EFFECT OF *NOCARDIA RUBRA* CELL WALL SKELETON ON INTERLEUKIN 1 PRODUCTION FROM MOUSE PERITONEAL MACROPHAGES

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Abstract — *Nocardia rubra* cell wall skeleton (N-CWS) was shown to augment interleukin 1 (IL-1) production from peritoneal resident and exudate macrophages in C3H/HeN mice. The mol. wt of the N-CWS-induced IL-1 product was about 17,000 daltons, which is a similar weight to that obtained by lipopolysaccharide stimulation. The stimulation of IL-1 production by N-CWS was seen as early as 8 h after the start of incubation and peak production was observed at 48 h. Profound effects were seen with 10 μg/ml or more of N-CWS. Experiments on the regulation of the N-CWS-augmented IL-1 production showed that prostaglandin E2 inhibited the augmentation, and indomethacin (cyclo-oxygenase inhibitor) further augmented it. Leukotriene B4 and AA861 (lipoxygenase inhibitor) had no effect.

Our findings suggest that the previously reported adjuvant effect of N-CWS may, in part, be mediated via its ability to stimulate IL-1 production; and that such a stimulation may be blocked by prostaglandins.

*Nocardia rubra* cell wall skeleton (N-CWS) is a potent reticuloendothelial and hemopoietic stimulant (Azuma, Taniyama, Yamawaki, Sugimura & Yamamura, 1976) that has been demonstrated to be effective in prolonging the survival of patients with lung cancer (Yamamura, Ogura, Sakatani, Hirao, Kishimoto, Fukuoka, Takada, Kawahara, Furuse, Kuwahara, Ikegami & Ogawa, 1983) and gastric cancer (Ochiai, Sato, Sato Hayashi, Asano, Isono & Suzuki, 1983) when administered to the tumor-associated site and subcutaneously.

The exact mechanisms of the antitumor effect of N-CWS are not yet fully elucidated, but are thought to be augmentation of general immune response by induction of specific cell-mediated cytotoxicity by T-lymphocytes (Yanagawa, Yasumoto, Ohta, Nomoto, Azuma & Yamamura, 1979; Kawase, Uemiy, Yoshimoto, Ogura, Hirao & Yamamura, 1981) and/or macrophages (Ogura, Namba, Hirao & Yamamura, 1979; Itho, Iizuka, Masuno, Yasunami, Ogura, Yamamura & Azuma, 1981; Sone & Fidler, 1982). Intraperitoneal (i.p.) or intravenous (i.v.) injection of N-CWS activates the cytotoxicity of peritoneal (Ogura et al., 1979; Itoh et al., 1981; Inamura, Fujitsu, Nakahara, Abiko, Horii, Hashimoto & Aoki, 1984) or alveolar (Sone, Pollack & Fidler, 1980) macrophages. Peritoneal exudate macrophages of mice and alveolar macrophages of rats were rendered tumoricidal after direct interaction with N-CWS in *vitro* (Sone et al., 1980; Inamura et al., 1984). It is known that several immunopotentiators, such as lipopolysaccharide (LPS) (Alexander & Evans, 1971), muramylpeptide (Sone & Tsubura, 1982), lymphokines (Meltzer, 1981) and interferons (Varesio, Blasi, Thurman, Talmadge, Willtrout & Herberman, 1984) stimulate interleukin 1 (IL-1) production (Dinarello, 1984). However, there has been little information on N-CWS concerning IL-1 production (Hayashi, Masuno, Hosoe, Kawase, Sakatani, Ogura, Kishimoto & Yamamura, 1986).

In the present study, we examined the effect of N-CWS on IL-1 production in murine peritoneal macrophages, and whether arachidonic acid metabolites modify the production of IL-1 by N-CWS-activated macrophages.

**EXPERIMENTAL PROCEDURES**

*Animals*

Male C3H/HeN mice (age 6–10 weeks) were obtained from Shizuoka Agricultural Cooperative
Association, Hamamatsu, Japan and male C3H/HeJ (age 5–8 weeks) from Jackson Laboratories, Bar Harbor, ME. They were kept under specific-pathogen-free conditions in our animal facility.

Culture media and reagents

Hank’s balanced salt solution (HBSS), RPMI 1640 and Eagles’ minimal essential medium (E-MEM) were purchased from Flow Laboratories, Irvine, Scotland. Fetal bovine serum (FBS) was obtained from Sigma Chemical Co., St Louis, MO. Phytohaemagglutinin (PHA) was obtained from Wellcome Research Laboratories, Beckenham, England; thioglycollate broth from Baltimore Biological, Cockysville, MD, U.S.A.; lipopolysaccharide (LPS) (E. coli 055:B5) from Difco Lab., Detroit, MI, U.S.A.; leukotriene B4 (LTB4) from Daiichi Pure Chemicals Co., Ltd, Osaka, Japan; and CR-human IL-1 from Collaborative Research, Inc., Bedford, MA, U.S.A. AA-861 (FR76372, lot. no. 806165F); placebo for N-CWS (lot no. 109865K) and squalene-attached N-CWS (FR900124, lot no. 101461K) were prepared by Fujisawa Pharmaceutical Co., Osaka, Japan.

IL-1 measurement

Individual macrophage culture supernatants (2 ml samples) were dialyzed overnight with HBSS at 4°C to remove low mol. wt substances which might affect thymocyte proliferation. The supernatants were later sterilized using a millipore membrane (0.45 µm, Sartorius, Gottingen, W. Germany). They were placed in flat-bottomed microtitre plates (Sumitomo Bakelite Co., Japan) with thymocytes (1 x 10^6 cells/well) from C3H/HeJ mice and PHA at a final concentration of 1 µg/ml. The cultures were incubated for 72 h in RPMI 1640 containing 10% FBS and 5 x 10^-5 M 2-mercaptoethanol, pulsed with 1 µCi/well of 3H-thymidine (spec. act., 6.7 Ci/m mol; ICN Radiochemicals, Irvine, CA) during the final 6 h and harvested onto glass fiber strips with a microharvester (Bellco Glass, Inc., Vineland, NJ). Data were plotted as 3H-thymidine incorporation (counts/min) vs the reciprocal of four serial dilutions of each sample and calculated as units/ml, compared to standard human IL-1 (CR-human IL-1).

Interleukin 2 (IL-2) determination

IL-2 activity was measured as the ability to support IL-2-dependent cytotoxic T-cell line (CTLL) proliferation, as reported by Gillis & Smith (1977).

Sephadex G-75 chromatography

Sephadex G-75 chromatography was performed essentially as described by Mizel & Mizel (1981). Columns of freshly prepared Sephadex were usually made for each run. The gel slurry (328 ml) was poured into a 26 mm diameter glass column (Pharmacia, C26/70). The height of the gel bed after setting was approximately 62 cm. The eluting fluid was run through the column before sample application. Forty millilitres of the supernatants was lyophilized, reconstituted with 4 ml of RPMI 1640, and passed through the millipore filter in order to remove undissolved residue. An aliquot diluted at 1/4 was retained for bioassay. The elution rate was approximately 25 ml/h and the fraction volume was 3.3 ml. The fractions were stored at –80°C until assayed.

RESULTS

Kinetics of IL-1 production from macrophages by N-CWS

Peritoneal exudate macrophages (PEM) were treated with 10 µg/ml N-CWS or placebo and the supernatants were collected after incubation for
Effect of *N. rubra* on IL-1 Production

Fig. 1. Kinetics of N-CWS-augmented IL-1 production by macrophages. Monolayer cultures were incubated with 10 μg/ml N-CWS (●) or placebo (○) for various times (h), and the supernatants were assayed for IL-1 activity. Each point represents the mean ± S.E. of IL-1 production in these macrophage culture supernatants.

various times. Their IL-1 activities are shown in Fig. 1. Augmentation of IL-1 levels was seen in the N-CWS-treated cultures as early as 8 h after starting incubation. Moreover, the production of IL-1 increased linearly and reached a peak at 48 h, followed by a plateau phase. Since there was no significant loss of IL-1 activity in the culture supernatant between 48 and 72 h, it was decided to measure IL-1 at a stable point during the plateau phase (48 h). Placebo had no effect on IL-1 production.

**Dose-dependent stimulation of IL-1 production by N-CWS**

To confirm the dose-dependency of IL-1 production in response to N-CWS activation *in vitro*, PRM and PEM were treated with N-CWS or placebo at a concentration range from 0.32 to 100 μg/ml (Table 1). A dose-dependent augmentation of IL-1 production was observed when the cultures were incubated in the presence of N-CWS at 3.2 μg/ml or more. However, placebo did not stimulate IL-1 production at any concentrations.

**IL-2 activity in culture supernatants of macrophage treated with N-CWS**

Since IL-2 as a T-cell growth factor can cause mouse thymocytes to proliferate, it is possible that IL-2 in macrophage culture supernatants was involved in the enhancement of IL-1 activity in this system. To ascertain whether this was the case, the supernatants were examined for IL-1 and IL-2 activity (Fig. 2). IL-1-rich supernatants treated with 10 μg/ml N-CWS showed no detectable IL-2 activity in any dilutions, either when resident or exudate peritoneal cells were used as macrophage source.

**Sephadex G-75 chromatography of N-CWS-induced IL-1**

IL-1-rich supernatants treated with 10 μg/ml N-CWS were collected and their mol. wt determined by Sephadex column chromatography. IL-1 activity of the supernatant was obtained at a position corresponding to a mol. wt of 17,000 daltons, which was confirmed in two independent experiments (Fig. 3). IL-1 obtained from LPS (10 μg/ml)-stimulated macrophages was also eluted in a fraction of the same mol. wt (data not shown). The IL-1 produced by the N-CWS-treated macrophages seemed to be similar to the IL-1 produced by the LPS-stimulated macrophages.

**Effect of PGE2 on N-CWS-augmented IL-1 production**

Macrophage monolayers were incubated with N-CWS, LPS or medium for 48 h. PGE2 was added

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<th>Concentration (μg/ml)</th>
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<tr>
<td>Placebo</td>
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<td>0 ± 0</td>
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<td>N-CWS</td>
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<td>14.1 ± 0.8</td>
<td>24.7 ± 3.9</td>
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<tr>
<td>N-CWS</td>
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<td>6.7 ± 3.8</td>
<td>50.3 ± 18.3</td>
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*PRM and PEM (1 × 10⁶) from C57/HeN mice were incubated with N-CWS or placebo at a concentration range from 0.32 to 32 μg/ml for 2 days. After dialysis and filter sterilization, the supernatants were tested for IL-1 activity against thymocytes proliferation assay.

*Mean of triplicate determinations ± S.E.
at the same time as the stimulants in a concentration range from $10^{-9}$ to $10^{-5}$ M. After 48 h, the cell-free culture supernatants were dialyzed to remove PGE$_2$ prior to determination of IL-1 activity. Increasing concentrations of PGE$_2$ suppressed N-CWS- and LPS-augmented IL-1 production (Fig. 4).

**Effect of indomethacin on N-CWS-augmented IL-1 production**

The effect of indomethacin at a concentration range from $10^{-9}$ to $10^{-5}$ M on IL-1 production was examined in the same way as that of PGE$_2$. As shown in Fig. 5, a dose-dependent augmentation of IL-1 production was observed when N-CWS- and LPS-stimulated macrophages were incubated in the presence of indomethacin.

**Failure of LTB$_4$ and AA861 to stimulate IL-1 production**

The effects of LTB$_4$ and AA861, a lipoxygenase inhibitor (Yoshimoto, Yokoyama, Ochi, Yamamoto, Maki, Asida, Terao & Shiraishi, 1982) on extracellular IL-1 production were determined. LTB$_4$ alone did not augment IL-1 production in the absence of both of stimulants nor augment N-CWS- or LPS-induced IL-1 production (Fig. 6). AA861 also had no effect on IL-1 production by N-CWS and LPS (Fig. 7). The findings suggest that lipoxygenase does not influence IL-1 production.

**Confirmation of dialysis**

Control experiments were performed to verify that the reagents added to the macrophage cultures were effectively dialyzed and did not suppress thymocyte proliferation in IL-1 assay. The highest concentrations of these reagents were added to aliquots of an IL-1-rich macrophage culture supernatant. These preparations were further

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Fig. 2. Absence of IL-2 activity in IL-1-rich macrophage culture supernatants. PEM or PRM were activated with 10 µg/ml N-CWS, and each of the supernatants was assayed for IL-1 activity against thymocyte proliferation (■, ○) and for IL-2 activity against CTLL proliferation (□, ○).

Fig. 3. Sephadex G-75 chromatography of N-CWS-induced IL-1 in culture supernatants. The supernatants were prepared by stimulation of PEM with 10 µg/ml N-CWS for 48 h and lyophilized. The samples were chromatographed on sephadex G-75 column. Each fraction was assayed for IL-1 activity.
Effect of N. rubra on IL-1 Production

Fig. 4. Inhibition by PGE₂ of IL-1 production by N-CWS-stimulated macrophages. Monolayer cultures were incubated with 10 μg/ml N-CWS ( ● ) or 10 μg/ml LPS ( ○ ) for 48 h and PGE₂ was added at the same time as the stimulants. The control cultures ( △ ) were incubated with PGE₂ in the absence of the stimulants. Each point represents the mean ± S.E. of IL-1 production in triplicate macrophage culture supernatants.

Fig. 5. Augmentation by indomethacin of IL-1 production by N-CWS-stimulated macrophages. Indomethacin was added to monolayer cultures, instead of PGE₂. IL-1 productions and assay are described in Fig. 4.

dialyzed and tested for IL-1 activity against that of control IL-1-containing samples to which the reagents had not been added. There was no significant suppression of thymocyte proliferation by any of the reagents tested, indicating that any inhibitory effects described were due to effects on IL-1 production by macrophages (Fig. 8).

DISCUSSION

In the present study, we show that N-CWS augments the production of IL-1 from peritoneal macrophages and that the production is regulated by a cyclo-oxgenase product.

Fig. 6. Effect of LTB₄ on the production of IL-1 by N-CWS-stimulated macrophages. LTB₄ was added to monolayer cultures in the same way as PGE₂ in Fig. 2.

Fig. 7. Effect of AA861 on the production of IL-1 by N-CES-stimulated macrophages. AA861 was added to monolayer cultures in the same way as PGE₂ in Fig. 2.

Fig. 8. Lack of suppression of thymocyte proliferation by reagents added to macrophage cultures. IL-1-rich macrophage culture supernatant was added to a dialysis tube, and various reagents were added. These preparations were dialyzed and tested in the IL-1 assay. 10⁻⁶ M IND. (indomethacin), 10⁻⁶ M PGE₂, 10⁻⁶ M LTB₄ and 10⁻⁶ M AA861 were compared with medium. Each figure represents the mean ± S.E. of IL-1 activity in triplicate macrophage culture supernatants.
The mol. wt of IL-1 produced from PEM treated with N-CWS was examined by Sephadex G-75 column chromatography and found to be about 17,000 daltons, which is comparable to that of murine IL-1 from LPS-stimulated PEM. This mol. wt was in accord with the data presented by others (Mizel, Oppenheim & Rosenstreich, 1978; Economou & Shin, 1978; Mizel, 1979). The mol. wt of murine IL-2 is approximately 30,000 daltons, as measured by gel filtration analysis (Watson, Gillis, Marbrook, Mochizuki & Smith, 1979) and no IL-2 activity is present in supernatants treated with N-CWS by CTLL assay in this study.

It is of special interest that IL-1 was produced from PRM as well as PEM, because it has been reported that the functional activities of resident macrophages are much lower than those of exudate macrophages in superoxide anion production (Cohn, 1978) and induction of cytocidal activity against tumor cell (Farr, Wechter, Kiely & Unanue, 1979; Inamura et al., 1984). The mechanism of the augmented IL-1 production may differ from that of these functions.

N-CWS at high concentration (more than 32 μg/ml) was toxic to PEM (data not shown). Gery, Davies, Derr, Krett & Barranger (1981) reported that damage of macrophages by injurious agents stimulated IL-1 production. The injurious effect of N-CWS on macrophages may act synergistically to induce a hyper-production of IL-1 at the high concentration of N-CWS.

Many investigators (Rola-Pleszczyski & Lemaire, 1985; Brandwein, 1986; Kunkel, Chensue & Phan, 1986) have shown that IL-1 production by LPS stimulation is regulated by metabolites of arachidonic acid. In a subsequent experiment, we examined the effect of PGE₂ on IL-1 production by N-CWS stimulation. Exogenous PGE₂ suppressed the effect of N-CWS on IL-1 production, but exogenous LTB₄ did not. Indomethacin (cyclooxygenase inhibitor) increased the IL-1 production in the presence of N-CWS, but AA861 (lipooxygenase inhibitor) did not. These patterns in N-CWS-induced IL-1 production were also obtained in LPS-induced IL-1 production. The data suggest that N-CWS-induced IL-1 production may be regulated through prostaglandins in a way similar to that of LPS. Augmentation of IL-1 production by indomethacin was reported by Oshika, Umesaki & Sugawa (1986), studying the effect of OK-432, a bacterial immunopotentiator. They showed, furthermore, that indomethacin augmented the in vivo efficacy of OK-432 and induced greater regression more markedly than OK-432 alone. Our study has now shown the effect of indomethacin on in vivo efficacy of N-CWS, nevertheless, similar therapeutical phenomena to Oshika’s observation with OK-432 is expected in case of N-CWS.

IL-1 mediates a large number of diverse immunostimulatory events. It acts on the lymphocyte to promote IL-2 generation and thus lymphocyte proliferation potentiates the immune response (Dinarello, 1984). These studies suggest that the augmentation of cellular immune response by N-CWS treatment (Yanagawa et al., 1979; Kawase et al., 1981) may be mediated by N-CWS-augmented IL-1 production.

Recent studies have demonstrated the involvement of IL-1 in the tumoricidal activity of macrophages (Onozaki, Matsushima, Aggarwall & Oppenheim, 1985; Lovett, Kozan, Hadam, Resch & Gemsa, 1986). Thus, our data suggest that the augmentation of IL-1 production after stimulation with N-CWS might, in part, mediate the antitumor activity of N-CWS.

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REFERENCES


Effect of N. rubra on IL-1 Production


