

Possible Involvement of a Tumor Necrosis Factor (TNF)-like Mediator as an Endogenous Pyrogen in Fever Induction by *Nocardia rubra* Cell Wall Skeleton (N-CWS)

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Tumor necrosis factor (TNF), a cytokine produced in macrophages, also acts as an endogenous pyrogen (EP). To investigate whether TNF has a role in the fever induced by *Nocardia rubra* cell wall skeleton (N-CWS), the relationship between fever and TNF production was studied in guinea pigs.

N-CWS injected i.v. to guinea pigs caused biphasic fever and had L-929 cell-killing activity which resembled that of TNF in the sera 30 min before the first phase of fever appeared. *In vitro*, L-929 cell-killing activity was demonstrated in the culture supernatant of guinea pig peritoneal macrophages pretreated with N-CWS, and the activity increased dependently on N-CWS concentration or culture duration. When the supernatant of the macrophages was fractionated by gel filtration and each fraction was assayed for fever-inducing and L-929 cell-killing activities, the fraction with the cell-killing activity also induced fever with characteristics similar to that by i.v. injection of N-CWS in guinea pigs.

These results suggest that TNF acts as an EP on the fever induced by N-CWS in guinea pigs.

Keywords N-CWS (*Nocardia rubra* cell wall skeleton); fever; TNF (tumor necrosis factor); endogenous pyrogen

N-CWS is the cell wall skeleton prepared from a gram-positive bacterium, *Nocardia rubra*. This substance has a potent anti-tumor activity in several experimental animal models^{1,2)} and in humans.^{3–6)} Fever was a side effect of N-CWS in clinical trials, and also in such experimental animals as rabbits and rats.

In this connection, it has been shown that endogenous pyrogen (EP) produced in phagocytes stimulated with microorganisms induces fever by enhancing the production of prostaglandins (PGs) in the hypothalamus or adjacent regions.^{7–9)} It has also been reported that EP has many other biological activities,^{10–16)} and that their profiles are similar to those of interleukin-1 (IL-1). Because of this, EP is accepted to be IL-1. Recently, Dinarello *et al.*¹⁷⁾ reported that tumor necrosis factor (TNF) had EP activity in an experiment in which fever was induced by i.v. injection of recombinant human TNF (r-TNF α) in rabbits. Furthermore, they used r-TNF α to induce IL-1 production *in vitro* in human monocytes, and accordingly, concluded that two EPs were involved in the fever induced by TNF; one, TNF itself, and the other IL-1 produced by TNF. This indicates the importance of TNF as an EP in the fever producing process.

In our previous study, i.v. N-CWS caused biphasic fever in rats, rabbits, and guinea pigs, and the fever resembled that induced by r-TNF in rabbits in the study by Dinarello *et al.*

The present study was performed to investigate whether TNF acted as an EP in the fever induced by N-CWS.

Experimental

Animals Slc:Hartley male guinea pigs weighing 350–700 g were purchased from Shizuoka Agricultural Cooperative Association. The animals were housed under room temperature of 20 \pm 5°C; relative humidity, 60 \pm 5%; and lighting controlled to give 12 h of light and 12 h of darkness.

Chemicals Cell wall skeleton of *Nocardia rubra* (N-CWS) was prepared¹⁸⁾ by Fujisawa Pharmaceutical Co., Ltd. and suspended in physiological saline. Lipopolysaccharide (LPS) contamination in N-CWS was assayed by using the Limulus amoebocyte lysate assay and was found to be negligible, i.e. 0.36 ng per mg of N-CWS. Human γ -globulin (h- γ -G; Sigma), bovine serum albumin (BSA; Sigma) and cytochrome c (cyt-c; Sigma) were used as molecular mass markers. Hank's balanced salt solution (HBSS; Nissui Pharmaceutical) was used as the culture medium.

Measurement of Rectal Temperature Guinea pigs were lightly restrained by hand, and a thermister-probe (TF-DN type; Termo Co., Ltd.) was inserted about 50 mm into the rectum. The rectal temperature was measured for 1 min at intervals of 30 min.

Blood Sampling Blood was taken by heart puncture 0.5, 0.75, 1.0, 1.5, 2.0, 2.5 or 3.0 h after an i.v. injection of N-CWS into guinea pigs, and sera was separated by centrifugation for assay of L-929 cell-killing activity.

Isolation and Culture of Macrophages Guinea pig macrophages were isolated by a modification of the method of Ohishi *et al.*¹⁹⁾ Liquid paraffin was injected i.p. into guinea pigs to release cells into the peritoneal fluid, and 3 or 4 d later, the cells were collected, suspended in HBSS without serum, and incubated at 37°C in a humidified atmosphere under a stream of 5% CO₂ for 2 h. The adherent cells (macrophages) were separated from the nonadherent ones by washing, and 5 \times 10⁶ cells/ml of macrophages were pretreated with N-CWS at 37°C in a humidified atmosphere under a stream of 5% CO₂. One hour later, N-CWS was removed by decanting the incubation medium, and the macrophages were washed with physiological saline, suspended again in HBSS without serum and cultured for 0.5, 1, 3, 4 or 24 h under the same conditions. The supernatant was separated by centrifugation at 1000 rpm for 10 min to assay L-929 cell-killing activity and fever-producing ability.

Gel Filtration Macrophages were pretreated with 100 μ g/ml of N-CWS for 1 h and cultured in HBSS without serum for 24 h after removing the N-CWS. The supernatant obtained was concentrated 6-fold by using a membrane filter (Amicon model 8200, YM5) and 2 ml of the solution was applied to a Sephadex G-200 column (i.d. 1.5 \times 82 cm); 2 ml fractions were collected by eluting with HBSS without serum and BSA at the rate of 4 ml/h at 4°C. Absorbance at 280 nm was spectrophotometrically measured for each fraction and 5 serial fractions were pooled for assay of L-929 cell-killing activity and fever-producing ability.

Assay of L-929 Cell-Killing Activity Killing of the L-929 cell line was used to measure the TNF-like activity of soluble factors essentially according to the method described by Fisch and Gifford.²⁰⁾ Serial 1:2 dilutions of the guinea pig sera samples, cultured supernatant of the macrophages or gel filtration fractions were placed in 96-well microtiter plates. Then 5 \times 10⁴ cells of L-929 were added to the microtiter plates in the presence of 2 μ g/ml of actinomycin D and 10% of fetal bovine serum (FBS). The cells were incubated at 37°C in a humidified atmosphere under a stream of 5% CO₂ for 18 h, and the medium was removed. The cells were stained with 0.5% crystal violet for 10 min and dried, and absorbance at 540 nm was measured. The cell-killing activity (unit) in the test samples was expressed as the reciprocal of the dilution which exhibited 50% cytotoxicity to the L-929 cells.

Results

In our previous studies,²¹⁾ N-CWS caused fever in guinea pigs at i.v. doses of 100 μ g/kg or more. To examine the relationship between the fever and TNF production, we used N-CWS at doses of 1 to 1000 μ g/kg in this study.

Pattern of Fever N-CWS induced fever at doses of more than 100 $\mu\text{g}/\text{kg}$. The fever appeared within 1–1.5 h and lasted about 4 h with peaks at 1.5 and 3–4 h (Fig. 1).

Fever and TNF-like Activity in the Sera N-CWS (1000 $\mu\text{g}/\text{kg}$ i.v.) was used in the experiment on TNF-like activity. Cytotoxic activity in the sera increased from 1 h after injection of N-CWS, peaked at 1.5 h, and returned to normal at 3 h (Fig. 2). On the other hand, fever appeared at 1.5 h and did not subside during the observation time. The fact that cytotoxic activity preceded the onset of fever suggests that a TNF-like mediator plays an important role as an EP in the first phase of fever induced by N-CWS, but not in the second phase or that from 2 h after the injection of N-CWS. Other factors such as IL-1 may participate in the latter phase of the fever.

Figure 3 shows the cytotoxic activity in the sera 1.5 h after an i.v. injection of 1–1000 $\mu\text{g}/\text{kg}$ of N-CWS. Cytotoxic activity could be detected in the sera of the animals given 100 and 1000 $\mu\text{g}/\text{kg}$ of N-CWS.

Production of TNF-like Cytokine in Guinea Pig Perito-

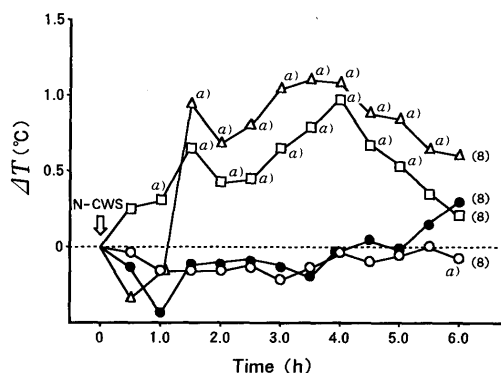


Fig. 1. Fever Pattern Induced in Guinea Pigs by N-CWS

A dose of 10 (\circ — \circ), 100 (\square — \square) or 1000 (\triangle — \triangle) $\mu\text{g}/\text{kg}$ of N-CWS, or physiological saline (\bullet — \bullet) was injected i.v. The arrow shows the time of injection. Values are expressed as mean \pm S.E. The numbers in parentheses are the number of guinea pigs used. Significantly different from the saline group, $a) p < 0.01$.

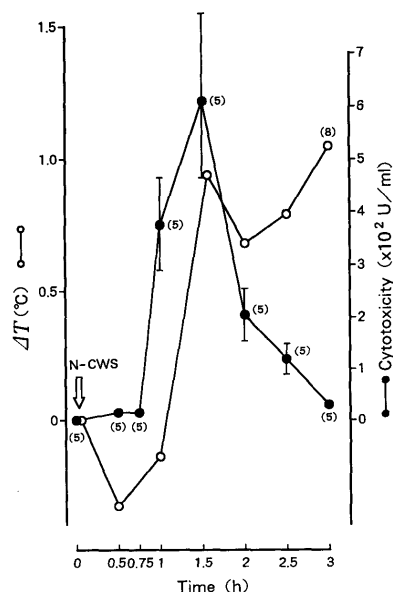


Fig. 2. Fever and Serum TNF-like Activity after i.v. Injection of N-CWS (1000 $\mu\text{g}/\text{kg}$) in Guinea Pigs

The arrow shows the time of injection. Values are expressed as mean \pm S.E. The numbers in parentheses are the numbers of guinea pigs used.

neal Macrophages Treated with N-CWS Since TNF is produced in macrophages,¹⁷⁾ we examined whether N-CWS would produce TNF in guinea pig peritoneal macrophages.

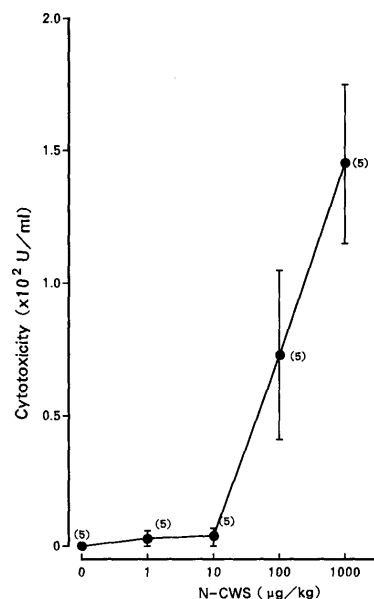


Fig. 3. Correlation between Serum TNF-like Activity and N-CWS Dose

TNF activity was measured 1.5 h after i.v. injection of N-CWS. Values are expressed as mean \pm S.E. The numbers in parentheses are the numbers of guinea pigs used.

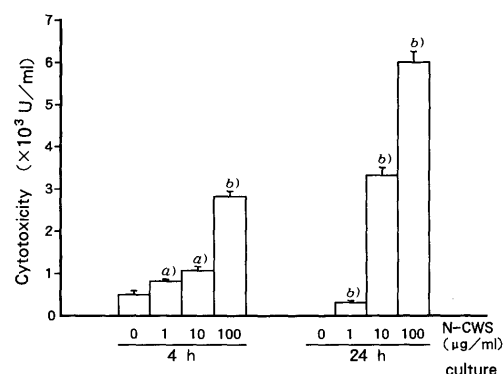


Fig. 4. TNF-like Activity in the Culture Supernatant of Guinea Pig Peritoneal Macrophages Pretreated with N-CWS

TNF-like activity was expressed as cytotoxicity of the culture supernatant to L-929 cells. Values are expressed as mean \pm S.E. Significantly different from the sample without N-CWS, $a) p < 0.05$, $b) p < 0.01$.

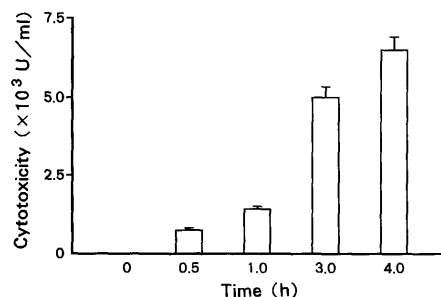


Fig. 5. TNF-like Activity in the Supernatant of Macrophages Cultured for a Short Time

Macrophages (5×10^6 cells/ml) were pretreated with 100 $\mu\text{g}/\text{ml}$ of N-CWS and cultured in HBSS without serum for 0.5–4.0 h after removing N-CWS. TNF-like activity was expressed as cytotoxicity of the culture supernatant to L-929 cells. Values are expressed as mean \pm S.E. TNF activity was not detected in the samples without N-CWS.

Macrophages were treated with N-CWS and cultured for 4 or 24 h. Cytotoxic activity was detected in all the supernatants of the macrophages treated with 1 $\mu\text{g}/\text{ml}$ or higher concentrations of N-CWS, and the activity was greater as the concentration was increased. Furthermore, the cytotoxic activity in the supernatants of the 24-h cultures was more than twice as strong as that of the 4-h cultures of macrophages treated with 10 or 100 $\mu\text{g}/\text{ml}$ of N-CWS. This suggests that cytotoxic activity also depends on culture duration (Fig. 4).

We propose that a TNF-like cytokine is produced in the macrophages immediately after treatment with N-CWS, because cytotoxic activity was detected in the supernatant of the macrophages as soon as 30 min after the start of incubation (Fig. 5).

Gel Filtration Pattern of the Cultured Supernatant In this study, 100 $\mu\text{g}/\text{ml}$ of N-CWS was used. When absorbance at 280 nm was measured for each fraction, 3 peaks of optical density (OD) were obtained, at fractions 27, 47 and 67. Cytotoxic activity was detected in fractions 41 to 55, and the greatest activity was in the pooled fractions 46 to 55. The molecular weight of materials in these pooled fractions was 32000–85000 as determined by gel filtration using molecular mass markers such as h- γ -G (Mol. wt. 160000), BSA (Mol. wt. 67000) and cyt-c (Mol. wt. 13000) (Fig. 6).

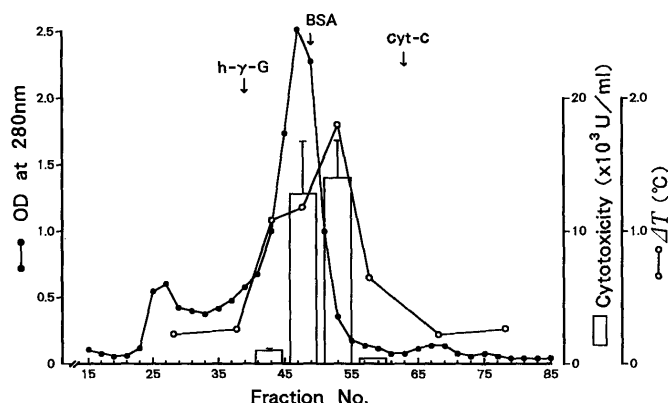


Fig. 6. Gel Filtration Pattern of the Culture Supernatant

The culture supernatant was applied to a Sephadex G-200 column (i.d. 1.5 \times 82 cm), and 2 ml fractions were collected by elution with HBSS. TNF-like activity and febrile response were measured for 5 pooled fractions. Febrile response was expressed as mean value of 3 guinea pigs 3 h after i.v. injection of 0.5 ml of the pooled fractions.

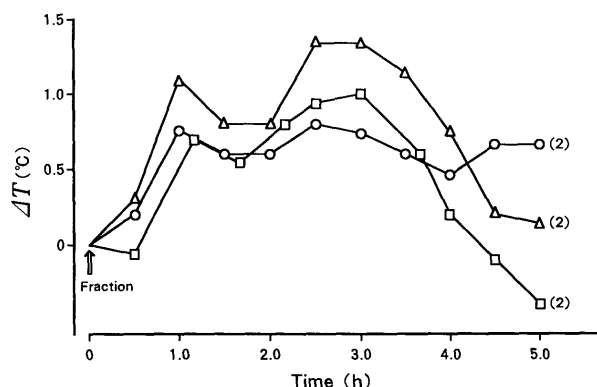


Fig. 7. Fever Response by Gel-Filtered Fractions

A dose of 0.1 (○—○), 0.5 (△—△) or 1.0 (□—□) ml of pooled fractions (fraction 46 to 50) was injected i.v. into guinea pigs. The arrow shows the time of injection. The numbers in parentheses are the numbers of guinea pigs used.

When 0.1, 0.5 or 1.0 ml of the pooled fractions 46 to 50 was injected i.v. into guinea pigs, biphasic fever with peaks at 1 and 2.5–3.0 h was observed in all the animals (Fig. 7). The pattern of the fever was similar to that induced by an injection of N-CWS, but its onset was 30 min earlier. In this case, the response was bell-shaped and the highest temperature was caused by 0.5 ml of the pooled fraction. In Fig. 6, febrile response 3 h after injection of 0.5 ml of each of the serially pooled fractions is shown. Clear fever of more than 0.5 $^{\circ}\text{C}$ was induced by fractions 41 to 60, and the highest fever was caused by the pooled fractions 51 to 55, in which cytotoxic activity was also greatest.

These results suggest that the fever induced by N-CWS is associated with TNF-like activity produced in the macrophages.

Discussion

For the reasons described below, it was suggested that TNF acted as an EP in the fever induced by N-CWS in guinea pigs. 1) TNF-like activity was detected in the sera 1 h after an injection of N-CWS and 30 min later, fever appeared. 2) When the guinea pig peritoneal macrophages were pretreated with N-CWS, increase of TNF-like activity in the culture supernatant depended on N-CWS concentration and culture duration. 3) The fraction with high TNF-like activity coincided with that with great fever-inducing ability, as determined by gel filtration.

Fever appeared within 1.5 h and lasted 5–6 h after an i.v. injection of N-CWS, whereas TNF-like activity appeared from around 1 h, peaked at 1.5 h, and then decreased. Dinarello *et al.*¹⁷⁾ reported that fever induced by an i.v. injection of r-TNF α occurred early and the febrile pattern was biphasic with peaks at 1 and 3–4 h in rabbits. They considered that TNF acted as an EP in the first phase of fever, but that in the second phase of fever, EPs other than TNF were involved, because more than 95% of r-TNF α injected in rabbits was cleared from the blood within 3 h.²²⁾ They suggested that the second phase was mediated by IL-1, because r-TNF α induced IL-1 in their experiment.¹⁷⁾ In our present study, the pattern of fever induced by N-CWS was similar to that induced by r-TNF α . Therefore, we propose that the first phase of fever was mediated by TNF produced by N-CWS, and that the EP on the second phase of fever would be IL-1, as suggested by Dinarello *et al.*, because the TNF induced by N-CWS disappeared quickly and was not detected in the second phase of fever in spite of this being higher. When macrophages were pretreated with N-CWS *in vitro*, the increase of TNF-like activity depended upon culture duration, and this pattern was quite different from that *in vivo*, in which TNF-like activity peaked 1 h after an injection of N-CWS and then quickly disappeared. We suggest that the cause of this difference lies in the clearance of TNF *in vivo*. When the supernatant of the cultured macrophages was gel-filtered, the fraction with TNF-like activity coincided with that with fever-inducing ability, and the patterns of the fever induced by the pooled fractions with TNF-like activity were similar to that of the fever induced by an injection of N-CWS. This may indicate mediation of the fever by TNF. The molecular weight of the pooled fractions with high TNF-like activity was determined to range from 32000–85000 by gel-filtration using molecular mass markers. Zacharchuk *et al.*²³⁾ report-

ed that the molecular weight of TNF from the sera, after challenge with an injection of LPS 14d after the injection of Bacillus Calmette-Guérin into guinea pigs, was about 45000 as determined by high performance liquid chromatography (HPLC), and our data coincide well with this finding.

The results of the present experiments suggest that TNF acts as an EP in the fever induced by N-CWS, but the possibility that the first phase of the fever is mediated by other factors such as IL-1 cannot be excluded.

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