Jpn. J. Cancer Res. 80, 1098–1105, November 1989

Augmentation of Murine Lymphokine-activated Killer Cell Induction by a Factor Produced by *Nocardia rubra* Cell Wall Skeleton-stimulated T Cells

Ichiro Kawase,[1] Takuma Shirasaka, Toshiyuki Ikeda, Hideki Hara, Yoshiro Tanio, Masatoshi Watanabe, Shin'ichi Saito, Tomiya Masuno, Susumu Kishimoto and Yuichi Yamamura

The Third Department of Internal Medicine, Osaka University Medical School, 1-1-50, Fukushima, Fukushima-ku, Osaka 553

Four-hour exposure of C3H/HeN mouse spleen cells to *Nocardia rubra* cell wall skeleton (N-CWS) before 4-day culture with a suboptimal dose of human recombinant interleukin 2 (rIL 2) augmented the induction of lymphokine-activated killer (LAK) cell activity, whereas the treatment with N-CWS alone induced no cytotoxicity. In accordance with this, the IL 2 binding activity of spleen cells was augmented by combined stimulation with N-CWS and rIL 2. The augmented cytotoxicity was mediated by Thy-1.2$^+$, Lyt-1.1$^-$, Lyt-2.1$^-$ and asialo GM$_1$$^+$ cells. Cell cultures in diffusion chambers revealed that N-CWS-treated spleen cells produced a LAK cell induction-helper factor (LAK-helper factor, LHF) when cultured with rIL 2. The LHF production required Thy-1.2$^+$, Lyt-1.1$^+$, Lyt-2.1$^+$ and asialo GM$_1$$^-$ cells, and the coexistence of unstimulated accessory cells was also essential for the LHF production. Cells responding to both LHF and rIL 2 to generate LAK activity were Thy-1.2$^-$, Lyt-1.1$^-$, Lyt-2.1$^-$ and asialo GM$_1$$^+$. The culture fluid of spleen cells stimulated with both N-CWS and rIL 2 contained no tumor necrosis factor (TNF) activity, and the additional stimulation with N-CWS caused no production of either IL 2 or interferon (IFN). Murine recombinant interleukin 1α (Mu-rIL 1α) could not replace the augmentative effect of N-CWS on LAK cell induction. These results suggest that in the presence of rIL 2, N-CWS stimulates murine T cells to produce LHF that is probably distinct from IL 1, IL 2, TNF and IFN.

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

There has been increasing evidence that the combined use of human recombinant interleukin 2 (rIL 2)[2] and other biological response modifiers (BRM) results in a potent augmentation of the antitumor efficacy of rIL 2.[1-3] In these reports, the augmented antitumor effects have been shown to be based on different effector mechanisms, depending on the biological activities of BRM used in combination with rIL 2. For example, tumor necrosis factor (TNF) augments the responsiveness of lymphocytes to IL 2 by increasing the number of IL 2 receptors on the cell surfaces, resulting in the augmentation of the efficacy of IL 2 to induce lymphokine-activated killer (LAK) cell activity.[4,5] On the other hand, interferon (IFN)-α or -β may cooperate with IL 2 in the augmentation of MHC-restricted, T cell-mediated cytotoxicity by enhancing the expression of MHC antigen on tumor cells.[2]

We recently demonstrated that *Nocardia rubra* cell wall skeleton (N-CWS), a bacterial immunoadjuvant, potentiates the antitumor efficacy of rIL 2 not only by accumulating LAK precursors at the injection site of N-CWS but also by increasing their responsiveness to rIL 2 for the generation of LAK activity. Furthermore, we showed that both natural killer (NK) and T cells are involved in the synergistic effect of rIL 2 and N-CWS on *in vivo* LAK cell induction.[6] To investigate more precisely the involvement of these cells in the augmented LAK cell induction, a possible role of a soluble factor produced by N-CWS-stimulated cells in the augmentative effect of N-CWS on LAK cell induction was analyzed *in vitro* using a diffusion chamber culture system. In this paper, we demonstrate that in the presence of rIL 2, N-CWS stimulates murine T cells to produce a LAK cell induction-augmenting factor that is probably distinct from IL 1, IL 2, TNF and IFN, and this factor cooperates with rIL 2 in LAK cell induction from NK cells.

MATERIALS AND METHODS

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

[1] To whom communication should be addressed.

[2] Abbreviations used are: rIL 2, recombinant interleukin 2; BRM, biological response modifier; CSF, colony-stimulating factor; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; IFN, interferon; IL 1, interleukin 1; IL 2, interleukin 2; IL 4, interleukin 4; KHF, killer-helper factor; LAK, lymphokine-activated killer; LHF, LAK-helper factor; MAF, macrophage-activating factor; Mu-rIL 1α, murine recombinant interleukin 1α; N-CWS, *Nocardia rubra* cell wall skeleton; NK, natural killer; NW, nylon wool; PBL peripheral blood lymphocytes; TNF, tumor necrosis factor.

and 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 passages *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 penicillin (Meiji Seika Co., Tokyo), and 100 μg/ml streptomycin (Meiji Seika). This medium was designated the complete medium.

Recombinant interleukin 1 (rIL 1), rIL 2 and N-CWS Murine rIL 1α (Mu-rIL 1α, 1000 units/ml) was purchased from Genzyme, Boston, MA. The derivation and the specific activity of human rIL 2 and N-CWS were described previously.[6]

Preparation and fractionation of spleen cells Spleen cells were prepared by teasing spleens in RPMI 1640 medium supplemented with 2% heat-inactivated FBS (2% FBS-RPMI 1640 medium), washing them twice, and suspending them in the complete medium. Nylon wool (NW)-nonadherent cells were obtained by 2 consecutive passages of spleen cells through NW columns (Wako Pure Chemicals Co., Osaka), as reported previously.[7] To obtain NW-adherent cells, vigorous rinsing and pressing of the first NW of the 2 NW columns was repeated 4 times in ice-cold 2% FBS-RPMI 1640 medium, and cells detached from the NW at the final rinsing were collected by centrifugation of the wash medium. The recovery and the purity of the fractionated cells were previously described.[7]

Treatment of cells with antibodies and complement As described previously,[6] cells were treated with 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; 1/50 dilution), and then with rabbit complement (Cedarlane; 1/8 dilution).

Treatment of cells with N-CWS Sixty million cells were suspended in 10 ml of the complete medium supplemented with 5×10^{-5} *M* 2-mercaptoethanol (Wako) and 10 μg/ml of N-CWS in a 25 cm^2 plastic flask (No. 25100, Corning, New York, NY), incubated for 4 h at 37°C in a humidified 5% CO_2 atmosphere, and then washed 3 times. Cells incubated in the medium without N-CWS seved as the control. In some experiments, spleen cells treated with antibodies and complement or fractionated with NW were used for the N-CWS treatment.

Incubation of cells with rIL 2 Three million medium- or N-CWS-treated cells were suspended in 1.5 ml of the complete medium supplemented with 5×10^{-5} *M* 2-mercaptoethanol, 10 m*M* HEPES buffer (GIBCO, Grand Island, NY) and rIL 2 at concentrations ranging from 0.025 to 0.2 unit/ml, and incubated for 4 days at 37°C in a humidified 5% CO_2 atmosphere in a well of a 24-well culture plate (No. 3424, Costar, Cambridge, MA). In some experiments, Mu-rIL 1α was added at concentrations ranging from 1 to 100 units/ml to the culture at the beginning of the 4-day culture.

Cell culture in diffusion chambers Five million medium- or N-CWS-treated spleen cells were suspended in 1.2 ml of the complete medium supplemented with 5×10^{-5} *M* 2-mercaptoethanol and 10 m*M* HEPES buffer, and added to a lower compartment of a 24-well double chamber culture plate (No. 3409, Costar). Eight hundred thousand normal spleen cells suspended in 0.3 ml of the same medium were added to an upper compartment possessing a microporous membrane (pore size: 0.4 μm) at the bottom. The upper compartment was placed in the lower compartment so that its lower half was steeped in 1.2 ml of the cell suspension. RIL 2 was added at a final concentration of 0.1 unit/ml, and the plate was incubated at 37°C for 4 days in a humidified 5% CO_2 atmosphere. In experiments involving the cellular reconstitution of N-CWS-treated spleen cells using NW-nonadherent and -adherent cells, 5×10^6 N-CWS treated, NW-nonadherent cells were mixed with 5×10^5 NW-adherent cells treated with N-CWS or with medium alone in 1.2 ml of the same medium as described above and then added to the lower compartment.

Cytotoxicity assay Spleen cells cultured for 4 days were tested for cytolytic activity against 3LL tumor cells by 4-h ^{51}Cr-release assay at an E/T ratio of 12.5/1, and analyzed statistically as described previously.[6]

IL 2 binding assay IL 2 binding assay was performed using 0.6 n*M* ^{125}I-labeled rIL 2 as described previously.[6]

IL 2, IFN and TNF assay IL 2 activity was determined by a conventional cell growth assay using CT6, an IL 2-dependent T cell line, with rIL 2 as a standard.[8] IFN activity was titrated as the ability to inhibit the cytopathic effects of vesicular stomatitis virus on a monolayer of L929 cells.[9] Assays of TNF activity were performed with a cytotoxicity test using L929 cells as a target, and the lytic activity was quantitated by a 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide dye reduction assay.[10]

RESULTS

Augmentation of *in vitro* LAK cell induction by N-CWS To investigate the effect of N-CWS on *in vitro* LAK cell induction from murine spleen cells, N-CWS was added to a 4-day culture of C3H/HeN spleen cells with rIL 2. It was found that N-CWS did not augment, but suppressed LAK cell induction when N-CWS had been present during the 4-day culture (data not shown). Therefore, N-CWS was removed by repeated washings of the cells before the start of 4-day culture. As shown in Fig. 1,

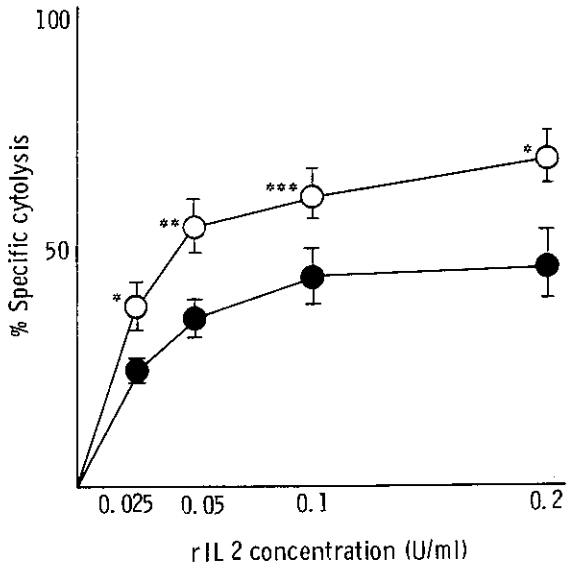

Fig. 1. Augmentation of LAK cell induction by N-CWS *in vitro*. C3H/HeN spleen cells were cultured in medium alone (●) or in the presence of 10 μg/ml of N-CWS (○) for 4 h, washed 3 times, and then cultured for 4 days in the presence of rIL 2 at a concentration indicated. After 4-day culture, cells were tested for cytolytic activity against 3LL tumor cells by 4-h ^{51}Cr-release assay at an E/T ratio of 12.5/1. Bars represent the mean±SD for triplicate cultures. The mean differences in the cytolytic activity between medium- and N-CWS-treated spleen cells were statistically significant (single asterisk; $P<0.02$, double asterisks; $P<0.01$, triple asterisks; $P<0.05$).

N-CWS-treated spleen cells generated a higher LAK activity than that shown by medium-treated cells when cultured in the presence of 0.025–0.2 unit/ml of rIL 2. In the absence of rIL 2, however, N-CWS-treated spleen cells did not generate any cytotoxic activity during the 4-day culture. Preliminary experiments revealed that a 4-h stimulation with N-CWS was the optimal period for the augmentation of LAK cell induction, that there was no difference in cell recovery after the 4-day culture with rIL 2 between N-CWS- and medium-treated spleen cells, and that the augmentative effect of N-CWS on LAK cell induction could no longer be detected when rIL 2 had been used at a concentration of more than 1 unit/ml, because LAK activity was fully induced by rIL 2 alone (data not shown). The cytotoxic effectors induced *in vitro* by both N-CWS and rIL 2 were found to be Thy-1.2$^+$, Lyt-1.1$^-$, Lyt-2.1$^-$ and asialo GM$_1$$^+$ (Table I).

Augmented IL 2 binding capacity of spleen cells stimulated by N-CWS and rIL 2 As shown in Fig. 2, a 4-day culture of spleen cells with rIL 2 alone resulted in a weak but distinct augmentation of the IL 2 binding capacity, compared with that shown by the cells cultured with medium alone. The effect of rIL 2 was further augmented when a 4-h stimulation with N-CWS preceded the 4-day culture with rIL 2, whereas the addi-

Table I. Phenotype of Splenic LAK Cells Induced by rIL 2 and N-CWS

Treatment with N-CWS[a)]	Addition of rIL 2[b)]	Treatment of effector cells[c)]	% Specific cytolysis[d)]
−	−	medium	−2.2±0.1
−	+	medium	27.3±2.5[e)]
+	+	medium	52.7±5.3[f)]
+	+	C	55.3±3.0
+	+	anti-Thy-1.2+C	15.1±1.3[g)]
+	+	anti-Lyt-1.1+C	44.6±4.1[h)]
+	+	anti-Lyt-2.1+C	45.2±3.0[i)]
+	+	anti-asialo GM$_1$+C	14.3±1.1[j)]

a) C3H/HeN spleen cells were incubated in medium alone or in the presence of 10 μg/ml of N-CWS for 4 h, and washed 3 times.
b) The cells were then cultured in medium alone or in the presence of 0.1 unit/ml of rIL 2 for 4 days.
c) After the 4-day culture, cells were treated with antibodies and complement. The number of cells was then counted, and the cell density adjusted to 6.25×10^5/ml. Cell depletion data showed that Thy-1.2$^+$ accounted for 50%, Lyt-1.1$^+$ for 21.4%, Lyt-2.1$^+$ for 17.9% and asialo GM$_1$$^+$ cells for 41.1%.
d) Cytotoxicity against 3LL tumor cells was determined by a 4-h ^{51}Cr-release assay at an E/T ratio of 12.5/1. Data represent the mean±SD for triplicate cultures. Statistical significance: *e*) vs. *f*), $P<0.01$; *f*) vs. *g*), $P<0.001$; *f*) vs. *h*), not significant; *f*) vs. *i*), not significant; *f*) vs. *j*), $P<0.001$.

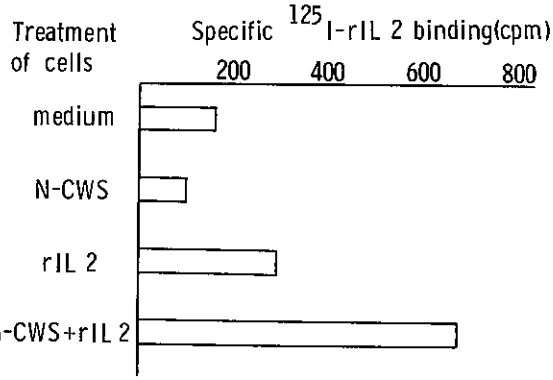

Fig. 2. Augmented IL 2 binding capacity of spleen cells stimulated with both N-CWS and rIL 2. Spleen cells treated with medium alone or with 10 μg/ml of N-CWS for 4 h were cultured in medium alone (medium, N-CWS) or in the presence of 0.05 unit/ml of rIL 2 (rIL 2, N-CWS+rIL 2) for 4 days. After culture, cells were washed 3 times, cultured in medium for 2 h to consume rIL 2 bound to IL 2 receptors, and then applied to the IL 2 binding assay. Data represent the mean of duplicate cultures.

tional N-CWS stimulation alone did not augment but rather weakly suppressed the IL 2 binding capacity.

Production of a factor capable of augmenting LAK cell induction by N-CWS-treated spleen cells in the presence of rIL 2 To investigate whether the augmentative effect of N-CWS on LAK cell induction could be mediated by a soluble factor, N-CWS-treated spleen cells were cultured alone for 2–4 days. The culture fluid was then tested for the ability to substitute for the augmentative effect of N-CWS on LAK cell induction. No further augmentation of LAK cell induction was observed when the culture fluid was added to the 4-day culture of normal spleen cells with rIL 2 (0.1 unit/ml), indicating that N-CWS-treated spleen cells did not produce any factors capable of augmenting LAK cell induction when cultured alone (data not shown). In the presence of rIL 2, however, N-CWS-treated spleen cells produced a factor mediating this response. Normal spleen cells were cultured for 4 days in the upper, and N-CWS-treated spleen cells in the lower compartment, each with 0.1 unit/ml of rIL 2 in a diffusion chamber culture plate (Fig. 3). In the lower compartment, N-CWS-treated spleen cells generated LAK activity more potently than did medium-treated spleen cells when cultured with rIL 2. At the same time, normal spleen cells cultured in the upper compartment generated LAK activity in response to rIL 2, and the LAK cell generation in the upper compartment was augmented only when the lower compartment had contained N-CWS-treated spleen cells, suggesting that N-CWS-treated spleen cells produced a factor capable of augmenting LAK cell induction (LAK-helper factor, LHF) in the presence of rIL 2.

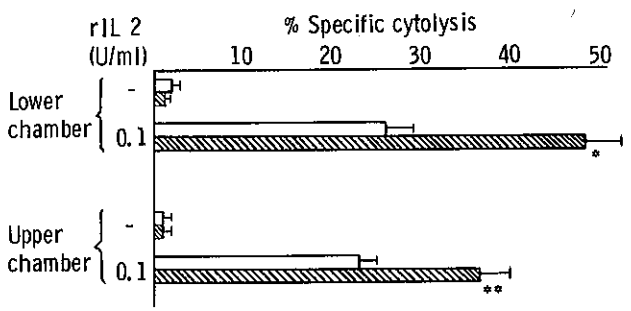

Fig. 3. Production of LAK-helper factor (LHF) by N-CWS-treated spleen cells in the presence of rIL 2. In a double-chamber culture plate, the upper compartment containing 8×10^5 normal spleen cells was placed in the lower compartment containing 5×10^6 N-CWS- (▨) or medium-treated spleen cells (□), and rIL 2 was added at a concentration of 0.1 unit/ml. The plate was cultured for 4 days, and then cells of the upper and the lower compartment were tested for cytolytic activity against 3LL tumor cells as described in the legend to Fig. 1. The mean differences in the cytolytic activity between medium- and N-CWS-treated spleen cells were statistically significant (single asterisk; $P<0.001$, double asterisks; $P<0.01$).

Cells responsible for the production of LHF To analyze the cells producing LHF in response to N-CWS, spleen cells were treated with antibodies and complement before N-CWS treatment, and tested for the ability to augment LAK cell induction occurring in the upper compartment. As shown in Fig. 4, anti-Thy-1.2, -Lyt-1.1, and -Lyt-2.1 treatment abrogated this activity of N-CWS-treated cells, while cells given anti-asialo GM_1 treatment still augmented LAK cell induction occurring in the upper compartment.

Cells essential for LHF production were further analyzed by the fractionation of cells with NW (Fig. 5). The ability of N-CWS-treated spleen cells to produce LHF was reduced by 2 consecutive passages of the cells through NW columns before N-CWS treatment, and was completely restored by adding a small number of N-CWS-treated, NW-adherent spleen cells to the lower compartment, whereas the adherent cells alone did not produce LHF even in the presence of rIL 2. This restoration was also observed when cells in the lower compartment had been reconstituted with N-CWS-treated, NW-nonadherent cells and medium-treated, NW-adherent

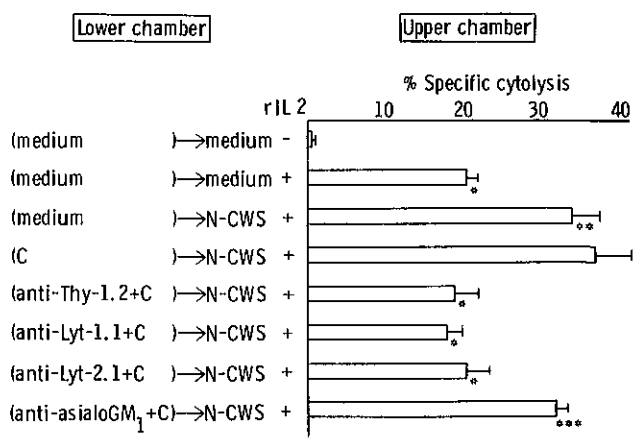

Fig. 4. Phenotype of cells producing LHF. Spleen cells treated with antibodies and complement were further treated with medium alone or with 10 μg/ml of N-CWS for 4 h, and then added to the lower compartment. The upper compartment containing normal spleen cells was placed in the lower compartment, and rIL 2 was added at a concentration of 0.1 unit/ml. The plate was cultured for 4 days, and then cells of the upper compartment were tested for cytolytic activity against 3LL tumor cells as described in the legend to Fig. 1. Data of cell depletion showed that Thy-1.2^+ accounted for 45.2%, Lyt-1.1^+ for 16.7%, Lyt-2.1^+ for 9.5%, and asialo GM_1^+ cells for 11.7%. Statistical significance: single vs. double asterisks; $P<0.01$, single vs. triple asterisks; $P<0.01$, double vs. triple asterisks; not significant.

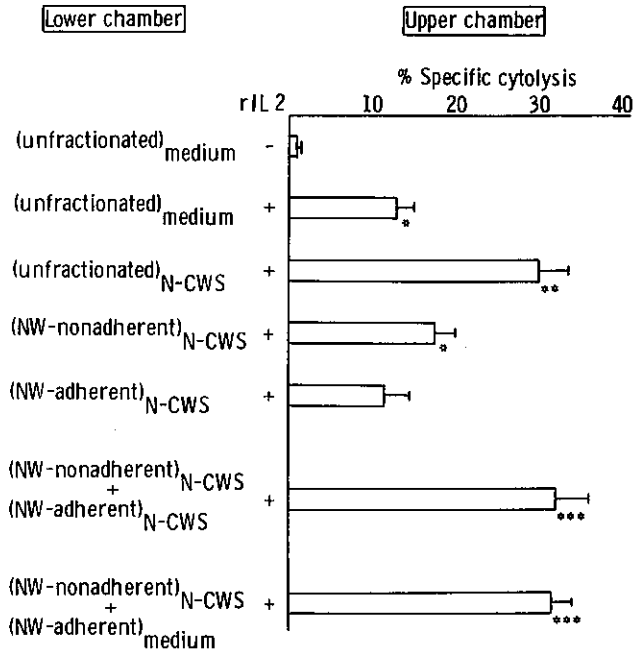

Fig. 5. Accessory cell requirement for LHF production. Unfractionated spleen cells or cells fractionated into NW-nonadherent and -adherent cells were treated with medium alone or with 10 μg/ml of N-CWS for 4 h, and then added to the lower compartment at the cell number of 5×10^6 (unfractionated or NW-nonadherent cells) or 5×10^5 cells (NW-adherent cells) per compartment. The upper compartment containing normal spleen cells was placed in the lower compartment, and rIL 2 was added at a concentration of 0.1 unit/ml. The plate was cultured for 4 days, and then cells of the upper compartment were tested for cytolytic activity against 3LL tumor cells as described in the legend to Fig. 1. Statistical significance: single vs. double asterisks, $P<0.02$; single vs. triple asterisks, $P<0.01$.

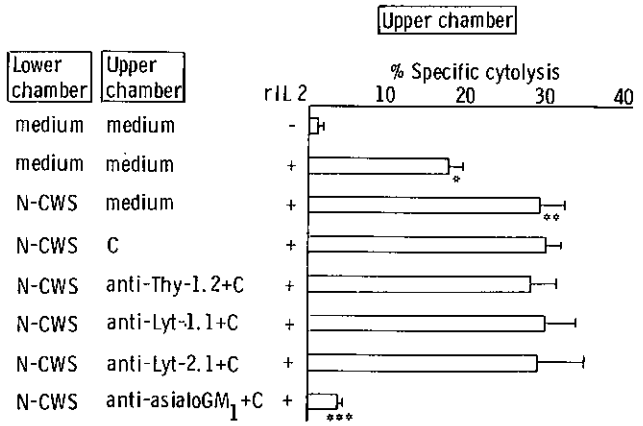

Fig. 6. Phenotype of LAK precursors responding to the combined stimulation with rIL 2 and LHF. Spleen cells treated with antibodies and complement were added to the upper compartment, and the compartment was placed in the lower compartment containing medium- or N-CWS-treated spleen cells. RIL 2 was added at a concentration of 0.1 unit/ml, and the plate was cultured for 4 days. After culture, cells of the upper compartment were tested for cytolytic activity against 3LL tumor cells as described in the legend to Fig. 1. Statistical significance: single vs. double asterisks, $P<0.01$; double vs. triple asterisks; $P<0.001$.

cells, indicating that LHF production induced by N-CWS required both NW-nonadherent and NW-adherent spleen cells, and the target of N-CWS stimulation was the NW-nonadherent but not the NW-adherent cell population.

LAK precursors responding to LHF and rIL 2 In a diffusion chamber culture plate, spleen cells treated with antibodies and complement were added to the upper compartment, and tested for the ability to generate LAK activity in response to both rIL 2 and LHF. The augmented LAK cell induction in the upper compartment was completely abrogated by anti-asialo GM_1 treatment of the responder cells, whereas none of the anti-Thy-1.2, -Lyt-1.1, -Lyt-2.1 treatments reduced this response (Fig. 6).

Table II. Combined Effect of N-CWS and rIL 2, or rIL 2 and Mu-rIL 1α on LAK Cell Induction

N-CWS[a] (10 μg/ml)	rIL 2[b] (0.1 U/ml)	Mu-rIL 1α[c] (U/ml)	% Specific cytolysis[d]
−	−	−	0.0±0.0
+	−	−	0.2±0.1
−	+	−	12.2±1.1[e]
+	+	−	39.1±2.5[f]
−	+	1	11.7±1.0[g]
−	+	10	12.0±0.9[h]
−	+	100	19.6±2.0[i]

a) See footnote *a*) in Table I.
b) See footnote *b*) in Table I.
c) Mu-rIL 1α was added at a concentration indicated to the culture at the beginning of 4-day culture.
d) See footnote *d*) in Table I. Statistical significance: *e*) vs. *f*), $P<0.001$; *e*) vs. *g*), not significant; *e*) vs. *h*), not significant; *e*) vs. *i*), $P<0.01$; *f*) vs. *i*), $P<0.001$.

Combined effect of rIL 2 and Mu-rIL 1α on LAK cell induction To investigate whether IL 1 could replace the activity of N-CWS, spleen cells were cultured for 4 days in the presence of both rIL 2 and Mu-rIL 1α, and then tested for LAK activity. As shown in Table II, Mu-rIL

Table III. LAK Cell Induction and IL 2, TNF and IFN Production by C3H/HeN Spleen Cells Stimulated with N-CWS and rIL 2

Cell culture[a]	LAK cell[b] induction	Activities in the culture fluids (unit/ml)[c]		
		IL 2	TNF	IFN
Medium	0.0±0.0	<0.012	0	<10
N-CWS	0.2±0.1	<0.012	0	<10
rIL 2	11.3±0.9[d]	0.354	0	<10
N-CWS+rIL 2	35.1±2.8[e]	0.354	0	<10

a) C3H/HeN spleen cells were incubated with medium alone or in the presence of 10 μg/ml of N-CWS for 4 h, washed 3 times, and then cultured with medium alone in the presence of 0.1 unit/ml of rIL 2 for 4 days.
b) After 4-day culture, cells were tested for cytolytic activity against 3LL tumor cells as described in footnote *d*) in Table I. Statistical significance: *d*) vs. *e*); $P<0.001$.
c) After 4-day culture, the culture fluids were obtained by centrifugation and then tested for IL 2, TNF and IFN activities.

1α only weakly augmented LAK cell induction even when used at a concentration of 100 units/ml.

IL 2, TNF, and IFN activities of the culture fluids Medium- or N-CWS-treated spleen cells were cultured for 4 days alone or in the presence of rIL 2. As shown in Table III, the pretreatment of spleen cells with N-CWS followed by a 4-day culture with medium alone resulted in no apparent production of IL 2. On the other hand, a 4-day culture of medium-treated spleen cells in the presence of rIL 2 (0.1 unit/ml) caused an apparent elevation of the IL 2 activity in the culture fluid (0.354 unit/ml), suggesting that IL 2, presumably of mouse origin, had been newly produced by spleen cells during 4-day culture with rIL 2. Indeed, ion exchange chromatography with DEAE-Sephacel revealed that the culture fluid contained 2 kinds of IL 2 with different isoelectric points (data not shown). The increase in the IL 2 activity in the culture fluid was also observed when N-CWS-treated spleen cells were similarly cultured in the presence of rIL 2, but it was exactly the same as that shown in the case of medium-treated spleen cells. No detectable TNF was present in any of the culture fluids. The IFN activity of these samples was also extremely low, and no differences among them were observed.

DISCUSSION

In our previous report demonstrating the augmentative effect of N-CWS on *in vivo* LAK cell induction, peritoneal lymphocytes induced by N-CWS injection were shown to possess a high ability to generate LAK activity in response to rIL 2, presumably resulting from their augmented IL 2 binding capacity.[6] In keeping with this observation, the results of the present study clearly show that the pretreatment of spleen cells with N-CWS potently augmented their responsiveness to rIL 2 for the generation of LAK activity in a 4-day culture. The phenotype of effector cells of the augmented cytotoxicity was Thy-1.2$^+$, Lyt-1.1$^-$, Lyt-2.1$^-$ and asialo GM$_1^+$, showing no difference from that of LAK cells induced by rIL 2 alone.[11] Consistently, the IL 2 binding capacity of spleen cells was also augmented by the combined stimulation with N-CWS and rIL 2.

In the present study, rIL 2 was used at a concentration lower than optimal for *in vitro* LAK cell induction, because *in vivo* LAK cell induction resulting from repeated sc injections of rIL 2 and its augmentation by N-CWS might occur at a low concentration of rIL 2 in mice.

The effect of the single use of N-CWS on LAK cell induction was different in *in vivo* and *in vitro* experiments. Peritoneal lymphocytes induced by an ip injection of N-CWS alone exhibited not only a high IL 2 binding capacity but also a weak though distinct LAK activity,[6] whereas these two responses could not be achieved *in vitro* unless N-CWS-treated spleen cells were further cultured in the presence of rIL 2. These results strongly suggest that the weak promotion of *in vivo* LAK cell induction by N-CWS might be achieved with the help of certain cytokines, including IL 2, which could be produced by N-CWS-induced, peritoneal cells *in vivo* but not by N-CWS-stimulated splenocytes *in vitro*. In fact, the present study revealed that N-CWS-treated spleen cells alone produced no detectable IL 2, TNF or IFN.

The combined effect of N-CWS and rIL 2 on LAK cell induction was further investigated *in vitro* with the diffusion chamber culture system, focusing on the possibility that this reaction could be mediated by a soluble factor. N-CWS-treated spleen cells produced a factor capable of augmenting LAK cell induction (LAK-helper factor, LHF), requiring the coexistence of rIL 2. Based on the results of cell depletion and fractionation experiments, it seems possible that N-CWS stimulates T cells to produce LHF in the presence of both rIL 2 and accessory cells, and LHF cooperates with rIL 2 in stimulating NK cells to generate LAK activity. However, the results of the present study do not rule out the possibility that N-CWS-stimulated T cells produce certain cytokines capable of stimulating accessory cells to produce LHF.

A small amount of N-CWS might have remained in the N-CWS-treated spleen cell suspension even after 3 washings. However, it seems unlikely that the augmented LAK cell induction in the upper compartment was due to the permeation of the contaminating N-CWS from the lower to the upper compartment, because spleen cells

depleted of T cells or of NW-adherent cells were found to be unable to induce this response, although they had been similarly treated with N-CWS.

It is of importance to investigate whether LHF is an already known cytokine capable of augmenting LAK cell induction. Indeed, a murine spleen cell culture with rIL 2 caused a new production of murine IL 2. However, there was no difference in the amount of newly produced IL 2 between the medium- and N-CWS-treated spleen cell culture. Recently, several investigators have demonstrated that TNF-α cooperates with rIL 2 in the increase in IL 2 receptors resulting in an augmented induction of their LAK activity,[4, 5] suggesting that LHF might be TNF-α. However, this seems unlikely, from the findings that the culture fluid containing LHF exhibited no TNF activity, and that the LHF production absolutely depended on T cell stimulation with N-CWS, whereas N-CWS-induced TNF production was shown to be directly mediated by macrophages.[10] In *in vivo* LAK cell induction, however, TNF produced by N-CWS-stimulated macrophages might play a role, in part, in the combined effect of N-CWS and rIL 2. The IFN assay of the spleen cell culture fluid also suggested that LHF could not be IFN. In a recent report by Eisenthal and Rosenberg, demonstrating the effect of various cytokines on the *in vitro* induction of antibody-dependent cellular cytotoxicity (ADCC) in murine cells,[12] a combined stimulation of thymocytes with human rIL 1α and rIL 2 was shown to result in a potent augmentation of both ADCC and direct cytotoxicity (LAK cell cytotoxicity), compared with the results of a culture with rIL 2 alone. However, their report also revealed that LAK cell induction from spleen cells was not affected by the addition of rIL 1α to the culture with rIL 2, whereas their ADCC activity was similarly augmented by the combined stimulation. Consistently with their result, our present study showed that Mu-rIL 1α only weakly augmented the effect of rIL 2 on LAK cell induction from spleen cells. Collectively, these results strongly suggest that LHF may be a cytokine that is distinct from IL 1, IL 2, TNF and IFN. Several investigators have reported that rIL 2-induced LAK cell generation from murine spleen cells is potently augmented by a combined use of T cell-derived cytokines, including murine interleukin 4 (IL 4)[13] and killer-helper factor (KHF).[14] Therefore, it is important to investigate whether LHF is also distinct from these cytokines.

Partial purification of LHF was tried using ion exchange chromatography and gel filtration. However, this trial was hampered by the coexistence of rIL 2 in the culture fluid, because murine LHF exhibited similar characteristics to those of rIL 2 regarding isoelectric point and molecular weight. Using peripheral blood lymphocytes (PBL), we have recently obtained similar findings that *in vitro* LAK cell induction from human PBL with a suboptimal concentration of rIL 2 is potently augmented by an addition of N-CWS, and that this augmented LAK cell induction is due to LHF production by N-CWS-stimulated PBL. Furthermore, preliminary experiments have revealed that N-CWS-stimulated PBL can produce LHF without the help of rIL 2, and that the culture fluid containing human LHF exhibits no IL 1, IL 2, TNF or IFN activity. Partial purification of human LHF is in progress.

ACKNOWLEDGMENTS

The authors wish to thank Ms. S. Izumi of the Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., Ibaraki, for assays of TNF and IFN activities. This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare.

(Received June 28, 1989/Accepted September 26, 1989)

REFERENCES

1) Iigo, M., Sakurai, M., Tamura, T., Saijo, N. and Hoshi, A. *In vivo* antitumor activity of multiple injections of recombinant interleukin 2, alone and in combination with three different types of recombinant interferon, on various syngeneic murine tumors. *Cancer Res.*, **48**, 260–264 (1988).
2) Rosenberg, S. A., Schwarz, S. L. and Spiess, P. J. Combination immunotherapy for cancer: synergistic antitumor interactions of interleukin-2, alfa interferon, and tumor-infiltrating lymphocytes. *J. Natl. Cancer Inst.*, **80**, 1393–1397 (1988).
3) McIntosh, J. K., Múle, J. J., Merino, M. J. and Rosenberg, S. A. Synergistic antitumor effects of immunotherapy with recombinant interleukin-2 and recombinant tumor necrosis factor-α. *Cancer Res.*, **48**, 4011–4017 (1988).
4) Owen-Schaub, L. B., Guttermann, J. U. and Grimm, E. A. Synergy of tumor necrosis factor and interleukin 2 in the activation of human cytotoxic lymphocytes: Effect of tumor necrosis factor α and interleukin 2 in the generation of human lymphokine-activated killer cell activity. *Cancer Res.*, **48**, 788–792 (1988).
5) Chouaib, S., Bertoglio, J., Blay, J-Y., Marchiol-Fournigault, C. and Fradelizi, D. Generation of lymphokine-activated killer cells: synergy between tumor necrosis factor and interleukin 2. *Proc. Natl. Acad. Sci. USA*, **85**, 6875–6879 (1988).

6) Kawase, I., Komuta, K., Shirasaka, T., Hara H., Tanio, Y., Watanabe, M., Saito, S., Ikeda, T., Masuno, T., Kishimoto, S. and Yamamura, Y. Synergy of *Nocardia rubra* cell wall skeleton and interleukin 2 in the *in vivo* induction of murine lymphokine-activated killer cell activity. *Jpn. J. Cancer Res.*, **80,** 1089–1097 (1989).
7) Kawase, I., Brooks, C. G., Kuribayashi, K., Olabuenaga, S. S., Newman, W., Gillis, S. and Henney, C. S. Interleukin 2 induces γ-interferon production: participation of macrophages and NK-like cells. *J. Immunol.*, **131,** 288–292 (1983).
8) Kern, D. E., Gillis, S., Okada, M. and Henney, C. S. The role of interleukin-2 (IL-2) in the differentiation of cytotoxic T cells: the effect of monoclonal anti-IL 2 antibody and absorption with IL 2-dependent T cell lines. *J. Immunol.*, **127,** 1323–1328 (1981).
9) Izumi, S., Ueda, H., Okuhara, M., Aoki, H. and Yamamura, Y. Effect of *Nocardia rubra* cell wall skeleton on murine interferon production *in vitro*. *Cancer Res.*, **46,** 1960–1965 (1986).
10) Izumi, S., Hirai, O., Hayashi, K., Konishi, Y., Okuhara, M., Kohsaka, M., Aoki, H. and Yamamura, Y. Induction of a tumor necrosis factor-like activity by *Nocardia rubra* cell wall skeleton. *Cancer Res.*, **47,** 1785–1792 (1987).
11) Kawase, I., Komuta, K., Hara, H., Inoue, T., Hosoe, S., Ikeda, T., Shirasaka, T., Yokota, S., Tanio, Y., Masuno, T. and Kishimoto, S. Combined therapy of mice bearing a lymphokine-activated killer-resistant tumor with recombinant interleukin 2 and an antitumor monoclonal antibody capable of inducing antibody-dependent cellular cytotoxicity. *Cancer Res.*, **48,** 1173–1179 (1988).
12) Eisenthal, A., and Rosenberg, S. A. The effect of various cytokines on the *in vitro* induction of antibody-dependent cellular cytotoxicity in murine cells. Enhancement of IL 2-induced antibody-dependent cellular cytotoxicity activity by IL 1 and tumor necrosis factor-α. *J. Immunol.*, **142,** 2307–2313 (1989).
13) Múle, J. J., Krosnick, J. A. and Rosenberg, S. A. IL 4 regulation of murine lymphokine-activated killer activity *in vitro*. Effects on the IL 2-induced expansion, cytotoxicity and phenotype of lymphokine-activated killer effectors. *J. Immunol.*, **142,** 726–733 (1989).
14) Fukuta, K., Sone, S., Kitahara, M., Okada, M. and Ogura, T. Enhancement of therapeutic effect of interleukin 2 on spontaneous pulmonary metastases of Lewis lung carcinoma by killer helper factor associated with increased induction of killer activity. *Jpn. J. Cancer Res.*, **80,** 562–569 (1989).