A Possible Mechanism of Fever Induced by *Nocardia rubra* Cell Wall Skeleton (N-CWS) in Experimental Animals

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The characteristics of fever elicited by the cell wall skeleton of *Nocardia rubra* (N-CWS) and by lipopolysaccharide (LPS) were compared in rabbits, and the possible involvement of the antigenicity of N-CWS was investigated in guinea pigs.

In rabbits, fever of more than 0.5 °C developed after an intravenous (i.v.) injection of 10 μg/kg or more of N-CWS, and was monophasic with 30—100 μg/kg but biphasic with the highest dose of 300 μg/kg. LPS elicited fever with similar characteristics at doses of 0.01—0.1 μg/kg. With both compounds, the fever was inhibited by indomethacin. Tolerance to N-CWS and LPS appeared after dosing with 30 or 0.1 μg/kg, respectively for 10 d.

In guinea pigs sensitized with N-CWS, challenge with 1 or 10 μg/kg of N-CWS 10 d later, which did not induce fever in the nonsensitized animals, caused fever of more than 0.5 °C, and delayed-type hypersensitivity (DTH) appeared. N-CWS also elicited fever in nonsensitized guinea pigs bearing N-CWS-sensitized lymphocytes or anti-N-CWS antibody; the fever was higher in the guinea pigs sensitized with the lymphocytes than in those with the anti-N-CWS antibody.

In brief, single injections of N-CWS and of LPS elicited fever with similar characteristics, although the potency of N-CWS was weaker. With N-CWS, the fever is proposed to be triggered by the antigenicity of the compound itself, because doses as low as 1 or 10 μg/kg elicited fever along with immunological response in N-CWS-sensitized animals, but not in nonsensitized ones.

Keywords *Nocardia rubra* cell wall skeleton; N-CWS; fever; gram positive bacteria; delayed-type hypersensitivity

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] but induced fever as a side effect both in clinical trials and in rabbits and rats.

As fever is caused by infection with microorganisms, its mechanism has been studied mainly using lipopolysaccharide (LPS)[7,8] derived from a gram-negative bacterium. Gram positive bacterium such as staphylococcus[9] can also elicit fever, however, and in this case the pyrogen is presumed to be a peptidoglycan or muramyldipeptide.[10,11] Since peptidoglycan is also a structural component of N-CWS,[12] the fever induced by N-CWS can be attributed to the peptidoglycan in N-CWS. In a previous study, we found that N-CWS had antigenicity with humoral and cellular immune responses,[13] and suggested that the immune responses are also involved in the N-CWS-induced fever. In addition to the nature of the fever induced by a single injection of N-CWS, we examined in the present work the possible involvement of an immune mechanism in guinea pigs.

Experimental

Animals Japanese white strain male rabbits weighing 1.8—2.5 kg and Slc: Hartley male guinea pigs weighing 350—700 g were used. The animals were reared at room temperature of 20 ± 5 °C and relative humidity of 60 ± 5%. Lighting was controlled to give 12 h cycles of light and darkness.

Chemicals and Treatment N-CWS was prepared[12] from *Nocardia rubra* by Fujisawa Pharmaceutical Company in Japan. A lyophilized N-CWS formulation and bulk N-CWS were used. The formulation contained 2 mg of N-CWS, 4 mg of squalene, 1 mg of polysorbate 80 and 28.2 mg of mannitol. Placebo was used as the control and contained the same ingredients except N-CWS. Distilled water or physiological saline was used for the N-CWS suspension. N-CWS was prepared under sterile conditions, and the content of LPS in the preparation was measured by means of the Toxicolor® test (Seikagaku Kogyo, Co., Ltd.). The content of LPS was found to be 0.36—0.66 ng per mg of N-CWS; that is, contamination by LPS was negligible. As a reference compound, LPS from *Escherichia coli* 0111: B4 (Seikagaku Kogyo Co., Ltd.) was used. Freund's incomplete adjuvant (FIA: Difco Laboratories) was used for sensitizing the

guinea pigs to N-CWS. Eagle's minimal essential medium (MEM: GIBCO) was filtered through a 0.20 μm plain membrane (Millipore®) and used for the cell suspension. N-CWS and LPS were injected *via* the ear vein in rabbits and N-CWS *via* the paw vein in guinea pigs.

Measurement of Rectal Temperature Rabbits were confined on a board with a pillory. Rectal temperature was measured with a thermister-probe inserted 90 mm into the rectum and recorded continuously on an automatic recorder. Guinea pigs were lightly restrained by hand, and the rectal temperature was measured for 1 min at intervals of 30 min by a thermister-probe inserted 50 mm into the rectum.

Sensitization and Challenge An emulsion of equal volumes of N-CWS and FIA was injected into the foot pad of guinea pigs. The dose of N-CWS was 100 μg/animal. The animals in the negative control group were given an emulsion of equal volumes of phsiological saline and FIA. Ten days later, 1 or 10 μg/kg of N-CWS was injected i.v. into the guinea pigs and rectal temperature was measured.

Skin Test and Assay of Anti-N-CWS Antibody Skin test and blood sampling were performed to examine DTH or production of anti-N-CWS antibody in the sensitized guinea pigs. In the skin test, 5 μg of N-CWS in 0.1 ml of physiological saline was injected i.d. into the clipped backs of the guinea pigs 10 and 14 d after sensitization and the diameter of erythema was measured 24 h later. Blood was taken from the paw vein 10 and 14 d after sensitization, and the serum was separated and pooled. Anti-N-CWS antibody titer in the pooled sera was assayed by means of a hemagglutination (HA) test using sheep red blood cells coated with a polysaccharide rich fraction (PS) extracted from N-CWS by the method of Azuma *et al.*,[14,15] and expressed as the $\log_2$ of the reciprocal of the highest serum dilution causing HA.

Preparation of Lymphocytes and Sera from Guinea Pigs Sensitized with N-CWS Lymphocytes: The axillar lymphnodes were collected from guinea pigs 18 d after sensitization and lightly homogenized in MEM. The lymphocytes were suspended in and washed three times with MEM by centrifugation at 1200 rpm for 10 min at 4 °C, and resuspended in MEM. Wright-Giemsa staining of the suspended cells confirmed that 97% of the cells were lymphocytes; their viability assayed by means of the neutral red (1%) dye exclusion test was 50.9—54.4%.

Sera: Blood was obtained from the femoral artery of the guinea pigs 18 d after sensitization, and the serum was separated and pooled. The titer of anti-N-CWS antibodies in the pooled sera was 7. When the sera were treated with 0.2 M 2-mercaptoethanol, the titer decreased to 2. Accordingly, the immunoglobulin class of the anti-N-CWS antibody was assumed to be immunoglobulin M (IgM).

Transfer of Lymphocytes or Sera to Normal Guinea Pigs and Challenge with N-CWS Normal guinea pigs were given 1×10^6 or 1×10^7 cells obtained from animals sensitized with N-CWS, 1×10^7 cells from non-

sensitized animals, or 0.1, 0.25, 0.5, 1.0 or 2.0 ml of the pooled anti N-CWS sera or nonimmune sera by i.v. injection. Twenty four hours later, 10 μg/kg of N-CWS was injected i.v., and the rectal temperature was measured.

Statistical Analysis The significance of differences between the control and experimental values was evaluated by the use of Student's t test.

Results

Fever Induced by a Single Intravenous (i.v.) Dose of N-CWS in Rabbits Rectal temperature was measured for 6 h after an i.v. injection of 1—300 μg/kg of N-CWS. Fever of more than 0.5 °C was elicited by a dose of 10 μg/kg or more, and was monophasic with a peak of about 1.5 °C at 3 h at doses of 30 and 100 μg/kg. In contrast, the fever induced by 300 μg/kg was biphasic with peaks at 2 and 4 h, although the peak levels were lower by about one-third than that with 100 μg/kg. The onset of fever was hastened dose-dependently.

LPS elicited fever of more than 1 °C at doses of 0.01 and 0.1 μg/kg (Fig. 1), and the patterns were monophasic and biphasic, respectively. The onset of fever tended to be earlier than that induced by N-CWS.

Tolerance to N-CWS by Repeated i.v. Dosing in Rabbits Since 30 μg/kg of i.v. N-CWS produced clear fever (Fig. 1), this dose was given daily for 10 d to find out whether tolerance would develop. The rectal temperature of the rabbits was measured on days 1, 4, 7 and 10 of dosing. The peak temperatures after repeated dosing were lower than those after a single dose.
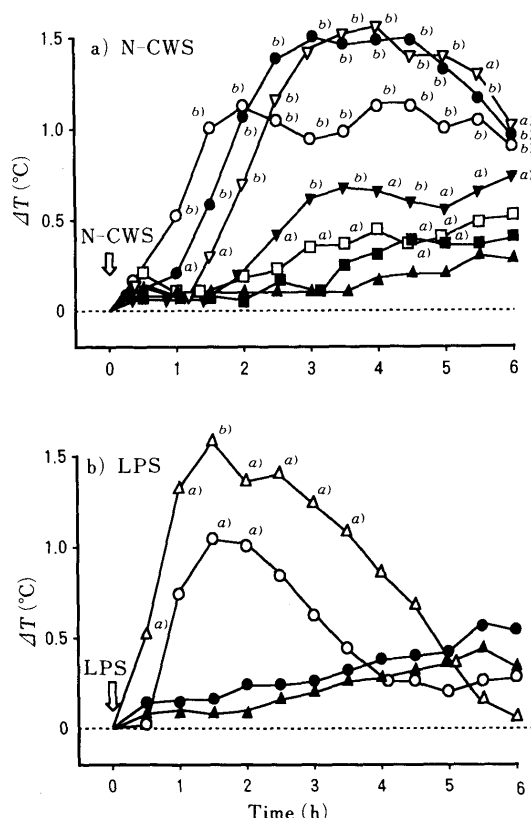
LPS was used at a dose of 0.1 μg/kg. In this case the fever on days 4 and 7 was slightly weaker than on day 1, and that on day 10 markedly declined to an almost negligible level (Fig. 2).

Effect of Indomethacin on Fever Induced by N-CWS N-CWS or LPS was given i.v. in a single dose of 30 or 0.1 μg/kg, respectively (at these doses the two compounds caused a similar rise in temperature). Indomethacin (2 mg/ kg) was given i.v. at the peak of fever and the rectal temperature declined immediately (Fig. 3).

Effect of Sensitization with N-CWS on Fever-Eliciting Capacity of N-CWS in Guinea Pigs Nonsensitized animals were injected with 10—1000 μg/kg of N-CWS and the rectal temperature was measured for 6 h. N-CWS elicited fever at doses of 100 and 1000 μg/kg, but not at 10 μg/kg. The fever appeared 1 or 1.5 h after the injection and lasted about 5 h with peaks at 1.5 and 3—4 h (Fig. 4). In contrast, when N-CWS was given to N-CWS-sensitized guinea pigs at a dose of 1 or 10 μg/kg 10 d after sensitization, the rise in temperature exceeded 0.5 °C. In the guinea pigs treated with FIA alone, the rectal temperature rose by only 0.5 °C or less (Fig. 5).

Skin Test and Determination of Anti-N-CWS Antibody All the guinea pigs showed pronounced erythema 10 and 14 d after sensitization with N-CWS, and the mean diameters were 9.6 and 8.9 mm, respectively. Very slight erythema was elicited in the nonsensitized guinea pigs.

No anti-N-CWS antibody was detected in the pooled sera from the N-CWS sensitized guinea pigs on day 10, but
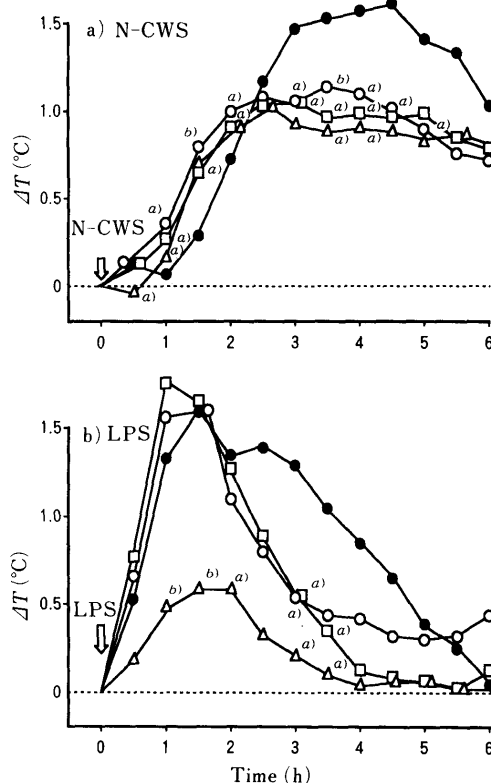
Fig. 1. Fever Induced by a Single i.v. Dose of N-CWS in Rabbits ($n = 3$)

Values are the mean increase of rectal temperature over predosing levels. The arrow shows the time of injection. a) ▲—▲, placebo; ■—■, 1 μg/kg; □—□, 3 μg/kg; ▼—▼, 10 μg/kg; ▽—▽, 30 μg/kg; ●—●, 100 μg/kg; ○—○, 300 μg/kg of N-CWS. b) ▲—▲, physiological saline; ●—●, 0.001 μg/kg; ○—○, 0.01 μg/kg; △—△, 0.1 μg/kg of LPS. Significantly different from placebo or saline control: a) $p < 0.05$; b) $p < 0.01$.

Fig. 2. Fever Induced by Repeated i.v. Dose of N-CWS in Rabbits ($n = 3$)

N-CWS and LPS were given at doses of 30 and 0.1 μg/kg i.v., respectively. Values are the mean increase of rectal temperature over predosing levels. The arrow shows the time of injection. ●—●, First day; ○—○, 4th day; □—□, 7th day; △—△, 10th day. Significantly different from levels on the first day: a) $p < 0.05$; b) $p < 0.01$.
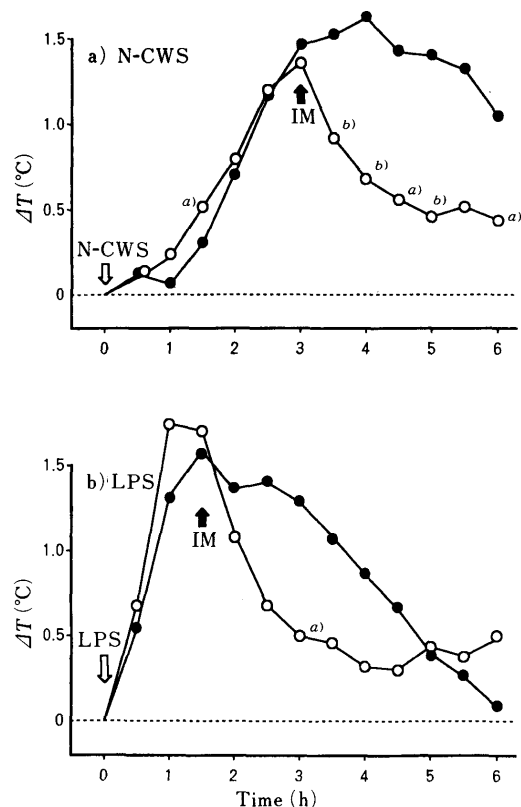
Fig. 3. Effect of Indomethacin on Fever Induced by N-CWS in Rabbits (n=3)

Indomethacin (2 mg/kg) was injected i.v. 1.5 or 3 h after i.v. injection of N-CWS (30 μg/kg) or LPS (0.1 μg/kg). The arrows show the time of injection. Values are the mean increase of rectal temperature over predosing levels. ●—●, N-CWS or LPS dosing; ○—○, indomethacin (IM) treatment. Significantly different from the treatment with N-CWS or LPS only: a) p<0.05; b) p<0.01.

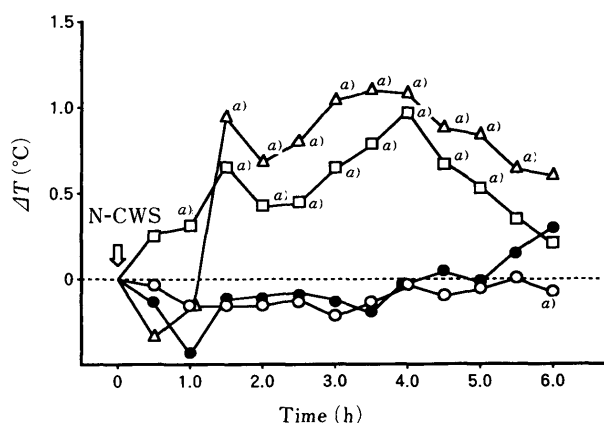Fig. 5. Fever Pattern after N-CWS Challenge to Guinea Pigs Sensitized with N-CWS (n=8)

A dose of 1 (●—●) or 10 (▲—▲) μg/kg of N-CWS was injected i.v. into sensitized guinea pigs, and 1 (○—○) or 10 (△—△) μg/kg of N-CWS into nonsensitized guinea pigs. The arrow shows the time of injection. Significantly different from the nonsensitized group: a) p<0.05; b) p<0.01.

TABLE 1. DTH and Determination of Anti-N-CWS Antibody

Sensitizing antigen (μg/animal)	Titer[a] of anti-N-CWS antibody (log$_2$)		Erythemic diameter in DTH[b] (mm; Mean ± S.E.)	
	Days after the first sensitization			
	10	14	10	14
Saline + FIA	N.T.[c]	N.T.	0 ± 0	2.3 ± 1.5
N-CWS (100) + FIA	< 1	4	9.6 ± 0.9	8.9 ± 0.6

a) Titer of anti-N-CWS antibody was measured by the passive hemagglutination method. b) N-CWS (5 μg/site) was injected i.d. into the back of guinea pigs (n=5), and after 24 h the diameter of erythema was measured. c) Not tested.

Fig. 4. Fever Pattern after a Single Injection of N-CWS to Normal Guinea Pigs (n=8)

A dose of 10 (○—○), 100 (□—□), or 1,000 (△—△) μg/kg of N-CWS, or saline (●—●) was injected i.v. and the rectal temperature was measured. The arrow shows the time of injection. Significantly different from the saline control: a) p<0.01.

Fig. 6. Fever Induced by N-CWS in Guinea Pigs (n=5 or 6) Given Lymphocytes of Guinea Pigs Sensitized with N-CWS

Lymphocytes sensitized with N-CWS (1 × 10^6 cells, ○—○ or 1 × 10^7 cells, △—△) or nonsensitized lymphocytes (1 × 10^7 cells, ●—●) were transferred by i.v. injection to normal guinea pigs. Then 24 h later, fever was induced by i.v. injection of 10 μg/kg of N-CWS. The arrow shows the time of injection. Significantly different from the group given nonsensitized lymphocytes: a) p<0.05; b) p<0.01.

it was detected with a titer of 4 on day 14 (Table I).

Fever Pattern after N-CWS Challenge to Guinea Pigs Treated with N-CWS-Sensitized Lymphocytes A 10 μg/kg i.v. dose of N-CWS caused fever in normal guinea pigs given lymphocytes from N-CWS-sensitized animals. The severity of the fever depended on the number of lymphocytes transferred and was greater than in the guinea pigs given lymphocytes from guinea pigs sensitized with FIA
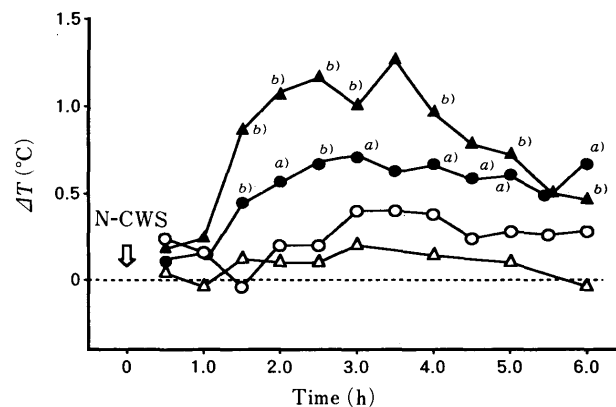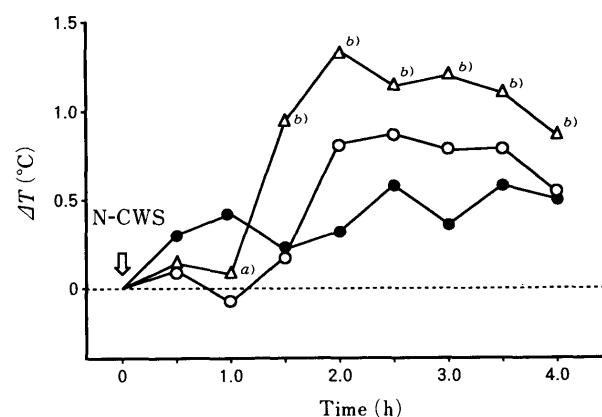
alone. With 1 × 10^7 lymphocytes, fever appeared 1.5 h after challenge with N-CWS and the peak rise in temperature was more than 1 °C. The onset of fever and the peak rise in temperature followed a pattern similar to that in guinea pigs sensitized actively with N-CWS (Fig. 6).

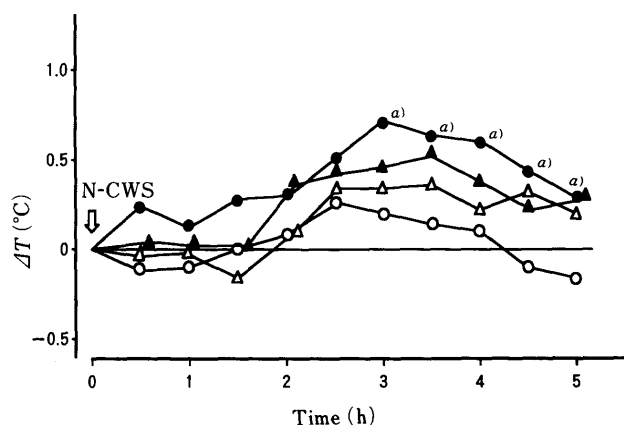**Fever Pattern after N-CWS Challenge to Guinea Pigs**

Fig. 7. Fever Pattern after N-CWS Challenge to Guinea Pigs $(n=5)$ Given Sera of Guinea Pigs Sensitized with N-CWS

An aliquot of 0.5 ml (●—●) or 2.0 ml (▲—▲) of immune serum or 0.5 ml (○—○) or 2.0 ml (△—△) of nonimmune serum was transferred by i.v. injection to normal guinea pigs. Then 24 h later, fever was induced by i.v. injection of 10 μg/kg of N-CWS. The arrow shows the time of injection. Significantly different from the group given nonimmune serum: a) $p < 0.05$.
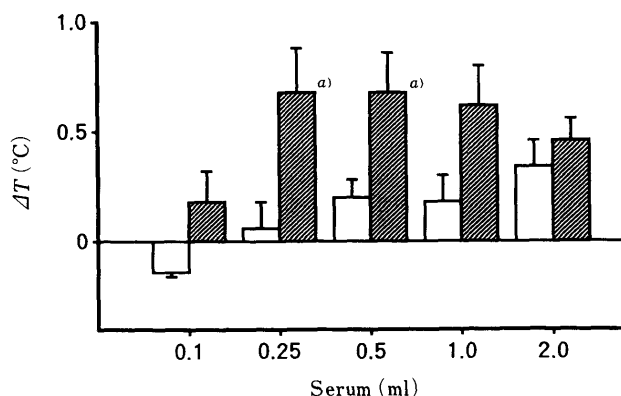

Fig. 8. Fever Induced by N-CWS in Guinea Pigs $(n=5)$ Given Sera of Guinea Pigs Sensitized with N-CWS

Immune serum (shaded), nonimmune serum (open). Immune sera were collected from guinea pigs sensitized with N-CWS. Values are the mean ± S.E. of temperature increase at 3 h after injection of 10 μg/kg of N-CWS. Significantly different from the group given nonimmune serum: a) $p < 0.05$.

Given Anti-N-CWS Sera Since the rise in temperature peaked 3 h after i.v. injection of 10 μg/kg of N-CWS and then declined (Fig. 7), the relationship between the fever and the volume of antiserum transferred was examined at the peak time.

Slight fever with a bell shaped dose-response curve was induced in the guinea pigs given 0.1—2.0 ml of antiserum. Rectal temperature increased by an average of 0.68 °C in the guinea pigs given 0.25 or 0.5 ml of antiserum, and the increase was significantly higher than in the guinea pigs given the same volumes of nonimmune serum (Fig. 8).

Discussion

N-CWS and LPS elicited dose-dependent fever in rabbits, with a few differences in the effects of the two compounds. First, the pyretic activity of N-CWS was weaker than that of LPS in terms of relative potencies of the two agents. LPS caused an increase in temperature by about 1.5 °C at an i.v. dose of 0.1 μg/kg, whereas 30 μg/kg i.v. of N-CWS was required to cause an equivalent increase. Second, the onset of fever after dosing with N-CWS was

slower than after LPS, and third, the fever induced by N-CWS was longer-lasting. These differences can be ascribed to the differences in the pyretic components of LPS and N-CWS. LPS is the main differences in the pyretic component in all gram-negative bacteria, although such organisms also contain a minor amount of peptidoglycan,[16,17] whereas peptidoglycan or its decomposition component muramyl-dipeptide is the pyretic component of gram-positive bacteria.[10,11] N-CWS also contains peptidoglycan.[18] Rotta[19] has shown that the pyretic effect of peptidoglycan is weaker and slower in onset than that of LPS. Since peptidoglycan and N-CWS are both insoluble in water and not easily metabolized, the pyretic activity of N-CWS would naturally be longer-lasting.

There were some common characteristics of the fever induced by N-CWS and LPS. First, the fever elicited by either compound was biphasic at the highest doses used,and second, repeated dosing with these compounds resulted in the development of tolerance to their fever-eliciting capacity. Tolerance to LPS is supposed to arise from 1) accelerated clearance of LPS from the blood owing to the activation of the reticulo-endothelial system by LPS,[20-22] and 2) the production of humoral antibody to LPS.[23,24] Since N-CWS can also activate the reticulo-endothelial system,[25] and has antigenicity leading to humoral and cellular responses,[13] the tolerance to N-CWS might have developed through a mechanism similar to that proposed for LPS. Third, the fever induced by N-CWS or LPS was inhibited by indomethacin, a cyclooxygenase inhibitor. This suggests that these fevers are mediated by prostaglandin. It is reported that N-CWS stimulates the production of cytokines such as tumor necrosis factor (TNF),[26] interleukin-1 (IL-1)[27] and interferon,[28] and TNF and IL-1 are known to produce prostaglandins. Thus, the pyretic activities of N-CWS would appear to be mediated by these cytokines and prostaglandins. In the experiment on guinea pigs sensitized with N-CWS, 1 and 10 μg/kg of N-CWS, which could not induce fever in nonsensitized guinea pigs, elicited fever and positive skin reaction, but not the production of anti-N-CWS antibody. Anti-N-CWS humoral antibody was not produced by day 10, but was produced 14 and 18 d after sensitization. Slight fever in guinea pigs given the anti-N-CWS sera showed a bell-shaped pattern with respect to serum volume, which indicates that the fever was not only associated with cellular immunity (DTH), but also humoral immunity at the time when anti-N-CWS antibody was produced, but the main mechanism would be cellular immunity because the fever-eliciting potency of N-CWS was higher in the guinea pigs given the lymphocytes than in the guinea pigs given anti-sera.

Atkins et al.[29,30] have reported that the lymphocytes of guinea pigs or rabbits sensitized with ovalbumin or bovine gamma globulin could release soluble substances, presumably "lymphokines," which may lead to the production of an endogenous pyrogen in the phagocytes. Accordingly, N-CWS may also elicit a kind of "lymphokine-mediated" fever via an immunological process based on its own antigenicity.

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