

## Possible Involvement of Lymphocyte Activating Factor (LAF) as an Endogenous Pyrogen in Fever Induced by the Cell Wall Skeleton of *Nocardia rubra* (N-CWS)

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We investigated whether interleukin-1 (IL-1) acts as an endogenous pyrogen (EP) on the fever caused by the cell wall skeleton of *Nocardia rubra* (N-CWS) in guinea pigs. IL-1 activity was expressed as potency of lymphocyte activating factor (LAF). When guinea pig peritoneal macrophages were pulse-stimulated with N-CWS (1—100 µg/ml), dose-dependent LAF activity was detected in the supernatants after culture for 4 h. Gel filtration of the culture supernatants on Sephadex G-200 showed that the fractions with LAF activity were not the same as those with cytotoxic activity for L-929 cells, which was measured as an index of tumor necrosis factor (TNF) in parallel with LAF activity. Pyretic activity was detected both in the fractions with LAF activity and in those with cytotoxic activity for L-929 cells. Furthermore, when these macrophages were pulse-stimulated again, this time with the supernatant obtained from macrophages previously pulse-stimulated with N-CWS, LAF and cytotoxic activity for L-929 cells continued to be released from the macrophages.

We suggest that IL-1 might be a possible EP in the process of fever elicited by N-CWS, and that such an EP stimulates the macrophages to release further IL-1 or TNF. The resultant long-lasting fever would thus be caused by the continuous release of an EP.

**Keywords** *Nocardia rubra* cell wall skeleton; fever; interleukin; endogenous pyrogen

Cell wall skeleton of *Nocardia rubra* (N-CWS) is a preparation of the cell wall skeleton of the gram positive bacteria, *Nocardia rubra*. It was found to have anti-tumor activities in several experimental animals<sup>1,2)</sup> and also was effective against cancer in clinical trials.<sup>3-6)</sup> In these trials, fever occurred as a side effect of N-CWS.

Fever caused by most exogenous agents is mediated by a hormone-like polypeptide produced by host macrophages or monocytes. This mediator, called an endogenous pyrogen (EP),<sup>7)</sup> induces fever through its action on the thermoregulatory area of the brain.<sup>8)</sup> Since interleukin-1 (IL-1),<sup>9)</sup> tumor necrosis factor (TNF)<sup>10)</sup> and interferon<sup>11)</sup> have been reported to be possible EPs in experimental animals, we investigated whether IL-1 is involved in the fever induced by N-CWS in guinea pigs.

### Experimental

**Animals** SLC: Hartley male guinea pigs weighing 350—700 g were purchased from Shizuoka Agricultural Cooperative Association.

**Chemicals** N-CWS was prepared<sup>12)</sup> in the laboratories of Fujisawa Pharmaceutical Co., Ltd. Lipopolysaccharide (LPS) contamination in N-CWS was assayed using the *Limulus* amebocyte lysate test and found to be negligible, i.e. 0.36 ng per mg of N-CWS. N-CWS suspensions in physiological saline and culture medium were prepared under sterile conditions. Human  $\gamma$ -globulin (H- $\gamma$ -glob.: Sigma Chemical Company), bovine serum albumin (BSA: Sigma Chemical Company) and cytochrome c (ct-c: Sigma Chemical Company) were used as molecular mass markers.

**Preparation of Cells** a) Macrophages: Peritoneal exudate cells were collected<sup>13)</sup> from guinea pigs 3 or 4 d after an intraperitoneal injection of liquid paraffin. The cells not adhering to a glass plate were rinsed away with physiological saline, and the adhering cells (macrophages) were collected under sterile conditions.

b) Thymocytes: Thymocytes excised from guinea pig thymus were homogenized gently with a glass homogenizer, and were collected by centrifugation at 1000 rpm for 10 min.

**Preparation of the Supernatant from the Macrophages** Macrophages ( $5 \times 10^6$ ) were suspended in Hanks balanced salt solution (HBSS) without fetal calf serum (FCS) and pulse-stimulated with 1—100 µg/ml of N-CWS at 37°C for 1 h. The N-CWS was washed away with physiological saline, and the macrophages were suspended again in HBSS and cultured for 4 h.

**Preparation of the Supernatant from the Macrophages Pulse-Stimulated with the Previously Obtained Supernatant (N-CWS 100 µg/ml)** Macrophages ( $5 \times 10^6$ ) were pulse-stimulated for 1 h with the supernatant ob-

tained in the above step; this supernatant yielded  $1.93 \times 10^5$  cpm/ml of LAF activity and  $7.35 \times 10^3$  U/ml of cytotoxic activity for L-929 cells. The stimulant was washed away with physiological saline, and the macrophages were cultured for a further 4 h.

**Assay for LAF Activity and Cytotoxic Activity for Cells** LAF activity was measured according to the method of Taniyama and Onoue.<sup>14)</sup> It was expressed as uptake (cpm) of [ $\text{methyl-}^3\text{H}$ ]thymidine ( $^3\text{H}$ -TdR: Amersham Japan) into the thymocytes by a 2-fold dilution of the supernatant. Cytotoxic activity for L-929 cells in the supernatant was measured according to the method described by Fisch and Gifford,<sup>15)</sup> and was expressed as the reciprocal of the dilution of the supernatant which exhibited 50% cytotoxicity toward L-929 cells.

**Gel Filtration** Macrophages ( $5 \times 10^6$ ) were pulse-stimulated with 100 µg/ml of N-CWS for 1 h. The N-CWS was washed away and the macrophages were cultured for a further 24 h. The culture supernatant was concentrated to 1/6 of the initial volume 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 at a 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 LAF activity, cytotoxic activity toward L-929 cells and fever-producing activity.

**Measurement of Rectal Temperature** A 0.5 ml aliquot of each of the pooled eluate fractions from the Sephadex G-200 gel column was injected i.v. into guinea pigs in groups of 3. Three hours later, the rectal temperature was measured and fever was expressed as the difference between rectal temperature at that time and before dosing.

**Statistical Analysis** Differences between the control and experimental values were analyzed by means of Student's *t* test.

### Results and Discussion

We investigated whether LAF has a role in the fever induced by N-CWS in guinea pigs by analyzing the cytokines released from the macrophages after treatment with N-CWS.

LAF activity in the culture supernatant of the macrophages increased dependently on N-CWS concentration (1—100 µg/ml) when the macrophages were pulse-stimulated for 1 h with N-CWS and cultured for a further 4 h after removing the N-CWS (Fig. 1). To investigate the relevancy of LAF to N-CWS fever, the culture supernatant was gel-filtered through Sephadex G-200, and LAF and pyretic activity in the eluates were measured for 5 pooled fractions. Cytotoxic activity for L-929 cells, which is an

marker of TNF, was also measured for the pooled fractions.

As shown in Fig. 2, the elution pattern of pyretic activity in the supernatant roughly fitted that of LAF and cytotoxic activity for L-929 cells; *i.e.*, peak activity was seen in fractions 46–50 (LAF) and 46–55 (cytotoxic activity for L-929 cells).

When the elution pattern was depicted in terms of specific activity of LAF and cytotoxic activity toward the L-929 cells (Fig. 3), these activities appeared in different fractions: peak LAF activity in 61–65 and cytotoxic activity for L-929 cells in 51–55. Fractions 61–65 induced fever of more than 0.5 °C, but they did not kill the L-929 cells. In this case we suppose that the fever was mediated by an EP such as IL-1; with fractions 41–60, however, it was impossible to demonstrate what the EP might be.

In the present study, LAF and cytotoxic activity for L-929 cells were measured as representative characteristic indices of IL-1 and TNF. Recently, it has been shown that IL-1 has not only LAF activity but also cytotoxic activity

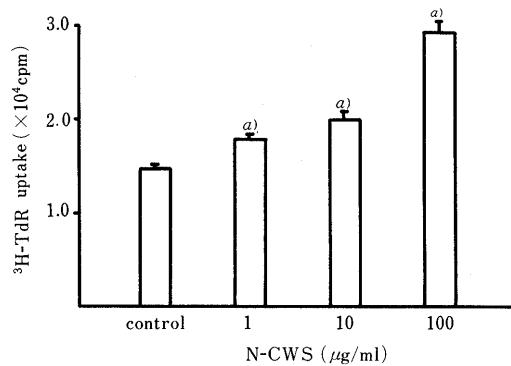


Fig. 1. LAF Activity in the Culture Supernatant of Macrophages Pulse-Stimulated with N-CWS

Guinea pig peritoneal macrophages ( $5 \times 10^6$ ) were pulse-stimulated with N-CWS for 1 h and then cultured in HBSS for 4 h after removing the N-CWS. LAF activity was measured in terms of the increase of  $^3\text{H}$ -TdR uptake into the thymocytes induced by 1  $\mu\text{g}/\text{ml}$  of PHA-P. The control value was the activity in the culture supernatant of macrophages without N-CWS. LAF activity was expressed as the mean  $\pm$  S.E. of cpm of  $^3\text{H}$ -TdR uptake into the thymocytes. Significantly different from the control  $a) p < 0.05$ .

toward L-929 cells, and that TNF has both activities. Accordingly, the LAF and cytotoxic activity in this study may not be necessarily regarded respectively as stemming from IL-1 and TNF. Onozaki *et al.*<sup>16)</sup> have reported that IL-1 showed cytotoxicity toward L-929 cells but the activity was less than 50% even at the highest concentration, whereas TNF showed concentration-dependent cytotoxicity toward L-929 cells and higher concentrations killed all of them.

As shown in Fig. 4, the cytotoxicity toward L-929 cells increased dependently on the volume of the culture supernatant which was obtained from the macrophages pulse-stimulated with N-CWS and cultured further without N-CWS, and around 100% cytotoxicity was obtained with a 4-fold dilution of the supernatant. Therefore, the cytotoxicity in our results may have been due to TNF.

Hurme<sup>17)</sup> reported that TNF showed LAF activity in the presence of phytohemagglutinin-P (PHA-P) at concentrations of 5  $\mu\text{g}/\text{ml}$  and more of PHA-P but not at 1  $\mu\text{g}/\text{ml}$ , the suboptimum concentration, whereas IL-1 showed LAF activity even in the presence of PHA-P at concentrations of 1  $\mu\text{g}/\text{ml}$  or less. Since LAF activity was measured using a concentration of 1  $\mu\text{g}/\text{ml}$  of PHA-P in the present study, the LAF activity in our results could have been derived from IL-1.

Recently it was reported that IL-1<sup>18)</sup> or TNF<sup>8)</sup> triggered the production of IL-1 in human monocytes. This prompted us to investigate whether the culture supernatant of N-CWS-stimulated macrophages would trigger the release of LAF- and TNF-like substances from guinea pigs macrophages. When the macrophages were pulse-stimulated with the culture supernatant of macrophages stimulated with N-CWS, LAF activity appeared in the supernatants and was dependent on the volume of the supernatant used. On the other hand, cytotoxic activity for L-929 cells, which also appeared in the supernatant, leveled off at a concentration of 7.5 dilution of the culture supernatant (Fig. 5). This supernatant yielded  $1.93 \times 10^5 \text{ cpm}/\text{ml}$  LAF activity and  $7.35 \times 10^3 \text{ U}/\text{ml}$  of cytotoxic activity for L-929 cells. Accordingly, we suggest that cytokines such as IL-1

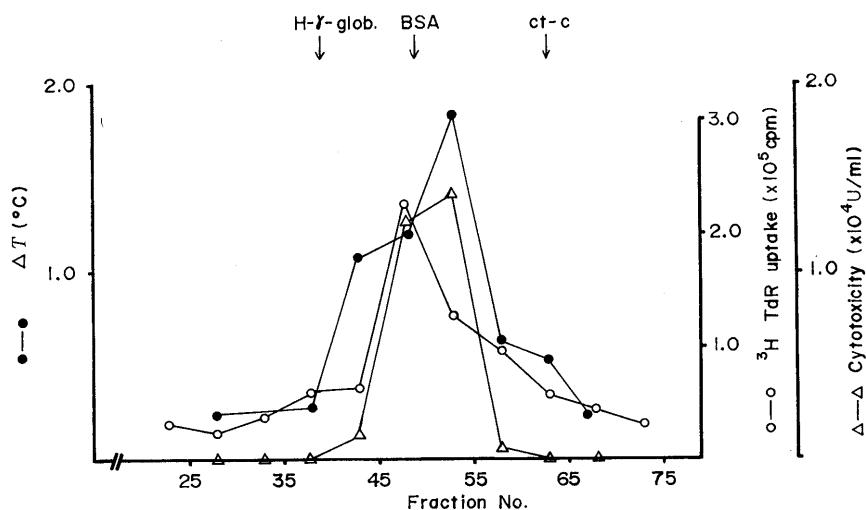


Fig. 2. Gel Filtration Pattern of the Culture Supernatant

The culture supernatant was applied to a Sephadex G-200 (i.d.  $1.5 \times 82 \text{ cm}$ ) column, and 2 ml fractions were collected by elution with HBSS. Activities of LAF and cytotoxicity toward L-929 cells, and fever were measured for 5 pooled fractions. Fever was expressed as mean temperature increase of 3 guinea pigs 3 h after i.v. injection of 0.5 ml of the pooled fractions.

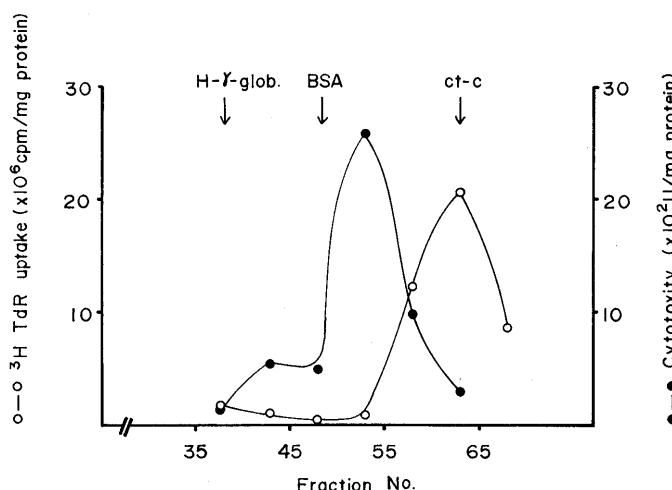


Fig. 3. Specific Activities of LAF and Cytotoxicity toward L-929 Cells in Each Pooled Fraction from Gel Filtration of the Culture Supernatant

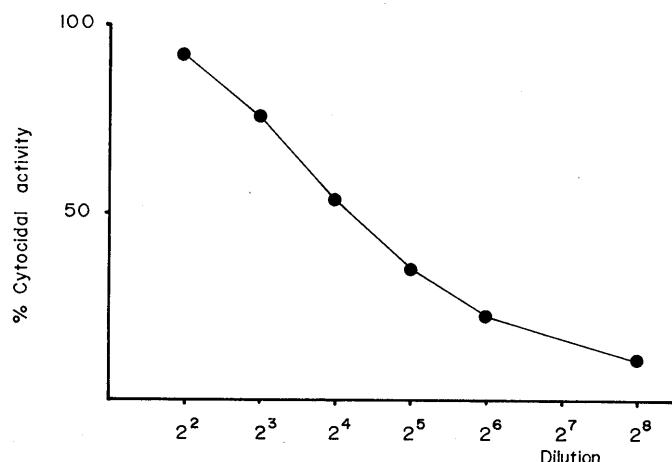


Fig. 4. Cytocidal Activity of the Culture Supernatant of Macrophages Pulse-Stimulated with N-CWS on L-929 Cells

Two-fold dilutions of the culture supernatant were added to  $5 \times 10^4$  L-929 cells, and incubated for 18 h at 37°C. The cells were stained with 0.5% crystal violet and absorbance at 540 nm was measured. Cytocidal activity is expressed as % of L-929 cells killed by the culture supernatant to that treated with the culture medium alone.

and TNF, which are products of macrophages stimulated with N-CWS, further act on the macrophages and trigger the release of IL-1 and TNF.

In a preliminary study, N-CWS caused fever lasting for 6 h in guinea pigs. This long-lasting fever may have been due to the continuous release of cytokines such as IL-1 and TNF from the macrophages stimulated with N-CWS.

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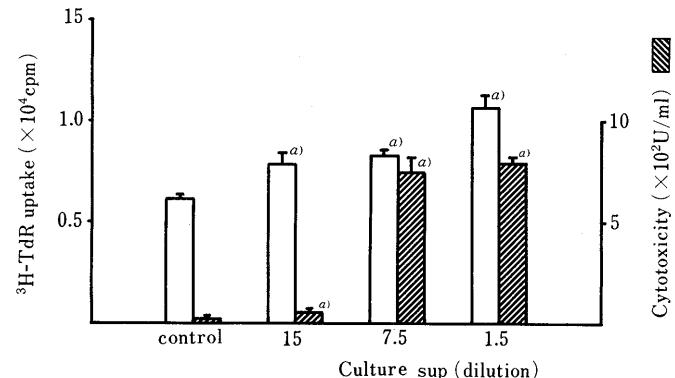


Fig. 5. Induction of LAF Activity and Cytotoxic Activity for L-929 Cells in Macrophages Pulse-Stimulated with the Culture Supernatant of N-CWS-Stimulated Macrophages

Guinea pig peritoneal macrophages ( $5 \times 10^6$ ) were pulse-stimulated with the culture supernatant for 1 h, and further cultured for 4 h without the supernatant. The open column shows LAF activity and the shaded column cytotoxic activity toward L-929. Controls were obtained from the supernatant of macrophages cultured with medium alone. Values are expressed as mean  $\pm$  S.E. of triplicate assays. Significantly different from the control a)  $p < 0.05$ .