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Synergistic Effect of *Nocardia rubra* Cell Wall Skeleton and Recombinant Interleukin 2 for *in Vivo* Induction of Lymphokine-activated Killer Cells

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ABSTRACT

C57BL/6 mice inoculated i.p. with 3LL tumor cells were treated by local combination therapy with *Nocardia rubra* cell wall skeleton (N-CWS) and recombinant interleukin 2 (rIL-2). The combination treatment significantly prolonged their survival and augmented lymphokine-activated killer (LAK) activity of peritoneal cavity lymphocytes (PCL), compared with treatments with rIL-2 alone or N-CWS alone. After *in vitro* culture of peritoneal exudate mononuclear cells with rIL-2, the nonadherent population derived from N-CWS-injected tumor-bearing mice showed a significantly higher LAK activity than did that population derived from saline solution-injected mice. When N-CWS-induced PCL were cocultured with either N-CWS-induced macrophages or control macrophages in the presence of rIL-2, their LAK activity was higher than that of control PCL. Therefore, it was suggested that N-CWS-induced PCL themselves have a more potent ability as precursors of LAK cells. Phenotypic analysis on PCL populations revealed that N-CWS-induced PCL contained increased proportions of $CD3^+CD4^-CD8^-$ cells and asialo $GM\text{-}1^+$ cells compared with control PCL. These results suggest that N-CWS selectively accumulates potent LAK precursors, namely, $CD3^+CD4^-CD8^-$ T-cells and asialo $GM\text{-}1^+$ natural killer cells, at the injection site and that LAK cells are efficiently induced by subsequent administration of rIL-2.

INTRODUCTION

Since the discovery of the LAK[3] phenomenon (1), IL-2 has been used clinically in cancer therapy. Systemic administration of IL-2, either alone or combined with *in vitro*-generated LAK cells, resulted in tumor regression in a substantial number of cases. However, the *in vivo* rapid clearance of administered IL-2 necessitated its high-dose administration, resulting in severe clinical toxicities with limited therapeutic benefits (2). To overcome such problems, Yasumoto *et al.* (3) induced LAK cells in the pleural cavity of patients with malignant pleurisy by local (*i.e.*, intrapleural) administration of IL-2, with a successful clinical response, such as the disappearance of malignant cells and effusion. In this context, Tanida *et al.* (4) proposed the concept of the site-selective induction of killer cells based on the results of their experiments in a murine tumor model. These authors accumulated LAK precursors by an i.p. injection of an irritant, proteose-peptone, locally in the peritoneal cavity of the mice which had been transplanted s.c. with fibrosarcoma cells having substantial degrees of tumor-specific antigenicity (4, 5). Injection of IL-2 i.p. following the above treatment caused an efficient induction of LAK cells or tumor-specific cytotoxic T-cells (4, 5). These killer cells were considered to possibly migrate into the tumor site and cause tumor regression.

Proteose-peptone, one of the culture media for bacteria, can not be used for clinical practice. On the other hand, N-CWS has no obvious toxicity when clinically applied to human patients and has been shown to activate various immune cells as a nonspecific immunostimulator or biomodulator (6–13). Antitumor effects have also been demonstrated in the clinical field (14–16). Moreover, the infiltration of inflammatory cells was found at the site injected with N-CWS (9).

In the present study, for a better control of malignant pleurisy and malignant peritonitis, we applied the idea of site-selective induction of LAK cells in a murine malignant peritonitis model. We used a tumor with a negligible degree of tumor-specific antigenicity in order to mimic a large majority of human cancers. We administered N-CWS i.p. as an irritant followed by IL-2. The results clearly demonstrated the synergistic effect of N-CWS and IL-2 on the prolongation of survival and the local induction of LAK cells in tumor-bearing mice. The mechanism of synergy between N-CWS and IL-2 in LAK cell induction was further investigated in this study.

MATERIALS AND METHODS

Mice. Seven- to 8-wk-old male C57BL/6 mice were purchased from Japan SLC, Shizuoka, Japan.

Media. The CM consisted of RPMI-1640 (Nissui Seiyaku Co., Ltd., Tokyo, Japan) supplemented with 10% fetal calf serum (Grand Island Biological Co., Grand Island, NY), 100 units/ml of penicillin G, and 100 μg/ml of streptomycin sulfate.

Tumors. Two tumor cell lines, syngeneic to C57BL/6 mice, were used in this study: Lewis lung carcinoma cells (3LL) and B16 melanoma cells. The tumor cells were maintained in CM.

Reagents. N-CWS was kindly provided by Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan. The lyophilized preparation contained N-CWS (2 mg), squalene (4 mg), Polysorbate 80 (1 mg), and mannitol (28.2 mg). Human rIL-2 was supplied by Takeda Chemical Industries, Ltd., Osaka, Japan. The specific activity was 3.5×10^4 units/mg of protein as determined by the ability to maintain NKC-3 cells (17). The dose was comparable to 1.2×10^7 units/mg of protein by the Biological Response Modifiers Program standard. Usually, 4 units/ml of this rIL-2 can induce optimal LAK activity *in vitro*.

Therapeutic Design. Normal C57BL/6 mice were inoculated i.p. with 1×10^5 3LL tumor cells per mouse on Day 0 and randomly allotted to one of the following groups: Group 1, no therapy; Group 2, i.p. injection of 100 μg of N-CWS on Day 1; Group 3, i.p. injection of 1000 units of rIL-2 on Days 1, 3, and 5; and Group 4, i.p. injection of 100 μg of N-CWS on Day 1, followed by 1000 units of rIL-2 on Days 1, 3, and 5. Each group consisted of 8 mice.

Preparation of PEMC, PCL, and PCM. C57BL/6 mice, inoculated i.p. with 3LL tumor cells, were treated according to the above-mentioned therapeutic design. On Day 6, the peritoneal cavity of each mouse was irrigated twice with 15 ml of HBSS containing 4 units/ml of heparin. The harvested cells were suspended in CM and subjected to two-layered (75% and 100%) Ficoll-sodium diatrizoate gradients (Lympholyte-M; Cedarlane Laboratories, Ltd., Ontario, Canada). The gra-

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[3] The abbreviations used are: LAK, lymphokine-activated killer; N-CWS, *Nocardia rubra* cell wall skeleton; rIL-2, recombinant interleukin 2; PCL, peritoneal cavity lymphocytes; PEMC, peritoneal exudate mononuclear cells; CM, complete culture medium; HBSS, Hanks' balanced salt solution; PCM, peritoneal cavity macrophages; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; PE, phycoerythrin; SA-DC, streptavidine-duochrome; IL-2, interleukin 2; N-PCL, *N. rubra* cell wall skeleton-induced peritoneal cavity lymphocytes; C-PCL, control peritoneal cavity lymphocytes; N-PCM, *N. rubra* cell wall skeleton-induced peritoneal cavity macrophages; C-PCM, control peritoneal cavity macrophages.

dient was centrifuged for 20 min at 500 × *g* and room temperature. Mononuclear cells were collected from the 100% interface. The cells were washed 3 times with HBSS and resuspended in CM. The cell suspension was placed in a plastic culture dish and incubated at 37°C in 5% CO_2 and 95% air. After 2-h incubation, nonadherent cells were recovered and used as PCL. More than 95% were lymphocytes, and the contamination of 3LL tumor cells was less than 1%, as assessed by Wright-Giemsa and Papanicolaou's stainings.

C57BL/6 mice were inoculated i.p. with 1×10^5 3LL tumor cells on Day 0 and then given injections i.p. of 100 μg of N-CWS or saline solution on Day 1. On Day 4, the peritoneal cavity of each mouse was irrigated, and peritoneal cavity cells were obtained as described above. The cells suspended in CM were subjected to two-layered (75% and 100%) Ficoll-sodium diatrizoate gradients (Lympholyte-M) and centrifuged for 20 min at 500 × *g* at room temperature. The mononuclear cells collected from the 100% interface were used as PEMC in further experiments. PEMC were incubated in plastic dishes at 37°C in 5% CO_2. After 2-h incubation, nonadherent cells were collected and used as PCL. Plastic adherent cells were detached by a jet stream of HBSS through a 26 gauge needle and used as PCM. The latter cells were cytologically identified as consisting of more than 95% macrophages, as assessed by Wright-Giemsa staining.

Culture of PEMC or PCL for *in Vitro* Induction of LAK Cells. PEMC or PCL were adjusted at 1×10^6 cells/ml in CM supplemented with 5×10^{-5} M of 2-mercaptoethanol and 0.1 unit/ml of rIL-2 and incubated in a plastic dish for 4 days at 37°C in 5% CO_2.

Cytolysis Assay. The cytotoxic activity of various cell populations was assessed by a standard 4-h ^{51}Cr release assay as described elsewhere (3). ^{51}Cr-labeled target cells (5×10^3 cells/well) were added to various numbers of effector cells in 96-well round-bottomed microtiter plates. After 4-h incubation at 37°C in 5% CO_2, the supernatants were harvested and counted for radioactivity with a gamma counter. Maximal and spontaneous releases of ^{51}Cr were obtained by incubation of the target cells with 0.1 N hydrochloric acid and medium alone, respectively. The percentage of specific cytolytic activity was calculated according to the following formula

$$\% \text{ of cytolysis} = \frac{\text{experimental (cpm)} - \text{spontaneous (cpm)}}{\text{maximal (cpm)} - \text{spontaneous (cpm)}} \times 100$$

All determinations were made in triplicate, and all data are given as the mean of triplicate determinations.

Phenotypic Analysis of PCL. The surface phenotype of the PCL population was determined by flow cytometric analysis. FITC-conjugated anti-CD3 mAb (145-2C11) was generously provided by Dr. J. A. Bluestone. FITC-conjugated anti-mouse immunoglobulin M antibody was purchased from Zymed Laboratories, Inc., San Francisco, CA. Rabbit anti-asialo GM-1 serum was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Biotin-conjugated anti-IL-2 receptor mAb was prepared from 7D4 culture supernatants (18). PE-conjugated streptavidine, PE-conjugated anti-CD4 mAb, biotin-conjugated anti-CD8 mAb, and SA-DC were purchased from Becton Dickinson, Mountain View, CA. FITC-conjugated goat anti-rabbit immunoglobulin G antibody was purchased from Tago, Inc., Burlingame, CA. Cells were stained with the above antibodies in either a direct or indirect manner and examined for fluorescence with a FACScan (Becton Dickinson).

Statistical Analysis. Statistical analysis was performed by Student's *t* test, and a *P* value less than 0.05 was judged as significant.

RESULTS

Therapeutic Effect of Combination Immunotherapy with N-CWS and rIL-2 on 3LL-bearing Mice. Mice, inoculated i.p. with 3LL tumor cells, received various immunotherapies consisting of i.p. injection of N-CWS and/or rIL-2. The effects on survival are shown in Fig. 1 and Table 1. N-CWS alone had no effect on survival. The mice treated with rIL-2 alone survived significantly longer than the control mice (mean, 30.0 days *versus* 22.8 days; $P < 0.01$). Furthermore, the combination treatment with N-CWS and rIL-2 resulted in a significant prolongation of survival, compared with the treatment with rIL-2 alone (mean, 40.5 days *versus* 30.0 days; $P < 0.01$). Three of 8 mice treated with N-CWS and rIL-2 remained alive for over 180 days after treatment.

Effect of Combination Immunotherapy with N-CWS and rIL-2 on Cytotoxicity of PCL. To analyze the synergistic effect of N-CWS and rIL-2 on survival, PCL were prepared from 3LL-bearing mice after various treatments and then were examined for *in vitro* cytotoxicity against 3LL and B16 tumor cells. The results are shown in Table 2. PCL from control mice or mice treated with N-CWS alone had no significant level of cytotoxicity. On the other hand, PCL from mice treated with rIL-2 exhibited a cytolytic activity against both 3LL and B16 targets. The combination treatment with N-CWS and rIL-2 produced a significantly higher cytotoxicity of PCL against both targets than the cytotoxicity which resulted in the treatment with rIL-2 alone. These results were compatible with those of the experiment on survival and suggest that the prolonged survival by the combination treatment with N-CWS and rIL-2 was caused by the augmentation of PCL cytotoxicity.

Characteristics of PEMC Induced by N-CWS. To investigate the role of N-CWS in synergy with rIL-2, PEMC were obtained from tumor-bearing mice on Day 3 after i.p. injection of either N-CWS or saline solution and then were used in further experiments. The yielded number and cellular classification of PEMC after an i.p. injection of N-CWS are shown in Table 3. The number of PEMC increased about 3.5-fold by N-CWS administration. An increase of the macrophage proportion was observed in N-CWS-induced PEMC accompanied with the relative decrease of lymphocytes, compared with control PEMC (saline solution i.p.). N-CWS-induced PEMC and control PEMC were cultured *in vitro* with a suboptimal concentration

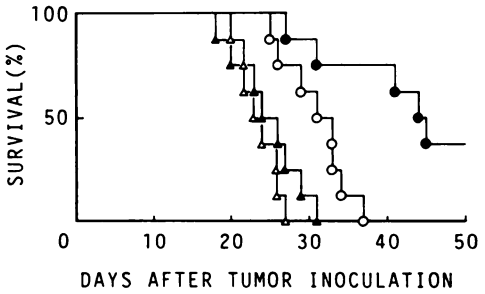

Fig. 1. Prolongation of survival time of 3LL-bearing C57BL/6 mice by combination treatment with N-CWS and rIL-2. C57BL/6 mice were inoculated i.p. with 1×10^5 3LL tumor cells on Day 0 and received: no treatment (△); i.p. injection of 100 μg of N-CWS alone on Day 1 (▲); i.p. injection of 1000 units of rIL-2 alone on Days 1, 3, and 5 (○); or i.p. injection of 100 μg of N-CWS on Day 1 and 1000 units of rIL-2 on Days 1, 3, and 5 (●). All mice were followed daily for survival.

Table 1 *Survival time of 3LL-bearing C57BL/6 mice*

Group[a]	Survival (days)[b] Range	Median	Mean ± SD
1. Control	19–26	22.5	22.8 ± 2.4
2. N-CWS alone	17–30	24.0	23.8 ± 4.4
3. rIL-2 alone	24–36	31.0	30.0 ± 4.1[c]
4. N-CWS + rIL-2	26–47	43.5	40.5 ± 8.2[d]

[a] 3LL-bearing C57BL/6 mice received various treatments described in Fig. 1.
[b] Survival time was evaluated on Day 47.
[c] $P < 0.01$ (control *versus* rIL-2); $P < 0.05$ (N-CWS *versus* rIL-2).
[d] $P < 0.01$ (control *versus* N-CWS + rIL-2); $P < 0.01$ (N-CWS *versus* N-CWS + rIL-2); $P < 0.01$ (rIL-2 *versus* N-CWS + rIL-2).

Table 2 *Augmentation of cytotoxicity of PCL from 3LL-bearing mice by combination treatment with N-CWS and rIL-2*

In vivo treatment[a]	% of cytotoxicity against the following[b] 3LL	B16
No treatment	7.4 ± 0.7[c]	0.5 ± 0.4
N-CWS alone	8.5 ± 0.2	0.6 ± 0.2
rIL-2 alone	57.1 ± 2.0[d]	28.7 ± 4.2[e]
N-CWS + rIL-2	64.9 ± 2.4	46.8 ± 1.3

[a] C57BL/6 mice were inoculated i.p. with 1×10^5 3LL tumor cells on Day 0 and then treated with an i.p. injection of 100 μg of N-CWS on Day 1 or 1000 units of rIL-2 on Days 1, 3, and 5. PCL were harvested and tested on Day 6.
[b] Effector:target ratio = 100:1.
[c] Mean ± SE.
[d] $P < 0.05$ (rIL-2 alone *versus* N-CWS + rIL-2).
[e] $P < 0.01$ (rIL-2 alone *versus* N-CWS + rIL-2).

Table 3 *Changes of number and cellular components of peritoneal exudate mononuclear cells after i.p. injection of N-CWS*

In vivo treatment[a]	Total cells/mouse	Lymphocyte (%)	Macrophage (%)
Saline solution i.p.	1.5×10^6	45.8	54.2
N-CWS i.p.	5.2×10^6	28.2	71.8

[a] C57BL/6 mice were inoculated i.p. with 1×10^5 3LL tumor cells on Day 0 and then given injections i.p. of 100 μg of N-CWS or saline solution on Day 1. Peritoneal exudate mononuclear cells were harvested and examined cytotologically on Day 4.

Table 4 *Augmentation of cytotoxicity of nonadherent cells in N-CWS-induced PEMC cultured with rIL-2 in vitro*

In vivo treatment[a]	% of cytotoxicity against the following[b] 3LL	B16
Saline solution i.p.	37.3 ± 1.0[c, d]	29.8 ± 0.5[e]
N-CWS i.p.	61.9 ± 2.5	50.3 ± 1.4

[a] C57BL/6 mice were inoculated i.p. with 1×10^5 3LL tumor cells on Day 0 and then given injections i.p. of 100 μg of N-CWS or saline solution on Day 1. Peritoneal exudate mononuclear cells were harvested on Day 4 and cultured *in vitro* with 0.1 unit/ml of rIL-2 for 4 days. Nonadherent cells were recovered and tested on Day 8.
[b] Effector:target ratio = 20:1.
[c] Mean ± SE.
[d] $P < 0.01$ (saline solution *versus* N-CWS).
[e] $P < 0.01$ (saline solution *versus* N-CWS).

of rIL-2 (0.1 unit/ml) for 4 days. After culturing, the nonadherent cells were harvested and examined for LAK activity. As shown in Table 4, nonadherent cells from N-CWS-induced PEMC exhibited a markedly higher LAK activity than those derived from control PEMC. Since N-CWS has been shown to be a potent activator of macrophages, the effect of PCM induced by N-CWS on the induction of LAK activity was next investigated. N-PCL or C-PCL were added with N-PCM or C-PCM in a criss-crossed manner (PCL:PCM = 4:1). The mixed cell populations were incubated in the presence of rIL-2 (0.1 unit/ml) for 4 days. The LAK activity of PCL from each culture is summarized in Table 5. The LAK activity of N-PCL was significantly higher than that of C-PCL, irrespective of the source of PCM added to the culture. These results indicated that PCL induced by i.p. administration of N-CWS had a greater potential to acquire LAK activity.

Surface Phenotype of PCL Induced by N-CWS. To further characterize N-PCL, a phenotypic analysis of PCL was performed. As shown in Table 6, N-PCL contained an approximately 1.6-fold higher proportion of asialo GM-1$^+$ cells than C-PCL. There was no difference in the proportion of CD3$^+$ T-cells between N-PCL and C-PCL. A further investigation on the subpopulation of CD3$^+$ cells by a three-color staining method revealed that the proportion of CD3$^+$CD4$^-$CD8$^-$ cells (double-negative T-cells) markedly increased in N-PCL (Fig. 2). Contrary to expectations, the proportion of IL-2 receptor-positive cells in N-PCL was the same as that in C-PCL (Table 6).

DISCUSSION

The present results suggest that N-CWS can be applied to clinical practice as an irritant in order to accumulate LAK precursors to the inflammatory site. The *in vivo* effect by combination therapy with N-CWS and rIL-2 on the survival of tumor-bearing mice may suggest its usefulness in cancer patients with comparable situations.

Further, we investigated the mechanism of synergy between N-CWS and IL-2 on LAK cell induction. After an i.p. injection with N-CWS, the PEMC increased in number by about 3.5-fold over the control PEMC (saline solution i.p.). When all PEMC were cultured with rIL-2 for 4 days, the nonadherent cells derived from N-CWS-induced PEMC acquired a significantly higher LAK activity than did those derived from control PEMC. In a culture experiment with mixed cell populations to determine which cell population was responsible for the higher LAK activity, the LAK activity of N-PCL was found not to be influenced by the different origins of added macrophages, namely, N-CWS-induced or control ones. These results suggested that N-CWS-induced PCL themselves have a very potent ability as precursors of LAK cells.

Phenotypic analysis of N-PCL revealed the increase in proportion of CD3$^+$CD4$^-$CD8$^-$ cells and of asialo GM-1$^+$ cells. Asialo GM-1$^+$ natural killer cells are generally accepted to be a major precursor of LAK cells (19, 20). Furthermore, it has been recently reported that CD3$^+$CD4$^-$CD8$^-$ cells (double negative T-cells), not classical T-cells, could lyse a wide variety of tumor cells after incubation with IL-2 (21, 22). Therefore, it is considered that N-CWS preferably accumulated potent precursors of

Table 5 *No augmentative effect of N-CWS-induced macrophages on the induction of LAK cells in vitro*

Group	PCL	Cultured with[a]	% of cytotoxicity against the following[b] 3LL	B16
1.	C-PCL	C-PCM	44.1 ± 3.9[c-e]	38.0 ± 3.1[f]
2.	C-PCL	N-PCM	39.6 ± 0.5	31.4 ± 0.4
3.	N-PCL	C-PCM	63.8 ± 1.3	58.3 ± 0.7
4.	N-PCL	N-PCM	67.7 ± 1.6	58.0 ± 0.8

[a] C57BL/6 mice were inoculated i.p. with 1×10^5 3LL tumor cells on Day 0 and then given injections i.p. of 100 μg of N-CWS or saline solution on Day 1. On Day 4, C-PCL and C-PCM were harvested from control mice (saline solution i.p.), and N-PCL and N-PCM were harvested from N-CWS-injected mice. PCL and PCM were reconstituted into 4 groups and cultured with 0.1 unit/ml of rIL-2 for 4 days.
[b] Effector:target ratio = 20:1.
[c] Mean ± SE.
[d] $P < 0.05$ (Group 1 *versus* Group 3).
[e] $P < 0.01$ (Group 1 *versus* Group 4).
[f] $P < 0.05$ (Group 1 *versus* Group 3, Group 1 *versus* Group 4).

Table 6 *Surface phenotypes of peritoneal cavity lymphocytes*

	In vivo treatment[a]	% of cells positive for CD3	Surface immunoglobulin	Asialo GM-1	IL-2 receptor
PCL	Saline solution i.p.	23.6	61.7	15.0	46.5
PCL	N-CWS i.p.	22.4	55.9	23.7	40.3

[a] C57BL/6 mice were inoculated i.p. with 1×10^5 3LL tumor cells on Day 0 and then given injections i.p. of 100 μg of N-CWS or saline solution on Day 1. On Day 4, PCL were harvested from each group and stained with the indicated antibodies. Stained cells were analyzed by FACScan.

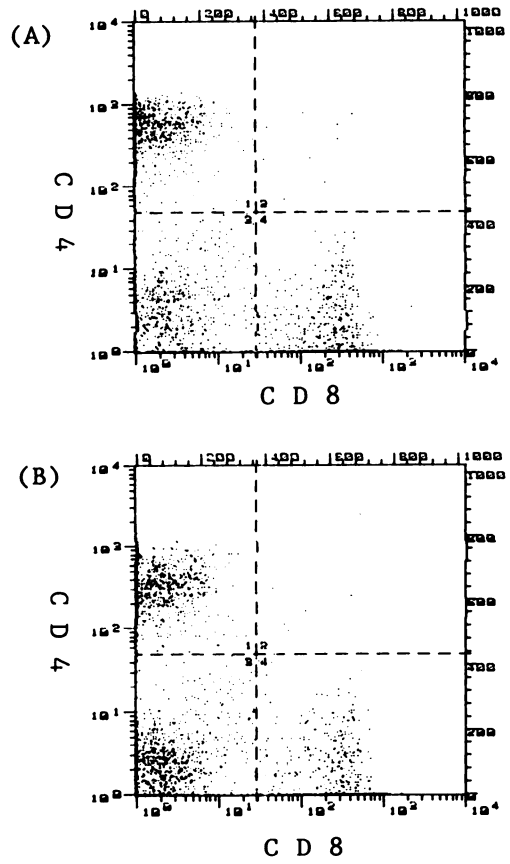

Fig. 2. Three-color cytometric analysis of surface CD4 and CD8 expression on CD3-positive cells of PCL from control mice and PCL induced by N-CWS. C57BL/6 mice were inoculated i.p. with 1×10^5 3LL tumor cells on Day 0 and then given injections i.p. of 100 μg of N-CWS or saline solution on Day 1. On Day 4, PCL were harvested from each group. PCL from control mice (saline solution i.p.) (*A*) and PCL from N-CWS-injected mice (*B*) were stained with FITC-conjugated anti-CD3 mAb, PE-conjugated anti-CD4 mAb, and biotin-conjugated anti-CD8 mAb followed by SA-DC and analyzed by FACScan. The analysis gate was set on $CD3^+$ cells, and profiles of fluorescence intensity of PE (CD4) and duochrome (CD8) were displayed. The proportions of $CD3^+CD4^+CD8^-$ cells, $CD3^+CD4^-CD8^+$ cells, and $CD3^+CD4^-CD8^-$ cells in control PCL were 50.3%, 28.7%, and 20.6%, respectively. The proportions of $CD3^+CD4^+CD8^-$ cells, $CD3^+CD4^-CD8^+$ cells, and $CD3^+CD4^-CD8^-$ cells in N-CWS-induced PCL were 40.2%, 26.3%, and 33.3%, respectively.

LAK cells at the injection site and resulted in the efficient induction of LAK cells. Recently, Kawase *et al.* (23) also reported the augmentative effect of N-CWS on the *in vivo* induction of LAK cells. They concluded that N-CWS did augment the IL-2 responsiveness of PCL. In our case, however, the proportion of IL-2 receptor-positive cells in N-PCL was not different from that in C-PCL. Thus, we consider that direct activation of the mechanism for expression of IL-2 receptors in PCL by N-CWS may not be involved in the augmentative effect on LAK cell induction as the principal mechanism. At present, we do not know the exact mechanism for the preferable accumulation of LAK precursor by N-CWS. It has been reported that various cytokines, including interferon (11), colony-stimulating factor (12), and tumor necrosis factor (13), can be endogenously produced by stimulation with N-CWS. It is still necessary to analyze exactly what cytokines are involved in this phenomenon.

We have not observed any significant peritoneal adhesions in the mice treated with N-CWS and rIL-2, compared with rIL-2 alone or control mice. Thus, in humans, malignant peritonitis can be treated with N-CWS and rIL-2. Further, this phenomenon can be clinically applied to accessible localized tumor lesions other than malignant peritonitis or pleuritis. For example, surgically residual tumors can be injected with N-CWS during an operation. By this procedure, the effect of following systemic administration of rIL-2 can be augmented at the tumor site.

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