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Author(s)	Kanoh, Seizaburo; Nishio, Akira; Kawasaki, Hironoshin
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STUDIES ON PYROGENIC TOLERANCE TO BACTERIAL LIPOPOLYSACCHARIDE IN RABBITS

SEIZABURO KANOH[1,2], AKIRA NISHIO[2] and HIRONOSHIN KAWASAKI[2]

Department of Virology, Research Institute for Microbial Diseases, Osaka University, Yamada-kami, Suita, Osaka[1] and Department of Pharmacology and Microbiology, National Institute of Hygienic Sciences, Osaka Branch, 6, Hoenzaka-cho, Higashi-ku, Osaka[2]

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SUMMARY The development of pyrogenic tolerance was studied in rabbits treated with varying doses of *E. coli* lipopolysaccharide (LPS). The following results were obtained.

1) Development of pyrogenic tolerance seemed to proceed in two steps: that is, the first in which tolerance appeared rapidly and the second in which tolerance proceeded more gradually or steadily in response to repeated injections of a constant dose.

2) Tolerance induced by the latter method was not absolute; the rabbits were still sensitive to increased doses of LPS.

3) Rabbits immunized with *E. coli* vaccine lost their pyrogenic sensitivity of parent LPS to some extent.

4) Following intracisternal injection of LPS into tolerant rabbit, pyrogenic response was not decreased but rather enhanced in comparison with control.

5) The contents of nor-epinephrine and serotonin in the brain did not differ between normal and tolerant rabbits.

6) The mechanisms of endotoxin tolerance remain to be further studied.

INTRODUCTION

In 1947, Beeson first reported that rabbits injected repeatedly with endotoxin could develop pyrogenic tolerance (Beeson, 1947a; b). The author indicated that the phenomenon did not depend on the immunological state but on the activation of the reticuloendothelial system (RES). The reports have been confirmed by some authors (Greissman, et al., 1964; Cluff, 1953; Urbaschel et al., 1968). On the other hand, Watson (1960), studying the pyrogenic tolerance between endotoxin and pyrogenic exotoxin derived from *Streptococcus pyogenes*, concluded from cross-tolerance experiments that immunological phenomena might be partially involved in pyrogenic tolerance.

Recently, Rietschel (1975) has shown that lipid A, the toxophore moiety of endotoxin, might contribute to the pyrogenic tolerance of endotoxin by stimulating lipid A antibody. These findings have suggested the partial importance of immunological modification of the host in the development of pyrogenic tolerance to endotoxin. However, tolerance phenomena including pyrogenic torelance, are complex

and the precise mechanisms are still obscure.

In the present report, we investigated the mechanism of development of pyrogenic tolerance and properties of the tolerant state with different methods from the pharmacological aspect of view.

MATERIALS AND METHODS

1. *Measurement of body temperature and Fever Index (FI)*

Male white rabbits weighing 1.8–2.5 kg were used throughout the experiments. Body temperature was estimated by automatic recorder with thermister from rectal temperature of rabbits. Fever indices were calculated from the fever curves for 3 or 5 hr as FI-3, FI-5 as previously reported by Kanoh et al. (1967).

2. *Measurement of ear skin temperature*

Ear skin temperature was also measured automatically by a thermister electrode attached to the back side of ear with tape avoiding large auricular vessels.

3. *Chemical analysis of the neurotransmitter amines*

Nor-epinephrine was extracted and purified from rabbit brain by the method of Shellenberger et al. (1971) and chemical analysis was done according to the method of Chang (1964). Serotonin was extracted and purified from rabbit brain by the method of Udenfriend et al. (1955) and estimated by the method of Ansell et al. (1968).

4. *Chemicals*

The following chemicals were used throughout the experiment; serotonin, norepinephrine and 2, 4-dinitrophenol (DNP). These chemicals were purchased from Katayama chemicals Co. Ltd. as "extra pure" reagents.

5. *Preparation of endotoxin (lipopolysaccharide; LPS) and leucocytic pyrogen (LP)*

Endotoxin (LPS) was prepared from the dried cells of *E. coli* UKT-b by the method of Westphal et al. (1954). Leucocytic pyrogen was prepared by the following method. Four hundred milliliters of sterilized saline were injected intraperitoneally into rabbit. Eighteen hours later the peritoneal exudate was collected and centrifuged at 800 rpm for 10 min. Cells were resuspended in sterile saline and counted by hemocytometer and then the suspension was adjusted to 10^8 cell/ml. The suspension was poured into a siliconized test tube and it was incubated in roller drum rotation with twelve rotating per hour at 41C for 1 hr. After incubation it was again centrifuged at 800 rpm for 10 min. The supernatant was used as leucocytic pyrogen.

RESULTS

I. *Development of pyrogenic tolerance to endotoxin (LPS) in rabbits*

1) Rapid development of pyrogenic tolerance during a single day.

0.2, 0.02 and 0.002 μg/kg of LPS were injected intravenously into each of three rabbits respectively four times a day. Their mean fever curves are shown in Fig. 1. It was shown that every response to the first injec-

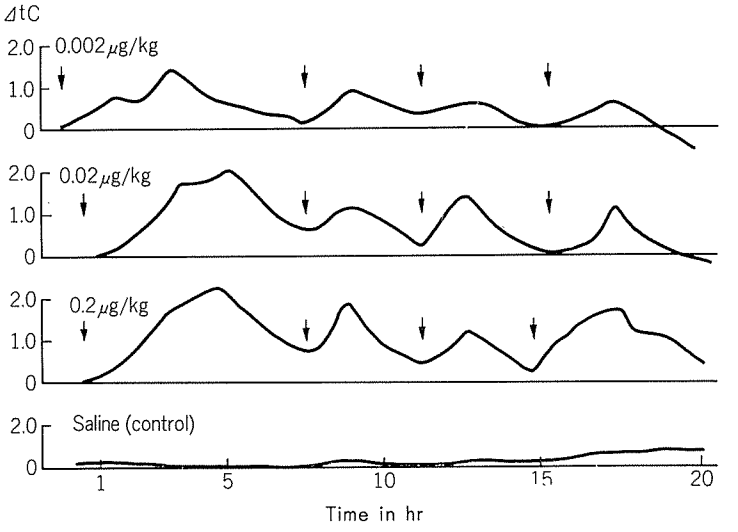

FIGURE 1. Pyrogenic tolerance by multiple injections at various doses of LPS during one day. Arrows represent the same dose of LPS iv as indicated. Fever curve represents the mean of three rabbits.

tions resulted in biphasic curves but subsequent responses were all monophasic curves. Further, the pyrogenic response diminished during the course of the experiments. This suggested that pyrogenic tolerance was related to the dose of LPS.

2) Development of pyrogenic tolerance induced by multiple injections of the same dose of LPS for three weeks.

Twelve rabbits were divided into two groups; one group received the same dose of 0.2 μg/kg of LPS three times a week, and the other group received 0.02 μg/kg of LPS intravenously three times a week, respectively. Their pyrogenic response to each injection was observed for 3 hr and expressed as the Fever Index for 3 hr (FI-3) at the indicated day during the experiment. As shown in Fig. 2, development of pyrogenic tolerance proceeded in two steps; one step which proceeded rapidly at the early time and the other in which the response was constant at the late time.

3) Development of pyrogenic tolerance by increasing doses of LPS for seven weeks.

As shown in the above experiments, pyrogenic tolerance was related to the dose of LPS, so it was thought that stepwise increased dose of LPS might affect the threshold of pyrogenic tolerance. In twelve rabbits, the stepwise increasing doses of LPS from 1 μg/kg to 20 μg/kg, were injected intravenously during seven weeks. During the development of tolerance, the degree of pyrogenic response of every rabbit was estimated by intravenous injection of 0.5 μg/kg of LPS at the indicated days and the responses were represented as Fever Index for 5 hr at the day of testing. The reasons of using FI-5 are as follows; first, it is possible to obtain a more precise value than that of FI-3, and secondly, the value of FI-3 is sometimes less sensitive in tolerant rabbits than the control. As shown in Fig. 3, pyrogenic tolerance developed gradually depending on the dose of LPS. At the end of the experiment, the pyrogenic response had almost entirely disappeared in the rabbits which had been inoculated with increasing dose from 1 μg/kg to 20 μg/kg for seven weeks. The body weights of the tolerant rabbits were less

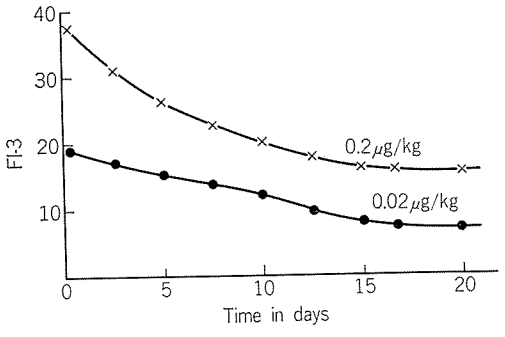

FIGURE 2. Development of pyrogenic tolerance induced by the two different doses of LPS during three weeks. Each curve represents the mean value of six rabbits. LPS was injected three times a week of the dose indicated. Each point represents the mean fever response for 3 hr of LPS injections as Fever Index-3 hr (FI-3) in ordinate.

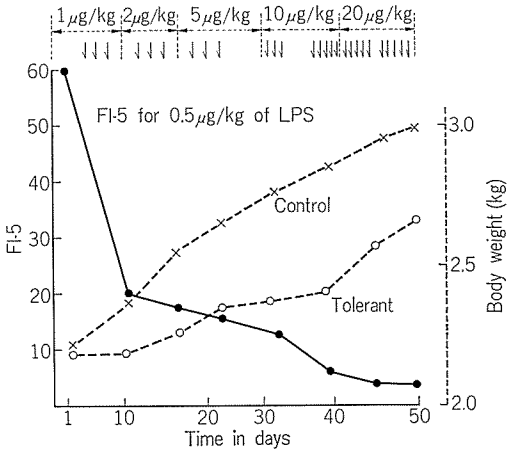

FIGURE 3. Development of pyrogenic tolerance induced by multiple injections of LPS during seven weeks and increase of body weight of control and tolerant rabbits. LPS was injected at the dose and time as indicated with arrow signs in the upper part of the figure. Mean degree of tolerance checked by injection of 0.5 μg/kg iv. of LPS at the indicated days in 12 rabbits (●—●), Mean body weight of six control rabbits (×·····×), Mean body weight of tolerant rabbits injected with LPS in 12 rabbits (○·····○).

than that of the controls.

4) Relationship between antibody titer to O-antigen and pyrogenic tolerance by *E. coli* vaccine.

During the development of pyrogenic tolerance induced by LPS, we were unable to detect antibody titers to LPS. Recent observation suggested that the immunological state might be coupled to the pyrogenic tolerance particularly to lipid A (Rietschel, 1975). In classical observations (Cluff, 1953), it has been noticed that there was no correlation between pyrogenic tolerance induced by LPS and its parent antibody titer. To disolve this problem, we injected *E. coli* vaccine containing 10^9 cells/ml as a pyrogen into rabbits twice a week for six weeks and determined the hemagglutinating titers to O-antigen of the cells. The results are shown in Fig. 4. As shown in the figure, the hemagglutinating titer increased in a biphasic manner while the pyrogenic response decreased gradually to half of the initial fever response for 3 hr (FI-3) and kept its value constant until the end of the experiment.

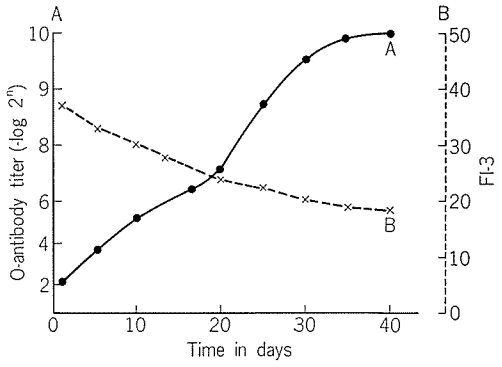

FIGURE 4. Relationship between development of pyrogenic tolerance and O-antibody titer in rabbits injected with *E. coli* vaccine. A: Mean O-antibody titer to *E. coli* cells in rabbits injected with 10^9 cell/ml/kg of *E. coli* vaccine. B: Mean pyrogenic response for 3 hr at injection of vaccine. The vaccine was injected into the rabbits three times a week.

2. *Response of E. coli or LPS induced pyrogenic tolerant rabbits to LP or DNP*

1) Pyrogenicity to lecocytic pyrogen (LP) or 2, 4-dinitrophenol (DNP) in the tolerant rabbit.

The response of tolerant rabbits to both LP, a pyrogenic mediator released from polymorphonuclear leucocytes (PMNL) and DNP, a peripheral pyrogen, were determined. After injection of the pyrogens iv, ear skin temperature and rectal temperature changes were measured in the tolerant rabbits and in control. As shown in Fig. 5, rectal temperature was increased following injection of both pyrogens. Response of ear skin temperature exhibited some different patterns, but there was no difference between normal and tolerant rabbits.

2) Response to LPS derived from *E. coli* in the rabbits vaccinated with *E. coli* vaccine.

After establishment of pyrogenic tolerance with *E. coli* vaccine which was described in Fig. 4, groups of the rabbits were challenged with *E. coli* LPS at doses of 0.2 and 0.02 μg/kg iv. Their pyrogenic responses were estimated and O-antibody could still respond to 0.2 μg/kg or 0.02 μg/kg of parent LPS, but the response was markedly less than the controls. This suggested that antibody could decrease the pyrogenic response to parent LPS but not completely.

To further investigate the mechanism of pyrogenic tolerance induced by intravenous injection of LPS, the response of LPS injected into the intracisternal cavity was studied. Doses from 0.005 μg/kg to 0.0001 μg/kg of LPS were injected intracisternaly into rabbits which had been made tolerant by intravenous injection of LPS as described in Fig. 3. There were significant differences in pyrogenic responses between normal and tolerant rabbits (Fig. 7). Tolerant rabbits responded more strongly than normal rabbits to intracisternal injection of LPS at end dose total.

3) Pyrogenicity of various dose of LPS in

tolerant rabbits

To check the degree of pyrogenic tolerance after seven weeks, the tolerant rabbits were injected with larger doses of pyrogen than the usual 0.5 μg/kg of LPS which was used for checking the degree of tolerance.

As shown in Fig. 8, the response to LPS in tolerant rabbits was markedly suppressed in comparison with controls. However, the more the dose was increased, the more the response increased.

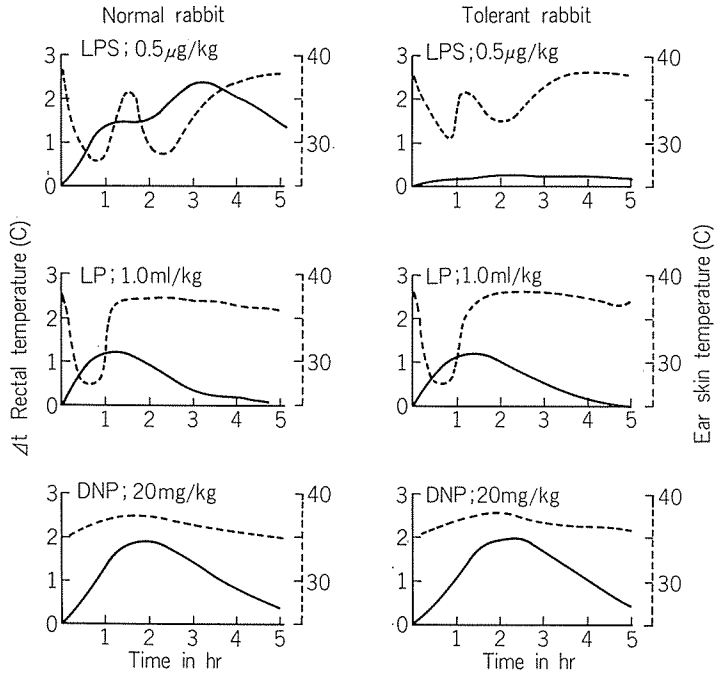

FIGURE 5. Comparison of the response of rectal and ear skin temperatures induced with LPS, leucocytic pyrogen (LP) and 2, 4-dinitrophenol (DNP) in normal and pyrogenic tolerant rabbits. The tolerant rabbits were prepared by multiple injections of LPS at dose of 1.0 or 20 μg/kg iv during seven weeks as indicated in Fig. 3. LP was prepared from the supernatant of incubation of rabbits PMNL at 37 C (See Materials and Methods.). Each curve represents the mean of six rabbits injected intravenously. Rectal temperature (——), Ear skin temperature (- - - -).

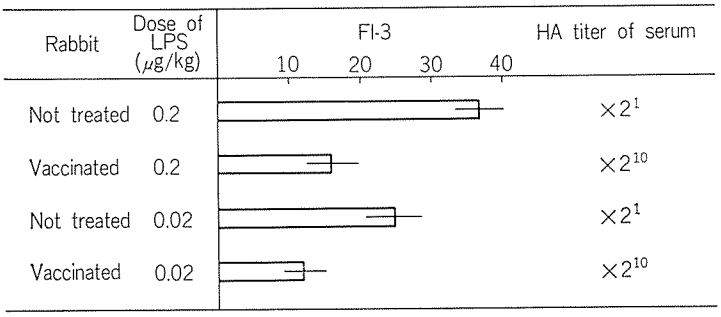

FIGURE 6. Pyrogenic response to LPS in rabbits vaccinated with *E. coli* vaccine. The values represent the mean value of six rabbits with S.D.

3. *Pharmacological state in the pyrogenic tolerant rabbit*

There have been published many reports concerning neurotransmitters in pyrogen induced fever. Particularly, the adrenergic nervous system may play an important role in pyrogen induced fever by releasing nor-epinephrine or serotonin (Raskova et al., 1964; Rosenberg et al., 1959). But there have been no reports of the content of these amines in pyrogenic tolerant rabbits. In the rabbits described in Fig. 3, nor-epinephrine and serotonin were extracted from the whole brain and the content of these amines was determined.

As shown in Table 1, there was no difference in the content of amines between normal and tolerant rabbits.

DISCUSSION

Pyrogenic tolerance induced with endotoxin first described by Beeson (Beeson, 1947a; b). He stated that this phenomenon was due to the activation of the reticuloendothelial system

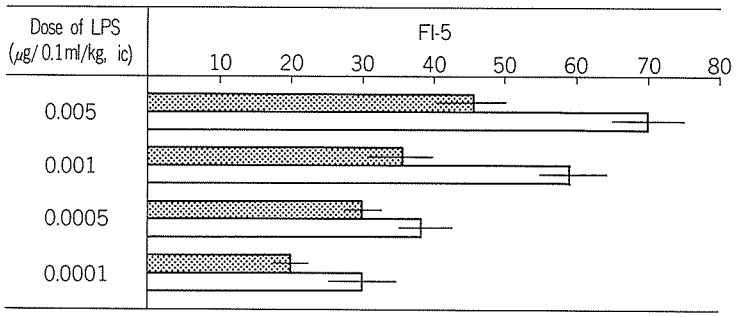

FIGURE 7. Pyrogenic response induced by intracisternal injection of LPS in normal and pyrogenic tolerant rabbits. ▒, Normal rabbits; □, Tolerant rabbits. Vertical bars represent S.D. of six rabbits.

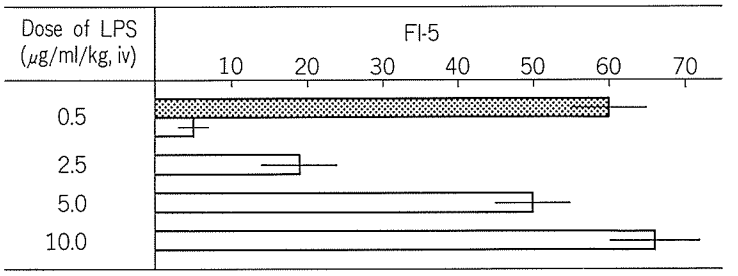

FIGURE 8. Pyrogenic response induced by intravenous injection of LPS in normal and pyrogenic tolerant rabbits. ▒, Normal rabbits; □, Tolerant rabbits. Each bar represents the mean value of six rabbits with S.D.

(RES). By blockade of RES with trypan-blue or Indian ink, tolerance could be reversed. Despite efforts to clarify the precise relationship between host RES activity and pyrogenic tolerance, the problem remained unresolved (Luzio et al., 1969).

TABLE 1. *Comparison of the content of Norepinephrine and Serotonin in normal and tolerant rabbit brain*

Rabbits	Number of rabbits	Norepinephrine	Serotonin
Normal	3	0.36±0.03[a]	0.35±0.04
Tolerant	3	0.38±0.03	0.36±0.01

[a] Data are expressed as μg/g of wet tissue with mean±S.D.

Watson postulated that endotoxin activities could be divided into two configurations; A configuration, on the sugar determinants of "O" polysaccharide, is not functional in biological activities and plays no role in the tolerance, while on the other hand, the B configuration including lipid A might affect the RES or delayed hypersensitivity (Watson et al., 1965).

In the present report, we described the two preparative methods of pyrogen tolerant rabbits. One is the method by repeated injections of a constant dose of LPS, and the other is the method by repeated injections of increasing doses of LPS.

By repeated injections of various doses of pyrogen during one day, development of pyrogenic tolerance proceeded rapidly depending on the doses. By injections two or three times a week for three weeks, development of pyrogenic tolerance proceeded more slowly than in the former experiment. This suggested that partial recovery might have occurred. By injections of increasing doses for seven weeks, the tolerance became more complete than in the former two experiments.

At the stage of complete tolerance after seven weeks, the response to intracisternal injection of LPS was different from the response to intravenous injection of LPS. However, the response to LP or DNP iv was the same as control (Fig. 5). The reason is unknown but it is possible that the receptor for these pyrogens may be different from each other.

Loss of body weight in tolerant rabbits might

be due to metabolic enhancement which is one of the pharmacological properties of endotoxin (Raskova, 1964). Berry et al. (1965) have demonstrated that the metabolic aspect of tolerance did not show any change including liver tryptophan pyrolase.

In the present experiment, the content of neurotransmitter amines was unchanged in both tolerant and normal rabbit brain. The reason for this is still unknown. But similar results have been reported by Hahn et al (1971) in morphine-tolerant mice.

From these data, we are still unable to discuss final conclusions concerning the mechanisms of pyrogenic tolerance because of the complexing of the phenomena. Further investigation is needed.

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