Involvement of Central Action of Lipopolysaccharide in Pyrogen Fever

Yoshiyuki OGAWA and Seizaburo KANOH
National Institute of Hygienic Sciences, Osaka Branch, Hoenzaka 1-1-43, Osaka 540, Japan
Accepted July 30, 1984

Abstract—To elucidate the mechanisms of fever response by bacterial pyrogen (lipopolysaccharide, LPS), we investigated both pyrogenicity and Limulus amoeboocyte lysate (LAL) gelation activity of cerebrospinal fluid (CSF) withdrawn from febrile rabbits induced by i.v. injection of LPS. One ml of CSF was withdrawn from donor animals 2 hr after i.v. injection of E. coli LPS at graded doses of 0, 1, 10 and 50 μg/kg, and its pyrogenicity was checked by administration into cisterna magna of recipient animals. Pyrogenicity was revealed only in the CSF withdrawn from the group injected with 50 μg/kg of LPS. Differences of both protein content and concentrations of ions (Na, K and Ca) were not obtained among the CSF withdrawn from control and LPS-injected groups. All of the above CSF had no LAL gelation activity, but the activity could be detected in the CSF withdrawn from animals injected with a higher dose of LPS (500 μg/kg, i.v.). LAL gelation activity of LPS dissolved in normal CSF was 2 orders less potent in comparison with that of LPS dissolved in saline, which suggested the presence of inhibitor(s) in CSF for LAL assay. Pyrogenicity was not revealed in the CSF withdrawn from hyperthermic rabbits induced by administration of reserpine after pretreatment with a monoamine oxidase inhibitor. These findings suggest that the central action of LPS is involved in pyrogen fever and that monoamines are not closely related to pyrogen fever.

Endotoxins of gram-negative bacteria possess various biological activities, and its pyrogenicity is the most prominent. The principle of endotoxicity is the lipid A portion (1) of the lipopolysaccharide (LPS) which is a major constituent of endotoxin. The theory of "endogenous pyrogens" has been widely accepted as one of many dominant theories on the mechanisms of pyrogen fever. The endogenous pyrogens are de novo synthesized after phagocytizing LPS both in vivo and in vitro from reticuloendothelial phagocytic cells such as polymorphonuclear leucocytes (2, 3) and especially macrophages (4). The endogenous pyrogens are suggested to directly stimulate the thermoregulatory center of the hypothalamus and cause fever.

On the other hand, there are many investigators who believe that there is involvement of the direct action of LPS on the central nervous system (CNS) in pyrogen fever. The reports supporting the central action of LPS are as follows: the inhibitory effect by LPS on activity of the thermosensitive neurons in the CNS (5–7) and the fever induction by injection of a minute amount of LPS as compared with peripheral administration into the subarachnoid space (8), lateral ventricles (9, 10) or hypothalamus (11). The best support for the central action of LPS is the report of Bennett et al. that after i.v. injection of endotoxin to dogs, a heatstable pyrogenic substance, LPS, could be detected in the CSF withdrawn from febrile animals. According to the method of Bennett et al., we (12–14) found pyrogenicity in the CSF withdrawn from febrile rabbits induced by i.v. injection of LPS. We (15–17) previously discussed the relation between the
penetration of LPS into CNS and febrile response based on the results that only LPS, of various pyrogens, was specifically bound in vitro to proteolipid apoprotein extracted from rabbit cerebrum.

There are also other theories on the mechanisms of pyrogen fever including the “amine theory” (18) that body temperature may be regulated by amounts of monoamines (catecholamines and serotonin) in the hypothalamus and the “ion theory” (19, 20) that the ratio of Na+/Ca2+ in the hypothalamus affects the thermoregulation.

In this report, we aimed to identify LPS in the CSF withdrawn from febrile rabbits induced by i.v. injection of LPS both by check of pyrogenicity and by the Limulus test, an in vitro specific assay for LPS (21, 22). Both protein content and concentrations of ions (Na, K and Ca) in the above CSF were determined at the same time. Furthermore, pyrogenicity was tested on the CSF withdrawn from hyperthermic rabbits induced by reserpine administration after pretreatment with monoamine oxidase (MAO) inhibitors.

Materials and Methods

1. Experimental animals: Male Japanese domestic white rabbits weighing 1.8 to 2.7 kg were used in all experiments.

2. Induction of febrile or hyperthermic response and record of body temperature: Ambient temperature was maintained at 25±1 °C; humidity was controlled at 45-55%. Rectal temperature was measured with a thermoelectric apparatus. LPS dissolved in saline was i.v. administered. Reserpine was i.v. or intracisternally (i.c.) administered 16 hr after s.c. pretreatment with MAO inhibitors (23).

3. Extraction of LPS: LPS was prepared from the hot-phenol extraction (24) of E. coli UKT-B strain and purified by ultracentrifugation (105,000×g for 4 hr, 2 times). The purified LPS was lyophilized.

4. Withdrawing CSF and measuring pyrogenicity of CSF: CSF was withdrawn from cisterna magna of rabbits without anesthesia by a tuberculin syringe. Pyrogenicity of CSF was measured by injection into cisterna magna of unanesthetized normal rabbits.

5. Chemical analyses: Protein was determined by the method of Lowry et al. (25). Sodium, potassium and calcium were determined by an atomic absorption spectrophotometer (Perkin Elmer, model 290) (26).

6. Limulus test: The Limulus test on LPS was performed by the use of “Pregel” (Seikagaku Kogyo Co., Ltd.). Incubation mixtures for the assay consisted of 0.1 ml of Limulus amoebocyte lysate (LAL) and 0.1 ml of the test sample and were incubated at 37°C for 1 hr. A definite increase in viscosity and turbidity, but not necessarily, progressing to adherent or a solid gel was considered as a positive assay. Negative assays remained clear or contained a slight flocculent precipitate suspended in a clear fluid medium. The LAL used in this experiment was sensitive to 1 ng per milliliter of E. coli UKT-B LPS.

7. Chemicals: Reserpine, β-phenylisopropyl hydrazine (PIH) and iproniazid phosphate were purchased from Sigma Chemical Co., Chugai Pharmaceutical Co. and Wako Chemical Co., respectively.

Results

1. Pyrogenicity and concentrations of ions in CSF withdrawn from febrile rabbits induced by i.v. administration of LPS: Pyrogenicity of CSF withdrawn from febrile rabbits induced by i.v. injection of LPS was measured. One ml of CSF was withdrawn from donors 2 hr after i.v. injection of LPS and administered into cisterna magna of recipients whose CSF was withdrawn approx. 1 ml just before receiving it. Figure 1 shows a typical febrile response in one recipient given the CSF from one donor injected with 50 iig/kg of LPS. As shown in the figure, the rectal temperature of the recipient rose approx. 1.2°C, 2 hr after receiving the CSF from the donor.

It was investigated whether the pyrogenic potency of the CSF might be dependent on the administered doses of LPS to donors. The concentrations of ions (Na, K and Ca) in the CSF were also determined. The results are shown in Table 1. Pyrogenicity was revealed significantly (P<0.05) in the CSF withdrawn from the group injected with...
50 μg/kg of LPS. Differences of concentrations of ions were not obtained among the CSF withdrawn from control and LPS-injected groups.

2. LAL gelation activity and protein content of CSF withdrawn from rabbits injected i.v. with LPS: LAL gelation activity was tested on the CSF withdrawn from rabbits injected i.v. with LPS. Protein content in the CSF was also determined. The results are shown in Table 2. The gelation activity was not detected in the CSF withdrawn from rabbits injected with 50 μg/kg of LPS, the same as with the rabbits injected with saline alone. However, the gelation activity could be detected in the CSF withdrawn from rabbits injected with a higher dose of LPS (500 μg/kg, i.v.). The protein contents of all the above CSF were within normal ranges of rabbit CSF (27), which excluded the possibility of blood contamination.

3. Inhibitory effect of CSF on LAL gelation activity of LPS: Although pyrogenicity was revealed in the CSF withdrawn from the rabbits injected i.v. with 50 μg/kg of LPS (Table 1), LAL gelation activity could not be detected in it (Table 2). These results suggested that an inhibitor(s) for the LAL test was also present in CSF, as in plasma (28). As shown in Table 3, as expected, the LAL gelation activity of LPS dissolved in fresh normal CSF was 2 orders less potent in comparison with that of LPS dissolved in saline. The inhibitory potency of normal CSF was considerably (1 order) reduced by tenfold dilution with saline.

4. Absence of pyrogenicity of CSF withdrawn from hyperthermic rabbits induced by reserpine administration after pretreatment with MAO inhibitors: Hyperthermia could be induced markedly in rabbits by i.v. injection of reserpine after pretreatment with MAO inhibitors. Therefore, the pyrogenicity was also checked on the CSF withdrawn from the animals 2 hr after i.v. injection of LPS at the dose indicated in the table. Pyrogenicity of CSF was checked by cisternal administration to recipients (1 ml/animal). Statistical significance: *P<0.05, **P<0.01, compared with each value in the LPS-nontreated group.

![Graph showing typical fever response induced by administration of LPS and pyrogenicity of the CSF withdrawn from the febrile rabbit. The donor received i.v., 50 μg/kg of LPS.](image)

**Table 1.** Pyrogenicity and concentrations of ions in CSF withdrawn from donor rabbits injected intravenously with LPS

| Dose of LPS (μg/kg, i.v.) | Fever response of donors at 2 hr (°C) | Pyrogenicity of CSF (°C) | Concentrations of ions (mEq/l) |
|--------------------------|--------------------------------------|--------------------------|-------------------------------|
| 0                        | 0.2±0.2 (5)                          | 0.3±0.2 (5)              | Sodium: 121.7±43 (12)  2.9±0.3 (9)  2.8±0.1 (15) |
| 1                        | 1.4±0.1** (5)                        | 0.2±0.3 (5)              | Potassium: 117.4±8.7 (9)  3.1±0.3 (5)  2.7±0.3 (9) |
| 10                       | 2.0±0.5** (5)                        | 0.4±0.1 (5)              | Calcium: 120.0±26.1 (3)  2.9±0.3 (3)  3.1±0.7 (3) |
| 50                       | 1.5±0.3** (5)                        | 1.0±0.2* (5)             |                               |
withdrawn from hyperthermic rabbits induced with reserpine.

The upper part of Fig. 2 shows a typical hyperthermic response in one rabbit induced by administration of reserpine (2.5 mg/kg, i.v.) after pretreatment with iproniazid phosphate (100 mg/kg, s.c.). As shown in the lower part of the figure, however, pyrogenicity was not demonstrated in the CSF withdrawn from the hyperthermic rabbit.

As summarized in Table 4, pyrogenicity could not be detected in the CSF withdrawn from hyperthermic rabbits induced by i.v. or i.c. injection of reserpine after pretreatment with a MAO inhibitor (iproniazid or PIH).

**Discussion**

Bennett et al. (8) reported the detection of endotoxin in CSF of febrile dogs based on its heat-stability. However, we (29) previously observed that an extremely dilute solution of LPS could be inactivated by heating. Consequently in this report, the *Limulus* test, an in vitro specific and highly sensitive assay for LPS (21, 22), was utilized in addition to the pyrogen test. The results of the test was positive only in the CSF withdrawn from the rabbits injected with a higher dose of LPS (500 μg/kg, i.v.) because of the presence of inhibitor(s) in CSF for this test (Tables 2 and 3). Accordingly, if the inhibitor(s) was not present, it would be possible to detect LPS in CSF even when LPS is administered at a dose lower than 50 μg/kg.

Trippodo et al. (30) described that after injection of a higher dose of endotoxin (5 mg/kg, i.v.) to dogs, endotoxin was detected in CSF by the *Limulus* test and concluded that endotoxin found in CSF resulted from

**Table 2.** LAL gelation activity and protein content of CSF withdrawn from rabbits injected intravenously with LPS

| Dose of LPS (μg/kg, i.v.) | Gelation of LAL | Protein content in CSF (mg/dl) |
|---------------------------|----------------|-------------------------------|
|                           | −              | 22.3                          |
|                           | −              | 41.4                          |
|                           | −              | 24.7                          |
|                           | −              | 26.1                          |
|                           | −              | 33.3                          |
| 0                         | −              | 38.0                          |
|                           | −              | 27.6                          |
|                           | −              | 30.0                          |
|                           | −              | 23.8                          |
| 50                        | −              | 20.0                          |
|                           | ±              | 28.5                          |
|                           | +              | 40.0                          |
|                           | +              | 25.2                          |

+, solid gel; ±, adherent granules; −, clear solution.

**Table 3.** Inhibitory effect of CSF on LAL gelation activity of LPS

| Medium            | Concentration of LPS (μg/ml) |
|-------------------|------------------------------|
|                   | 10  | 1   | 10⁻¹ | 10⁻² | 10⁻³ | 10⁻⁴ | 10⁻⁵ |
| Saline            | +   | +   | +    | +    | +    | ±    | −    |
| Undiluted CSF     | +   | +   | +    | +    | ±    | −    | −    |
| Diluted CSF (1/10)| +   | +   | +    | +    | +    | ±    | −    |

+, solid gel; ±, adherent gel; −, clear solution.
blood contamination. In their results, however, the endotoxin level did not parallel the numbers of red blood cells in CSF, and one exceptional result that high endotoxin level was revealed in the CSF without blood contamination was not clearly explained. Also, it is possible to check blood contamination in CSF by determining the concentrations of protein and ions, since they are lower than in plasma (27). In our present experiments, there would be little possibility of blood contamination into the CSF samples, since the concentrations of protein and ions (Na and Ca) were within normal ranges of rabbit CSF (27) (Tables 1 and 2).

Myers and Tytell (31) observed that after administration of typhoid vaccine to the unanesthetized cat, $^{45}\text{Ca}^2+$ efflux into the third cerebral ventricle increased while $^{22}\text{Na}^+$ was retained in hypothalamic tissue at the same time that the set-point temperature began to rise. They claimed that this result supported the "ion theory" (19, 20) that a change in the set-point temperature is determined by an alteration in the inherent ratio of Na$^+$ to Ca$^{2+}$ in the hypothalamus. In our present results, however, concentrations of ions (Na, K and Ca) in the CSF did not change after administration of LPS to rabbits (Table 1).

Hollon states in his review (32) that the accumulated evidence for the theory so far cannot be said to have established that local concentrations of Na and Ca ions in a

---

**Table 4. Absence of pyrogenicity in the CSF withdrawn from hyperthermic rabbits induced by reserpine administration after pretreatment with MAO inhibitors**

| MAO inhibitor | Reserpine | Donors | Recipients |
|---------------|-----------|--------|------------|
|               | Dose (mg/kg) | Route | Time at withdrawing (hr) | Temperature at withdrawing ($\Delta T^\circ C$) | Dose of CSF (ml/animal, i.c.) | Maximal temperature ($\Delta T^\circ C$) |
| Iproniazid    | 4.0       | i.v.   | 1.0          | 3.5       | 0.5       | 0.3       |
|               | 4.0       | i.v.   | 1.0          | 2.6       | 0.5       | 0.4       |
|               | 2.5       | i.v.   | 1.0          | 4.5       | 1.0       | 0.3       |
|               | 2.5       | i.v.   | 1.5          | 1.8       | 1.0       | 0.5       |
|               | 2.5       | i.v.   | 1.0          | 2.0       | 1.0       | 0.3       |
|               | 0.25      | i.c.   | 2.5          | 2.4       | 1.0       | 0.4       |
| PIH           | 1.0       | i.v.   | 1.0          | 4.0       | 0.5       | 0.3       |
|               | 1.0       | i.v.   | 1.0          | 3.7       | 1.5       | 0.2       |

Abbreviations used: MAO, monoamine oxidase; PIH, $\beta$-phenylisopropyl hydrazine. Pretreatment with MAO inhibitors: Iproniazid phosphate, 16 hr before 100 mg/kg, s.c.; PIH, 16 hr before 5 mg/kg, s.c.
particular part of the posterior hypothalamus determines the set point for temperature. In addition, the author claims that the data could equally well be interpreted in terms of a nonspecific action of the ions when their concentrations are changed in the vicinity of some neurons which govern the control actions for heat loss and heat conservation. From the present results, it also does not seem that Na\(^+\) and/or Ca\(^{2+}\) might be closely related to pyrogen fever.

Although marked hyperthermia was induced in rabbits by administration of reserpine after pretreatment with MAO inhibitors, the CSF withdrawn from them did not show pyrogenicity in contrast with the case of LPS-induced fever (Fig. 2 and Table 4). Feldberg and Myers (18) put forward the “amine theory” of thermoregulation, and many reports of the role of monoamines in pyrogen fever have been presented (32). However, Feldberg (33) himself denies this theory, since there is considerable uncertainty about the significance of results, particularly the variations between species. Hellon (32) also states that any monoamine involvement in fever would be as a consequence rather than as a cause. Therefore, it would be likely that marked hyperthermia induced by reserpine administration after pretreatment with MAO inhibitors might be mainly due to a peripheral effect; that is, the heat conservation by skin vasoconstriction.

References

1. Nowotony, A.: Molecular aspects of endotoxic reactions. Bacteriol. Rev. 33, 72–98 (1969)
2. Atkins, E.: Pathogenesis of fever. Physiol. Rev. 40, 580–646 (1960)
3. Kanoh, S., Kawasaki, H., Yoshida, M., Nishio, A. and Mochida, K.: Studies on the pyrogen (IV). Some factors influencing the production of leucocytic pyrogen from polymorphonuclear leucocytes in vitro. Japan. J. Pharmacol. 17, 125–132 (1967)
4. Hanson, D.F., Murphy, P.A. and Windle, B.E.: Failure of rabbit neutrophiles to secrete endogenous pyrogen when stimulated with Staphylococci. J. Exp. Med. 151, 1360–1371 (1980)
5. Cabanac, M., Stolwijk, J.A.J. and Hardy, J.D.: Effect of temperature and pyrogens on single-unit activity in the rabbit’s brain stem. J. Appl. Physiol. 24, 645–652 (1968)
6. Wit, A. and Wang, S.C.: Temperature-sensitive neurons in preoptic/anterior hypothalamic region: Actions of pyrogen and acetylsalicylate. Am. J. Physiol. 215, 1160–1169 (1968)
7. Eisenman, J.S.: Pyrogen-induced changes in the thermosensitivity of septal and preoptic neurons. Am. J. Physiol. 216, 330–334 (1969)
8. Bennett, I.L., Jr., Petersdorf, R.G. and Keene, W.R.: The pathogenesis of fever: Evidence for direct cerebral action of bacterial endotoxins. Trans. Assoc. Am. Physicians 70, 64–73 (1957)
9. Sheth, U.K. and Borrison, H.L.: Central pyrogenic action of S. typhosa lipopolysaccharide injected into the lateral cerebral ventricle in cats. J. Pharmacol. Exp. Ther. 130, 411–417 (1960)
10. Villablanca, J. and Myers, R.D.: Fever produced by microinjection of typhoid vaccine into hypothalamus of cats. Am. J. Physiol. 208, 703–707 (1965)
11. Myers, R.D.: Hypothalamic mechanisms of pyrogen action in the cat and monkey. In Pyrogens and Fever, Edited by Wolstenholme, G.E. and Birch, J., p. 131–153, Churchill Livingstone, Edinburgh and London (1973)
12. Ogawa, Y., Kanoh, S. and Takagi, H.: Studies on the pyrogen factor appeared in the cerebrospinal fluid of the febrile rabbit (Report II). Japan. J. Pharmacol. 22, Supp. 74P (1972)
13. Mochida, K., Ogawa, Y. and Kanoh, S.: Studies on the pyrogenic factor appearing in the cerebrospinal fluid of febrile rabbits. Folia Pharmacol. Japon. 70, 359–363 (1974) (Abs. in English)
14. Kanoh, S. and Watson, D.W.: Pyrogenic specificity of endotoxin and exotoxin. A. J. Med. Sci. Biol. 30, 64–67 (1977)
15. Kanoh, S. and Ogawa, Y.: Interactions between bacterial pyrogen and proteolipid extracted from the cerebrum (I). Japan. J. Pharmacol. 31, 419–424 (1981)
16. Ogawa, Y. and Kanoh, S.: Interactions between bacterial pyrogen and proteolipid extracted from the cerebrum (II). Japan. J. Pharmacol. 31, 425–431 (1981)
17. Ogawa, Y. and Kanoh, S.: Interactions between bacterial pyrogen and proteolipid extracted from the cerebrum (III). Variation in affinity of proteolipid proteins derived from rabbit, rat and chicken cerebrums to bacterial pyrogen. Japan. J. Pharmacol. 32, 189–194 (1982)
18. Feldberg, W. and Myers, R.D.: A new concept of temperature regulation by amines in the hypothalamus. Nature 200, 1325 (1963)
19. Feldberg, W., Myers, R.D. and Veale, W.L.: [Further references]
Perfusion from cerebral ventricles to cisterna magna in the unanaesthetized cat. Effect of calcium on body temperature. J. Physiol. (Lond.) 207, 403–416 (1970)

20 Feldberg, W. and Saxena, P.N.: Mechanism of action of pyrogen. J. Physiol. (Lond.) 211, 245–261 (1970)

21 Levin, J. and Bang, F.B.: The role of endotoxin in the extracellular coagulation of Limulus blood. Bull. Johns Hopkins Hosp. 115, 265–274 (1964)

22 Rojas-Corona, R.R., Skarnes, R., Tamakuma, S. and Fine, J.: The Limulus coagulation test for endotoxin. A comparison with other assay methods. Proc. Soc. Exp. Biol. Med. 132, 599–601 (1969)

23 Bächtold, H. and Pletscher, A.: Einfluss von Isonikotinsäurehydraziden auf den Verlauf der Körpertemperatur nach Reserpine, Monoaminen und Chlorpromazin. Experientia 13, 163–165 (1957)

24 Westphal, O. and Lüderitz, O.: Chemische Erforschung von Lipopolysacchariden gramnegativer Bakterien. Angew. Chem. 66, 407–417 (1964)

25 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: Protein measurement with Folin phenol reagent. J. Biol. Chem. 193, 265–275 (1951)

26 Chutkow, J.G.: Magnesium and calcium in the cerebrospinal fluid of the rat. Proc. Soc. Exp. Biol. Med. 128, 555–558 (1968)

27 Davson, H.: Chemical composition and secretory nature of the fluid. In Physiology of the Cerebrospinal Fluid, p. 35–54, J & A. Churchill Ltd., London (1967)

28 Levin, J., Tomasulo, P.A. and Oser, R.S.: Detection of endotoxin in human blood and demonstration of an inhibitor. J. Lab. Clin. Med. 75, 903–911 (1970)

29 Kanoh, S., Mochida, K. and Ogawa, Y.: Studies on heat-inactivation of pyrogen from Escherichia coli. Biken J. 13, 233–239 (1970)

30 Trippodo, N.C., Jorgensen, J.H., Priano, L.L. and Traber, L.L.: Cerebrospinal fluid levels of endotoxin during endotoxemia. Proc. Soc. Exp. Biol. Med. 143, 932–937 (1973)

31 Myers, R.D. and Tytell, M.: Fever: Reciprocal shift in brain sodium to calcium ratio as the setpoint temperature rises. Science 178, 765–767 (1972)

32 Hellon, R.F.: Monoamines, pyrogens and cations: Their actions on central control of body temperature. Pharmacol. Rev. 26, 269–321 (1975)

33 Feldberg, W.: On the mechanism of action of pyrogens. In Pyrogens and Fever, Edited by Wolstenholme, G.E. and Birch, J., p. 115–129, Churchill Livingstone, Edinburgh and London (1973)