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Analysis of Endotoxin Fever in Rabbits by Using a Monoclonal Antibody to Tumor Necrosis Factor (Cachectin)

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A murine monoclonal antibody to rabbit tumor necrosis factor (TNF; cachectin) was injected intravenously into an endotoxin-treated rabbit to examine the role of endogenous TNF in fever. Both early and late peaks of biphasic fever evoked by the endotoxin injection were suppressed by the antibody. TNF activity was detected in an endotoxin dose-dependent manner in the blood 1 h after the endotoxin injection, which was coincident with the early-peak fever. Although the late-peak fever responded to the antibody, no significant TNF activity was detected in the blood obtained 1 h before the peak response. The blood was found to contain endogenous pyrogen activity, which was stable after heating at 70°C for 30 min and resistant to in vitro treatment with the antibody. Rabbit TNF injection also elicited biphasic fever in rabbits, the second phase of which was found to be mediated by the similar endogenous pyrogen. These results suggest that endogenous TNF plays an important role in eliciting a febrile response to endotoxin.

Pyrogens can be classified as exogenous or endogenous (5, 8). It is well known that an exogenous pyrogen elicits a febrile response by stimulating the production of endogenous pyrogen (EP), although some exogenous pyrogens such as bacterial endotoxin (3, 4, 26) and peptidoglycans (24) are believed to act directly on the putative hypothalamic thermoregulatory center in addition to stimulating EP production.

Recent investigations on the pathogenesis of fever have been focused primarily on the characterization of EP (5, 11, 12). So far, such cytokines as interleukin-1 (IL-1) (7, 8, 10) and tumor necrosis factor (TNF; cachectin) (8, 9) are the most likely candidates for EP. However, the role of these cytokines in febrile response in physiological conditions has not yet been elucidated.

In this study, we examined the contribution of endogenous TNF in endotoxin-induced fever (endotoxin fever) in rabbits, using both highly purified rabbit TNF (RaTNF) and monoclonal anti-RaTNF (14). The results indicate that TNF is one of the EPs that plays a major role in endotoxin fever and suggest that the late-peak response in endotoxin fever was mediated by an undefined EP produced by endogenous TNF stimulation.

MATERIALS AND METHODS

Animals. Male New Zealand White rabbits (weighing 2.0 to 3.8 kg) from a single colony were used in these experiments.

TNF. Natural RaTNF (14) (more than 3×10^6 IU/mg of protein) purified from sera of rabbits treated with *Propionibacterium acnes* and endotoxin was kindly supplied by Dai Nippon Pharmaceutical Co. Ltd., Tokyo, Japan.

Polymyxin B treatment of RaTNF. RaTNF (5×10^5 IU) was incubated at 37°C for 30 min with 1 mg of polymyxin B sulfate (Taito Pfizer Co., Tokyo, Japan) in 1 ml of saline in a sterile plastic tube, as reported previously (15, 16), and then it was diluted with saline to an appropriate concentration. RaTNF solution incubated without polymyxin B under the same conditions was used as a control.

Endotoxin. Boivin-type lipopolysaccharide from *Escherichia coli* O127:B8 was purchased from Difco Laboratories, Detroit, Mich.

Treatment of rabbits with anti-RaTNF. Ascites fluid from BALB/c mice with hybridoma-secreting monoclonal anti-RaTNF (immunoglobulin G1) (14) was used as a source of anti-RaTNF. RaTNF of 11,200 IU/ml was completely neutralized in an in vitro L929 cytotoxicity assay by the addition of an equal volume of 1/100-diluted ascites fluid. The ascites fluid was injected intravenously (i.v.) into rabbits 10 min before the injection of endotoxin. Ascites fluid from BALB/c mice with immunoglobulin G1 monoclonal antitrinitrophenol (anti-TNP) was used as a control antibody.

Rabbit pyrogen assay. Pretrained rabbits were used for a pyrogen assay as described previously (16). Briefly, the rectal temperatures of rabbits were monitored by a thermistor probe for 5 h after an i.v. injection of the material to be tested, and the rectal temperature change (ΔT) was calculated by subtracting the temperature immediately before the injection from the temperature at each time point.

EP assay by plasma transfer. EP activity in blood was assayed by transferring 20 ml of rabbit plasma to a recipient whose rectal temperature was monitored. Pyrogen-tolerant rabbits were used as recipients to get rid of the effect of trace amounts of endotoxin remaining in the blood (2). The pyrogen-tolerant state (13) in a rabbit was induced by a single i.v. injection of 125 ng of endotoxin per kg of body weight, as reported previously (15). Donor rabbits were bled by cardiac puncture, using a heparinized syringe under ether anesthesia. The blood plasmas were immediately separated by centrifugation and pooled. A sample of the plasma was heated at 70°C for 30 min, and another sample was incubated overnight with anti-RaTNF (0.1 ml/ml of plasma) at 4°C.

TNF assay. A 2-ml sample of blood was obtained from the ear artery. TNF activity in the serum was assayed by the dye exclusion cytotoxicity test in the presence of dactinomycin

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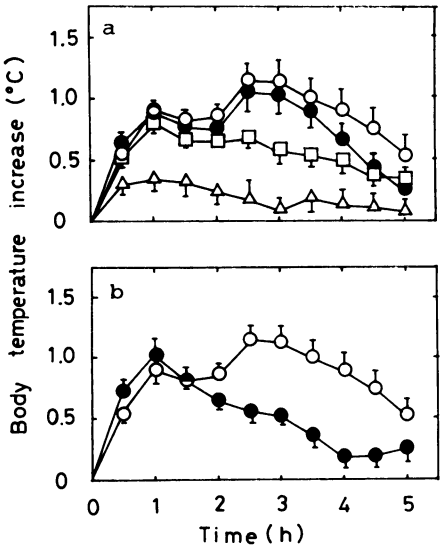

FIG. 1. Febrile responses of rabbits to an i.v. injection of RaTNF. (a) Body temperature changes (ΔT) of normal rabbits injected with RaTNF (3×10^2 [△], 3×10^3 [□], or 3×10^4 [○] IU/kg) or polymyxin B-treated RaTNF (3×10^4 IU/kg [●]). (b) Febrile responses of rabbits to 3×10^4 IU of RaTNF per kg which had been injected with the same amount of RaTNF on the previous day. The responses on the first (○) and the second (●) days are illustrated. Mean $\Delta T \pm$ SEM values ($n = 5$) are shown.

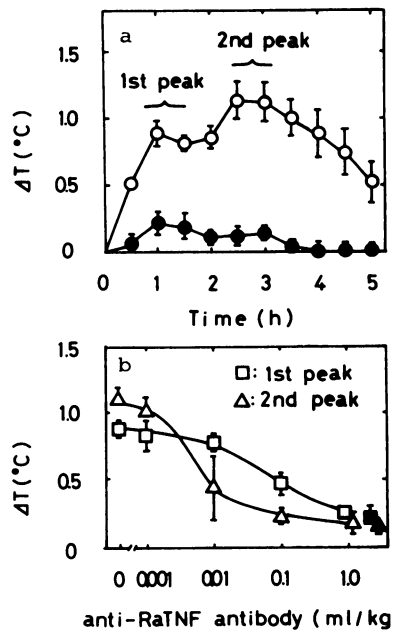

FIG. 2. Effect of anti-RaTNF on RaTNF-induced fever. (a) Body temperature changes (ΔT) of rabbits injected with 3×10^4 IU of RaTNF per kg alone (○) and along with 1 ml of anti-RaTNF antibody per kg (●). (b) Dose-response relationship between amounts of anti-RaTNF injected and ΔT in rabbits injected with 3×10^4 IU of RaTNF per kg. The results for the early- and late-peak fevers are shown. Febrile responses at 1 (■) and 3 (▲) h of rabbits injected with 1 ml of antibody per kg alone are also shown. Results are presented as the mean $\Delta T \pm$ SEM ($n = 3$).

(1 mg/ml) by using TNF-sensitive, interferon (IFN)-resistant mouse L929 cells (25, 29) after the serum was heated at 60°C for 30 min, since our previous experiments showed that TNF activity in the sera of rabbits injected with endotoxin alone could be detected only in heated specimens (15). TNF activity was determined by the serum dilution which showed 50% killing of the L929 cells and was expressed (in international units) in terms of a human reference standard (J-PS5K01) of the National Institute of Health, Tokyo, Japan.

RESULTS

Febrile responses of rabbits to exogenous TNF. Figure 1a shows the febrile responses of rabbits that received a single injection of different amounts of RaTNF. (All results are expressed as mean ± standard error of the mean [SEM].) A low dose of RaTNF (3×10^2 to 3×10^3 IU/kg of body weight) evoked monophasic fever, with a peak response at 1 h. A larger amount of RaTNF (3×10^4 IU/kg) induced biphasic fever, with a second peak 3 h after the injection. The trace amount of endotoxin possibly contaminating the TNF preparation could have modified the febrile response. However, pretreatment of RaTNF with polymyxin B affected neither the early-peak nor the late-peak fever, indicating that RaTNF itself induces a biphasic febrile response in rabbits.

Tolerance induced by RaTNF. It is well known that an injection of a high dose of endotoxin induces an early-phase pyrogen tolerance (13) or a refractory state for producing late-peak fever in response to a successive dose of endotoxin. Although an injection of 3×10^3 IU or a smaller amount of RaTNF per kg of body weight did not affect the febrile responses of the rabbits to the successive injection of RaTNF (data not shown), an injection of a larger amount of RaTNF (3×10^4 IU/kg) induced refractoriness in the rabbits to another RaTNF injection in terms of the late-peak febrile response (Fig. 1b). These results indicate that a high-dose RaTNF injection induces in rabbits a tolerant state that is similar to the early-phase tolerance to endotoxin.

Effect of anti-RaTNF on the febrile response to RaTNF. Figure 2a shows the febrile responses of rabbits injected with 3×10^4 IU of RaTNF per kg of body weight and the effect of the injection of anti-RaTNF on the response. The dose of RaTNF induced a biphasic fever with early-peak and late-peak fevers at 1 and 3 h, respectively, after the injection. An injection of 1 ml of anti-RaTNF per kg before the RaTNF injection abrogated both responses. The suppressive effects of various amounts of anti-RaTNF to 3×10^4 IU of RaTNF per kg are summarized in Fig. 2b. Since the dose of RaTNF elicited a biphasic febrile response, the effects of the antibody on the early- and late-peak fevers are separately illustrated. Antipyretic effects of the antibody on the early- and late-peak responses were dose dependent, although the late-peak response was suppressed more effectively than the early-peak response. Monoclonal anti-TNP showed no inhibitory effect on the febrile response to 3×10^4 IU of RaTNF per kg at any dose tested, from 0.001 to 1.0 ml/kg (data not shown).

Effect of anti-RaTNF on endotoxin fever. Endotoxin is one of the well-known exogenous pyrogens which elicit a strong febrile response in rabbits (11, 12, 28). In our experimental system, 5 ng or a smaller amount of endotoxin per kg induces monophasic fever in rabbits, with a peak response at 1 to 1.5 h after the injection, whereas doses of more than 25 ng per kg cause biphasic fever with peaks at 1 to 1.5 h and 3 to 4 h after the injection (15).

Pretreatment of rabbits with a small amount of anti-RaTNF (0.1 ml/kg) significantly suppressed the monophasic response induced by the injection of 5 ng of endotoxin per kg

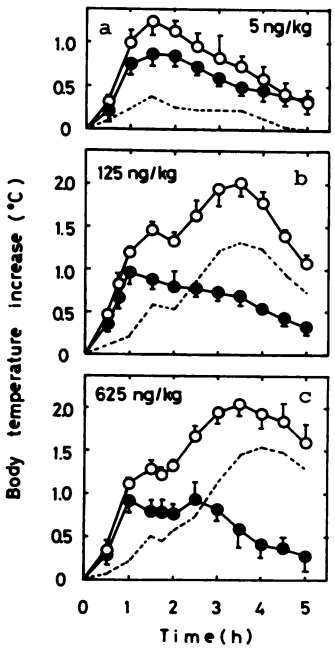

FIG. 3. In vivo effects of anti-RaTNF on endotoxin fever in rabbits. Febrile responses of rabbits given endotoxin alone (○) or along with the antibody (●) are shown. The following amounts (per kilogram) were administered: 5 ng of endotoxin and 0.1 ml of anti-RaTNF (a), 125 ng of endotoxin and 1 ml of antibody (b), or 625 ng of endotoxin and 1 ml of antibody (c). The responsiveness of the fever to anti-RaTNF, obtained theoretically by subtracting the mean ΔT of rabbits given endotoxin along with anti-RaTNF from that of rabbits given endotoxin alone at each time point, is shown (– – –). Results are presented as the mean $\Delta T \pm$ SEM (n = 3 to 5).

(Fig. 3a). The suppressed febrile response still reached a peak at 1.5 h after the endotoxin injection.

Figure 3b and c shows the suppressive effect of 1 ml of antibody per kg on the biphasic fever induced by 125 to 625 ng of endotoxin per kg. The antibody significantly suppressed both the early- and late-peak fevers, although an initial phase of the early response remained unaffected. These results suggest that both fevers are mediated by endogenous TNF directly or indirectly, although the initial part of the early-peak fever does not seem to be mediated by TNF.

TNF activity in blood. TNF activities in blood were examined 0.5 to 1 h before each peak response of the biphasic fever, i.e., 1 and 2.5 h after the endotoxin injection. TNF activity was detected in the serum obtained 1 h after the endotoxin injection (1-h serum) and increased with the dose of endotoxin, whereas serum obtained 2.5 h after the endotoxin injection (2.5-h serum) contained only 3 to 5% of the TNF activity of that in 1-h serum (Table 1). The activity observed was confirmed to be due to TNF-α because anti-RaTNF caused a decrease in the activity (data not shown). These results suggest that the antibody-sensitive response in the early-peak fever is mediated directly by the endogenous TNF but that the late fever is not.

It was striking that TNF activity in 2.5-h serum was lower than that in 1-h serum, since 2.5-h serum had to contain material(s) responsible for the late-peak fever which responded to the injection of anti-RaTNF. These findings strongly suggest that the late-peak fever is mediated indirectly by endogenous TNF, presumably by EP(s) generated by the TNF stimulation. Therefore, plasma transfer experiments were done to study the putative EP responsible for the late-peak fever.

TABLE 1. TNF activity in blood from normal rabbits given endotoxin alone

Endotoxin dose (ng/kg)	Mean TNF activity[a] ± SEM (n) at:	
	1 h	2.5 h
0	0.47 ± 0.15 (5)	NT
1	0.74 ± 0.06 (3)	NT
5	1.16 ± 0.22[b] (3)	NT
125	30.9 ± 7.8[c] (6)	1.64 ± 0.79 (6)
625	176 ± 33[c] (4)	5.36 ± 1.07[b] (3)

[a] Activity given as international units per milliliter of serum. NT, Not tested.
[b] $P < 0.05$ (versus 0 ng of endotoxin at 1 h) by Student's t test.
[c] $P < 0.01$.

EP activity responsible for the late peak of endotoxin fever. Donor rabbits were injected i.v. with 625 ng of endotoxin per kg of body weight and bled 1 h before the late-peak response, or 2.5 h after the endotoxin injection. EP activity was detected in the plasmas from rabbits injected with 625 ng of endotoxin per kg and was found to be stable after heating at 70°C for 30 min (Fig. 4), suggesting that the late-peak endotoxin fever was due mostly to material(s) other than IL-1 or IFN, since rabbit IL-1 (7) and IFN (19) have been reported to be heat labile. In addition, TNF was shown not to contribute to the EP activity in the plasma, because the activity was unaffected by the in vitro treatment with anti-RaTNF. These results suggest that the late-peak endotoxin fever is mediated by unknown material(s) other than IL-1, IFN, or TNF, although the generation of the activity is mediated by endogenous TNF.

EP activity in blood after RaTNF injection. To examine whether TNF could induce in vivo the undefined EP mentioned above, RaTNF was injected into rabbits and the plasmas were assayed for EP activity by transfer experiments. Blood was obtained 1 h before the late-peak fever, or 2 h after the RaTNF injection. The plasmas from rabbits which received 3×10^4 IU of RaTNF per kg of body weight contained EP activity (mean $\Delta T \pm$ SEM in the recipient =

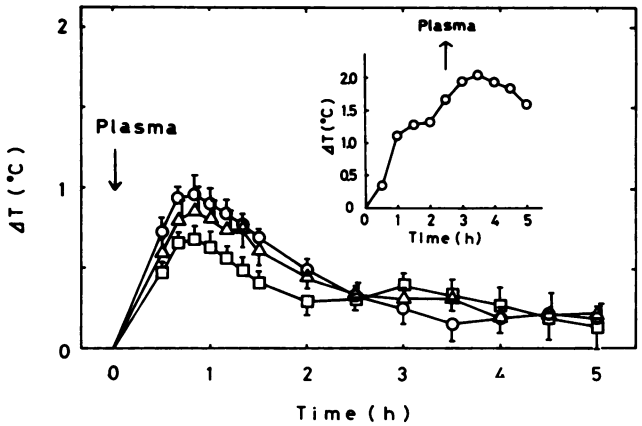

FIG. 4. EP activity in plasmas from febrile rabbits which received 625 ng of endotoxin per kg. The plasmas were obtained from donor rabbits 2.5 h after the endotoxin injection (inset, ↑) and transferred to the recipients at time zero (↓). EP activities of nontreated (○), heated (△), and anti-RaTNF-treated (□) plasmas are shown. Results are presented as the mean $\Delta T \pm$ SEM (n = 5).

0.77 ± 0.06°C; $n = 5$) which was heat stable ($\Delta T = 0.71 \pm 0.15$°C; $n = 5$). The febrile response of the recipients was a monophasic type, with a peak 1 h after the transfer. In another experiment, the plasma heated at 70°C for 30 min produced monophasic fever in rabbits 1 h after the transfer ($\Delta T = 0.53 \pm 0.05$°C; $n = 3$), and the plasma treated in vitro with anti-RaTNF gave rise to a similar febrile response ($\Delta T = 0.53 \pm 0.09$°C; $n = 4$). These results indicate that the late-peak fever caused by exogenous RaTNF is mediated by material(s) that are similar to those induced by endogenous TNF.

DISCUSSION

TNF is one of the well-characterized macrophage-derived cytokines with multiple biological activities (17). Recent studies have revealed that it has potent pyrogenicity in rabbits (9), suggesting that TNF is a candidate for EP, as is IL-1 (5–7, 10–12).

Nowadays, monoclonal antibodies to various cytokines are available, so we examined the in vivo role of cytokines. Using monoclonal anti-RaTNF in combination with purified RaTNF, we demonstrated that endogenous TNF contributes to both the early- and late-peak fevers evoked by endotoxin, possibly by different mechanisms.

First, anti-RaTNF suppressed both the early- and late-peak fevers caused by endotoxin; second, endogenous TNF activity was detected in 1-h blood in an endotoxin dose-dependent manner coincident with the early-peak fever but was not detected in 2.5-h blood; third, exogenous TNF evoked biphasic fever, the second peak of which could not be attributed to the direct action of TNF. Recently, Nagai et al. (20) reported that an injection of antibody against rabbit TNF reduced the late-peak fever caused by endotoxin. Our results showed that the injection of anti-RaTNF also affected the early-peak fever.

The early-peak fever induced by endotoxin has been considered to be due to the direct action of endotoxin on the hypothalamic thermoregulatory center (3, 4, 26). However, anti-RaTNF efficiently suppressed the early-peak fever, although the antibody did not affect the fever within 1 h after endotoxin injection (Fig. 3). The antibody-sensitive response in the early-peak fever reached a peak consistently at 1.5 h, regardless of the endotoxin dose. Since significant activity of TNF was demonstrated in the sera obtained 1 h after the endotoxin injection, the antibody-sensitive fever at around 1.5 h would be caused by the direct action of endogenous TNF on the thermoregulatory center. Therefore, it is possible that the early-peak fever induced by endotoxin consists of at least two responses caused by different mechanisms: one by the direct action of endotoxin on the thermoregulatory center, the peak of which appears at 1 h, and the other mediated by endotoxin-induced endogenous TNF, whose peak appears at 1.5 h.

It is worth noting that significant activity of TNF was detected in the sera obtained from normal rabbits 2 h after the injection of minute amounts of endotoxin. The quick and short response in TNF generation is consistent with the time course of TNF activity in the blood of mice injected with *Serratia marcescens* polysaccharide reported by others; in that report, the TNF activity in blood appeared soon after the injection, lasted for the first 1 h, and then declined (23).

Skarnes et al. reported (27) that prostaglandin E_2 was found in sheep blood during the early-peak fever but not during the late fever. The present results showing that an injection of endotoxin evoked a transient increase in TNF activity in blood may support their findings on the kinetics of prostaglandin E_2 in blood, since TNF has been reported to trigger prostaglandin E_2 production (9). However, the possibility was not examined in the experiments described here.

The endogenous TNF also seems to play an important role in the elicitation of late-peak fever, since the administration of anti-RaTNF abrogated the response almost completely. The endogenous TNF appears to mediate the production of the late-peak fever by the generation of another EP(s) which was heat stable and resistant to anti-RaTNF treatment. The contribution of endogenous TNF in the late-peak fever was examined further, using highly purified RaTNF. We demonstrated that a large amount of RaTNF induced a late-peak fever through the generation of EP that was similar to that induced by endotoxin. Therefore, it is possible that the late-peak fever caused by endotoxin is mediated by EP produced by the stimulation of endogenous TNF.

A previous injection of an amount of endotoxin that elicits biphasic fever induces a refractoriness for the production of late-peak fever in rabbits; this state is called an early-phase pyrogen tolerance (13). The early-phase pyrogen tolerance is believed to be due to the decreased ability to produce EP. In this study, we showed that at least two kinds of EP could be generated in vivo by the endotoxin injection. One is TNF, and the other is an undefined EP; the generation of the latter was triggered by TNF. Furthermore, a previous injection of an amount of RaTNF that evokes biphasic fever (3×10^4 IU/kg of body weight) inhibited the onset of late-peak fever. These results suggest that the early-phase pyrogen tolerance induced by an injection of endotoxin is partly due to the failure in ability to produce the putative EP in response to endogenous TNF, though the influence of the reduction in TNF production itself reported previously (15) could not be ruled out.

Although the characteristics of EP remain to be studied, the present results are inconsistent with the findings of other investigators that a heat-labile material was responsible for the late-peak fever caused by endotoxin (1, 5, 12). These investigators used such a huge amount of endotoxin or organisms to demonstrate EP activity in the blood that various types of heat-labile cytokines with pyrogenic activity, such as IFNs (18, 19, 21) or IL-1, might well have been generated.

Our results on the pyrogenicity of RaTNF agree somewhat with the findings of others that human TNF-α (HuTNF) produces monophasic or biphasic fever in rabbits, depending on the amount injected (9, 22). They have also shown that a large amount of HuTNF triggered in vivo production of another EP to elicit biphasic fever. However, EP induced by HuTNF in their experiments was shown to be heat labile (9). In addition, a previous injection of a large amount of HuTNF was shown in both reports not to inhibit the production of the late-peak fever in rabbits in response to another shot of HuTNF. Since HuTNF is obviously xenobiotic to rabbits despite the low species specificity in terms of in vitro cytotoxicity, it may work as an exogenous pyrogen in rabbits and cause fever through mechanisms different from those of RaTNF.

The mechanisms of endotoxin fever are postulated from the results described above as follows. First, endotoxin directly stimulates the putative thermoregulatory center in the hypothalamus to produce weak fever within 1 h after injection. At the same time, endotoxin stimulates macrophages or other cells to release TNF. Second, the endogenous TNF triggers the thermoregulatory center to produce moderate fever at around 1.5 h; then a large amount of TNF

stimulates the production of undefined EP. Third, the EP elicits the late-peak fever at around 3.5 h. A restriction of the ability to produce either TNF or the putative EP by a previous endotoxin stimulation leads to the early-phase pyrogen tolerance.

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