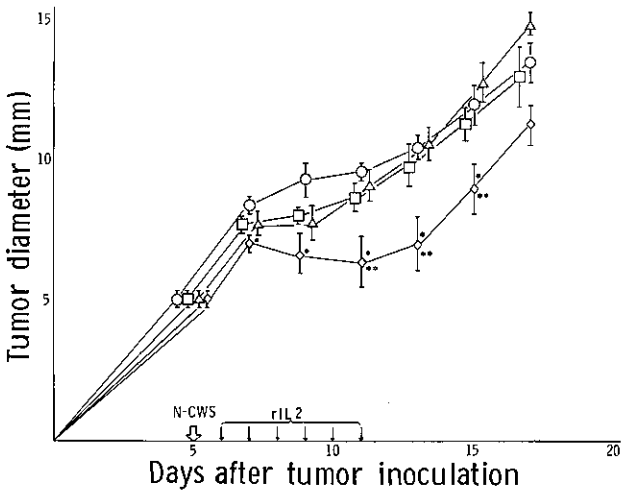

Fig. 3. Tumor growth suppression by the combined therapy with N-CWS and rIL 2. C57BL/6N mice were inoculated sc with 10^6 3LL tumor cells on day 0, and given an intratumoral injection of 100 μg of N-CWS on day 5 (△), 6 daily sc injections of 100 units of rIL 2 from day 6 to 11 (□), or a combination of the two treatments (◇). Control mice were given the medium used for preparing the N-CWS or rIL 2 solution in the same manner (○). Each group consisted of 6 mice. Each point indicates the mean tumor diameter. Bars represent SE. The difference in the mean tumor diameters between the control mice and mice given the combined therapy was statistically significant on days 7 ($P<0.01$), 9 ($P<0.02$), 11 ($P<0.02$), 13 ($P<0.01$) and 15 ($P<0.02$) after tumor inoculation (a single asterisk). The difference between mice given N-CWS or rIL 2 alone and those given the combined therapy was also statistically significant on days 11 ($P<0.05$, $P<0.05$), 13 ($P<0.01$, $P<0.05$) and 15 ($P<0.01$, $P<0.05$) (double asterisks).

N-CWS, developed for use as an immunotherapeutic agent for cancers,[4] has been shown to act as an immunoadjuvant by stimulating host cells, including macrophages, NK and T cells.[5-8] An ip injection of N-CWS into C3H/HeN mice resulted not only in an increase in the number of PC but also in a moderate induction of their LAK activity. By combining N-CWS and rIL 2 treatments, both the increase in the number of nonadherent PC and the induction of their LAK activity were further augmented. This augmented LAK cell induction was observed in the peritoneal cavity but not in spleen, indicating that the combined effect occurred only at the injection site of N-CWS. Peritoneal LAK activity induced by both N-CWS and rIL 2 was mediated by nonadherent and nonphagocytic cells whose cell surface phenotype was Thy-1.2$^{+\sim-}$ and asialo GM$_1^+$, suggesting that the cytotoxic effectors possessed quite similar characteristics to those induced by repeated ip injections of rIL 2 alone.[11]

The IL 2 binding assay showed that the IL 2 binding capacity of the nonadherent PC induced by an ip injection of N-CWS was augmented, compared with that of control cells. In keeping with this observation, additional *in vitro* stimulation with rIL 2 revealed that N-CWS-induced, nonadherent PC exhibited a high responsiveness to rIL 2 resulting in a potent LAK activity, while this treatment induced only a weak LAK activity from control, nonadherent PC. These results strongly suggest that the synergism between N-CWS and rIL 2 in the *in vivo* LAK cell induction is based, at least in part, on the augmentation of the IL 2 binding capacity of peritoneal lymphocytes by N-CWS, resulting in an increase in their

responsiveness to rIL 2. In this study, however, it remained unclear whether the augmented IL 2 binding capacity of N-CWS-induced, nonadherent PC is derived from the increase in the number of IL 2 receptors per cell, or from the accumulation of IL 2 receptor-positive cells in the peritoneal cavity. Furthermore, it is also important to investigate whether N-CWS-induced, nonadherent PC possess high-affinity receptors for IL 2.

It has been generally accepted that the majority of LAK precursors belongs to non-T cell populations.[16] We also reported that the phenotype of unstimulated murine LAK precursors was Thy-1.2$^-$ and asialo GM$_1^+$.[11] In fact, this study showed that nonadherent PC of BALB/c *nu/nu* mice, possessing a high NK, but no mature T cell activity, generated LAK activity more potently than did BALB/c *nu/+* mouse cells when stimulated with rIL 2 alone. On the other hand, the peritoneal LAK precursors induced by N-CWS were shown to be mainly asialo GM$_1^+$, but in part, Thy-1.2$^+$. It seems possible that N-CWS stimulates cells of both NK- and T-derived LAK precursors, although the major part of the precursors may be derived from the NK cell lineage. Indeed, C57BL/6 *bg/bg* mice, lacking normal NK cell activity, could not respond to N-CWS and thus could not generate LAK precursors with a high responsiveness to rIL 2. On the other hand, N-CWS also failed to augment the rIL 2 responsiveness of nonadherent PC in BALB/c *nu/nu* mice. This failure can not be ascribed only to a defect in T-derived LAK precursors, and therefore suggests that mature T cells may be involved in the induction of LAK precursors with an elevated rIL 2 responsiveness.

It was further investigated whether the synergy between N-CWS and rIL 2 on LAK cell induction could result in an *in vivo* antitumor effect. A therapeutic experiment using C57BL/6N mice bearing a LAK-sensitive 3LL tumor revealed that the combination of an intratumoral injection of N-CWS and daily sc injections of rIL 2 produced apparent retardation of the tumor growth, while neither N-CWS nor rIL 2 brought about any noticeable antitumor effect when used alone. Histological examination showed abundant cellular infiltration, including lymphocytes, macrophages and neutrophils, in 3LL tumors receiving both N-CWS and rIL 2 (data not shown). These results strongly suggest that N-CWS can augment the antitumor efficacy of rIL 2 by accumulating LAK precursors with an elevated IL 2 responsiveness at the injection site of N-CWS. In preliminary experiments, LAK activity augmented by N-CWS and rIL 2 declined 2 days after rIL 2 injections were stopped (data not shown). Consistent with this finding, regrowth of the 3LL tumor was observed a few days after the last injection of rIL 2 in the therapeutic experiment. This also strengthens the possibility that LAK cells play an important role in the augmented antitumor interaction between N-CWS and rIL 2, although this study did not produce direct evidence that the augmented antitumor effect is mediated by LAK cells.

More potent antitumor effect might be obtained by repeating the combined therapy. In this study, however, repeated injection of N-CWS was avoided to minimize the effect of activated macrophage involvement in the antitumor mechanism of the combined therapy, because N-CWS strongly activates macrophages when administered repeatedly.[17]

It would be of interest to investigate whether combining systemic administration of rIL 2 with local N-CWS therapy is effective against tumor metastasis. In this study, however, the anti-metastatic effect of the therapy was not investigated, because the 3LL tumors maintained *in vitro* in our laboratory produced no apparent pulmonary metastasis when inoculated sc into the back.

The effect of synergy between rIL 2 and other cytokines on *in vivo* antitumor activities has been shown using a variety of animal tumor models. McIntosh *et al.*[18] reported that a combined therapy consisting of a single iv injection of TNF-α followed by repeated ip injections of rIL 2 produced a marked tumor growth suppression resulting in the prolongation of survival, whereas TNF-α or rIL 2 alone did not. The synergistic antitumor interaction between rIL 2 and TNF-α has been suggested to be based, at least in part, on the increase in IL 2 receptors on TNF-α -stimulated LAK precursors, resulting in the augmentation of rIL 2 effect on LAK cell induction.[19, 20] This is quite similar to the results of the present study. Therefore, it is important to investigate whether TNF mediates the synergistic effect of N-CWS and rIL 2 on LAK cell induction and on antitumor activities, because N-CWS has been shown to be an inducer of TNF.[8] Similarly, IFNs have been shown to act synergistically with rIL 2 on *in vivo* antitumor activities. Iigo *et al.*[21] demonstrated that repeated sc injections of both rIL 2 and IFN-α or -β resulted in the significant suppression of established murine tumors. The combination of rIL 2 and IFN-α was also shown to be effective against established pulmonary metastases of murine tumors.[22] These reports suggest that T cells rather than NK cells are required for the development of the synergistic antitumor effect of rIL 2 and IFNs, since the synergy was diminished by depleting Lyt-2$^+$ cells from mice, and was seen in beige but not in athymic mice. Furthermore, they suggest that IFNs may cooperate with rIL 2 mainly in the augmentation of MHC-restricted, T cell-mediated cytotoxicity rather than of LAK activity, by increasing the expression of MHC antigen on tumor cells.[22] Indeed, no further elevation of splenic LAK activity has been achieved by the additional injection of IFN-α than that shown by rIL 2 alone.[21] Therefore, it seems unlikely that IFNs play a major role

in the augmentation of LAK cell induction by the combined use of N-CWS and rIL 2. To investigate these possibilities, further studies were performed using *in vitro* stimulation of murine spleen cells with N-CWS and rIL 2, the results of which will be reported in detail in the next paper.

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