TUMOR NECROSIS FACTOR (CACHECTIN) IS AN ENDOGENOUS PYROGEN AND INDUCES PRODUCTION OF INTERLEUKIN 1

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From the initial observations of Menkin and the pivotal experiments of Beeson, Wood and Atkins (1), the postulated mechanism for fever in a variety of diseases was based on the ability of various substances, usually of microbial origin, to stimulate phagocytes to synthesize and release a heat-labile protein called endogenous pyrogen (EP).[1] It was later shown that EP initiated fever by increasing prostaglandin PGE_2 synthesis in or near the anterior hypothalamus (reviewed in 2), and that antipyresis was the result of reduced cyclooxygenase activity there rather than in the EP-producing cells (3, 4). During the last decade, it became increasingly clear that EP, in addition to its ability to induce fever, possessed a great number of biological activities (5). Following tedious protein purifications, homogeneous EP was shown to stimulate T cells (6–9), increased hepatic acute-phase protein synthesis (10), activate neutrophils (11), stimulate prostaglandin production in vitro (12), and in general, mediate many components of the generalized acute-phase response (5). Because of its multiple biological activities, and particularly its ability to activate lymphocytes, renaming EP interleukin 1 has become accepted (13).

Attributing many diverse biological properties to a single molecule created a dilemma for investigators. Despite convincing evidence of the homogeneity of various preparations (8, 9, 14, 15), considerable doubt remained that a single polypeptide possessed such diverse activities. The controversy has now been resolved; two cDNAs coding for IL-1 have been cloned, a neutral form (16) and an acid form (17). Recombinant IL-1s of both forms have now been used to study the multiple biological properties attributed to IL-1. Although the two IL-1 forms, representing the two charged species at pI 7 and pI 5 (18), share little

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[1] *Abbreviations used in this paper:* EP, endogenous pyrogen; LAL, limulus amebocyte lysate; MNC, mononuclear cell; TNF, tumor necrosis factor.

amino acid homology, each recombinant form can induce the same broad spectrum of responses, including fever (19).

During the early investigation into the pathogenesis of fever, there was speculation that another leukocyte product, interferon, also caused fever. From the first clinical trials using partially purified IFNs, fever had become the major side effect of IFN therapy (20). Current clinical trials using recombinant forms have confirmed the observations that IFNs, particularly IFN-α, cause fever. Recently (21, 22) it was shown that IFN-α produces fever not because it is contaminated with endotoxins or induces IL-1, but rather because IFN-α is intrinsically pyrogenic by its direct action on the thermoregulatory center (21, 22). Thus, from a conceptual point of view, a second molecule exists with endogenous pyrogen activity. rIFN-α meets the criteria for being an endogenous pyrogen: it produces a brisk, monophasic fever following intravenous injection into rabbits and mice, is free of endotoxins, and increases the production of PGE_2 from brain tissue in vitro and in vivo.

We now report that another leukocyte product, tumor necrosis factor (TNF; cachectin) (23), is also intrinsically pyrogenic. Recombinant human tumor necrosis factor (rTNFα) produces a brisk, monophasic fever after intravenous injection into rabbits, and the fever is not due to contaminating endotoxin. However, unlike IFN-α, rTNFα also induces IL-1 in vivo and in vitro. These results, along with those observed with IFN-α and IL-1, support an expanded hypothesis for the pathogenesis of fever involving several endogenous pyrogens.

Materials and Methods

Materials. Human rTNFα was expressed in *Escherichia coli* and purified to homogeneity (24). *Limulus* amebocyte lysate (LAL) (Mallinckrodt, Inc., St. Louis, MO) testing employed an *E. coli* standard (Mallinckrodt, Inc.) and detected 10 pg/ml (0.1 endotoxin unit). Various lots of rTNFα were tested and revealed 200 pg or less of endotoxin per milligram of rTNFα. Adding rTNFα to known quantities of endotoxin did not interfere with the LAL assay. Using gas chromatography/mass spectrometry (5840A and 5985B, respectively, Hewlett-Packard Co., Palo Alto, CA), β-hydroxymyristic acid in rTNFα preparations was not detected above background levels (sterile water was <40 pg/mg). Adding known amounts of endotoxin to rTNFα resulted in complete recovery of the calculated amount of β-hydroxymyristic acid (25). The human rIL-1 used in these studies was the pI 7 form (16), provided by Cistron Technology, Inc. (Pine Brook, NJ). rIL-1 was expressed in *E. coli* and consisted of amino acids 112–269 of the precursor sequence. LAL testing used lysate from Associates of Cape Cod (Woods Hole, MA) with a sensitivity of 20 pg/ml (0.2 endotoxin unit) using the *E. coli* standard EC-5 (Bureau of Biologics, Bethesda, MD). The rIL-1 contained 40–80 pg endotoxin per milligram of protein. Adding rIL-1 to known quantities of endotoxin did not interfere with the LAL assay. Human rIFN-γ was produced in *E. coli* and purified to >98% purity (26). IFN-γ concentrations were determined in a cytopathic inhibition assay using A549 cells challenged with encephalomyocarditis virus. rIFN-γ used in these studies has a sp act of 1–2 $\times 10^7$ U/mg. Ibuprofen formulated for intravenous injection was provided by Upjohn Co., Kalamazoo, MI.

Trypsin Treatment. rTNFα (2 mg) was incubated with TPCK-treated trypsin (Cooper Biomedical, Freehold, NJ) at 37°C. Trypsin (20 μg) was added at 0, 12, 24, and 36 h. After 48 h of incubation, soybean trypsin inhibitor (160 μg; Sigma Chemical Co., St. Louis, MO) was added to each sample. Limulus lysate testing of the trypsinized rTNFα revealed 1 ng/mg endotoxin.

Pyrogen Testing. New Zealand–derived female rabbits weighing ~2.5–3.0 kg (Pine

Acres, Burlington, VT) were trained in restraining devices for 1 wk before pyrogen testing. Core temperature was measured using indwelling rectal thermistors (Yellow Springs Instruments, Yellow Springs, OH) and a Kaye Digistrip Model II recorder (Bedford, MA). Rectal temperatures were measured every minute, as described previously (21). The murine fever studies used C3H/HeJ mice, individually caged in an ambient temperature of 33–34°C. Body temperatures were measured with thermosensitive radiotelemetry devices (XM-FH; Minimitter Co., Inc., Sunriver, OR) implanted subcutaneously. After 2 h of stabilization, each mouse was injected with 0.1 ml of either PBS or a dilution of rTNF in PBS (six mice per group). Body temperature was measured at 10-min intervals before and after injection by determining the frequency emitted by each telemeter. The signal was detected with a Minimitter RA-1000-TH receiver coupled to a digital frequency counter (Heathkit SM 2420, Benton Harbor, MI) and converted to temperature using a previously determined calibration index.

PGE_2 Production and Measurements. Rabbit hypothalami were removed, minced, and incubated for 15 min at 37°C. After washing to remove PG induced by tissue trauma, aliquots were incubated for 60 min with various concentrations of rTNFα or rIL-1 in the presence of clinical grade polymyxin B (25 μg/ml) (Pfizer, Groton, CT) as previously described (21, 28). The supernatants were frozen at −70°C and later assayed for PGE_2 using a specific RIA (Seragen, Boston, MA).

Human Mononuclear Cell Incubations. Blood was obtained from healthy donors, and the mononuclear cells (MNC) were isolated as previously described (29). Methods for stimulating endogenous pyrogen activity for rabbit pyrogen testing using 5-ml volumes of MEM (Microbiological Associates, Walkersville, MD) have been described in detail (29). MNCs were also incubated with IFN-γ and rTNFα for 48 h in 1-ml wells with 2.5×10^6 cells/ml. For these experiments, MNCs were suspended in MEM (Gibco, Grand Island, NY) containing 10% FCS (Hyclone, Logan, UT). Supernatants were diluted and assayed for IL-1 activity on thymocytes.

IL-1 Murine Thymocyte Assay. Thymocytes from C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME) were removed and used to test IL-1 activity in the supernatants of human MNC using augmentation of [^{3}H]thymidine incorporation in response to suboptimal concentrations (1 μg/ml) of PHA-P (Burroughs Wellcome, Research Triangle, NC) as previously described (9, 30). Purified human monocyte IL-1 was used as a standard in these assays (9).

Western Blot Analysis. Methods for western blot analysis were used as previously described (37). Blots were developed using goat anti-rabbit IgG followed by ^{125}I-labeled protein A (Amersham Corp., Arlington Heights, IL).

Cytotoxicity Assay. Cytotoxicity of the murine fibroblast L929 line was used to assay rTNFα activity (31).

Results

Pyrogenicity of rTNFα. Rabbits were given an intravenous bolus injection of freshly thawed rTNFα diluted and mixed with 1.0 ml of 0.15 M NaCl containing 250 μg/ml of polymyxin B. As shown in Fig. 1*A*, rTNFα and rIL-1 at a dose of 1 μg/kg produce febrile responses characteristic of endogenous pyrogens, with monophasic fevers reaching mean peak elevations 48–54 min after the injections. The injection of 0.1 μg/kg of rTNFα did not induce significant fever (<0.3°C), whereas 0.1 μg/kg of rIL-1 induced small but significant fever (>0.3°C; data not shown). The fevers produced by either rTNFα or rIL-1 are unlike those produced by endotoxin in which the monophasic fever reaches peak elevation at ~90 min after the injection (1). In addition, the amount of endotoxin that was detected in either the rTNFα or rIL-1 was clearly below the minimum pyrogenic threshold for endotoxins in rabbits (3–5 ng/kg) (1). As shown in Fig. 1*B*, the fever induced by rTNFα is blocked by prior treatment with the cyclooxygenase

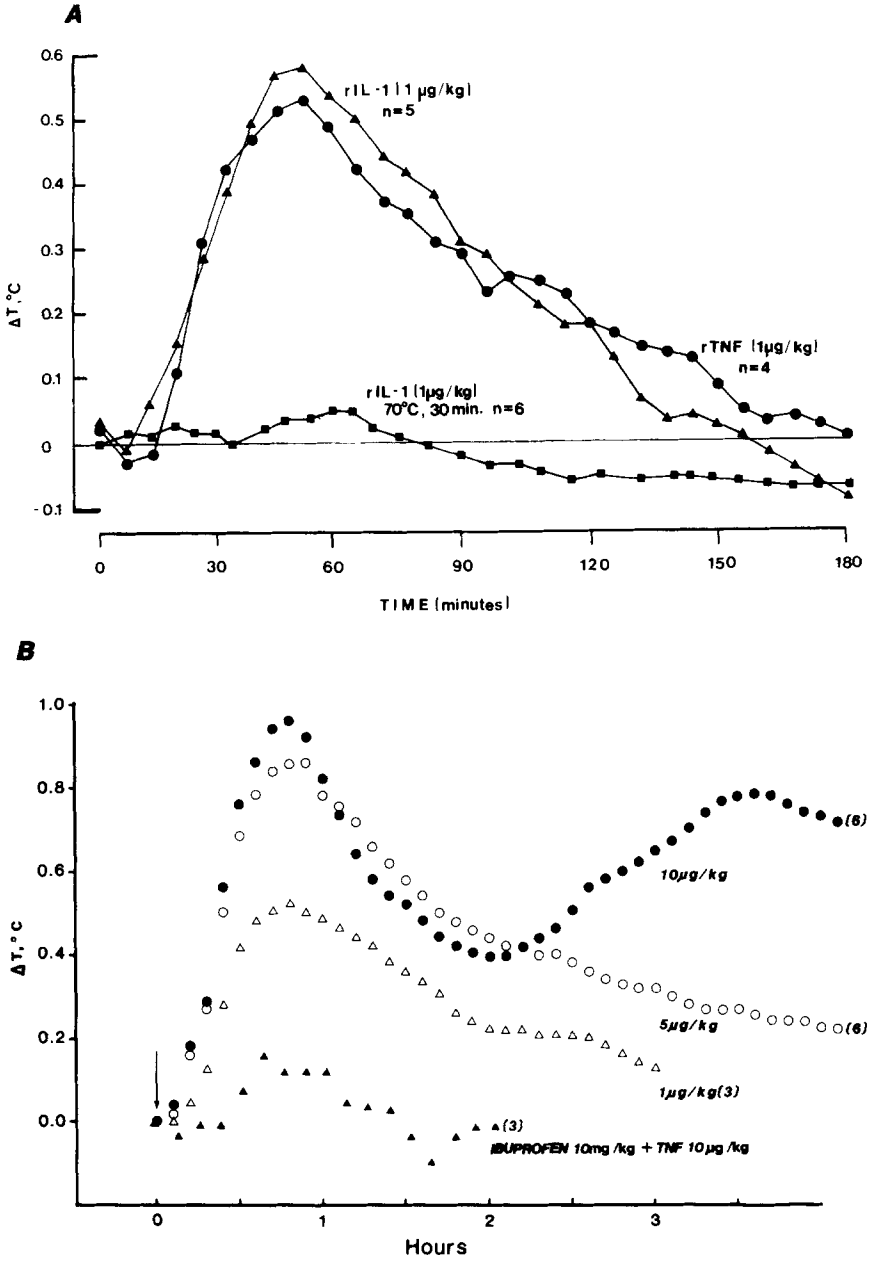

FIGURE 1. (*A*) Mean fevers of rabbits injected with either rTNFα or rIL-1. The recombinant materials were thawed, diluted in polymyxin B and injected intravenously. The numbers in parentheses indicate the number of rabbits in each group. (*B*) Dose-response of rTNFα in rabbits. rTNFα was treated as indicated in *A*. Ibuprofen was injected intravenously 10 min before rTNFα.

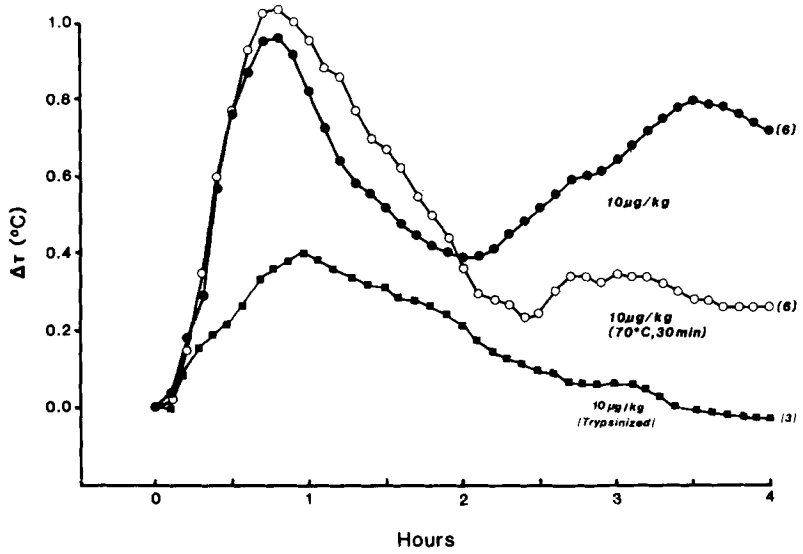

FIGURE 2. Mean fevers in rabbits injected with rTNFα (10 μg/kg) treated with various conditions, as indicated. The numbers in parentheses represent the number of rabbits in each group.

inhibitor and antipyretic, ibuprofen (32). Higher doses of rTNFα (10 μg/kg) induce biphasic fevers with the second fever peak occurring after 3.5 h. As shown in Fig. 2, heating rTNFα for 30 min at 70°C reduced the second fever peak without affecting the initial febrile response. However, the pyrogenicity of rTNFα was markedly reduced by trypsin treatment. The cytotoxicity assay of trypsinized rTNFα revealed that the treatment reduced biological activity of the rTNFα by ~80% and that heat treatment reduced activity by 50% (data not shown).

Although the concentrations of endotoxin in the rTNFα (see Materials and Methods) were below that which would induce fever in rabbits, we employed the endotoxin-resistant C3H/HeJ mouse for additional pyrogen testing. As shown in Fig. 3, rTNFα produces fever in these mice at 1 and 10 μg/kg. Significant elevations of body temperature occurred 40 min after intraperitoneal injections.

Rabbits injected daily with endotoxins develop progressive pyrogenic unresponsiveness (pyrogenic tolerance). In an attempt to induce pyrogenic tolerance to rTNFα, injections of rTNFα (10 μg/kg) were given to rabbits on four consecutive days. In general, no demonstrable decreases in febrile responses were observed. Three representative individual rabbit febrile responses are shown in Fig. 4. Rabbit 2 did show decreasing fever with successive injections of rTNF. However, this rabbit became ill (diarrhea and lethargy) after the second injection and died before the fourth injection. During the course of these studies, 56 rabbits received repeated injections of rTNFα (all intravenous), and 6 rabbits (11%) were found dead in their cages. In contrast, no rabbits receiving repeated injections of rIL-1 died.

To determine the cause of the second fever peak, rabbits injected with rTNFα were bled during the uprise of their second fever peak, and the heparinized

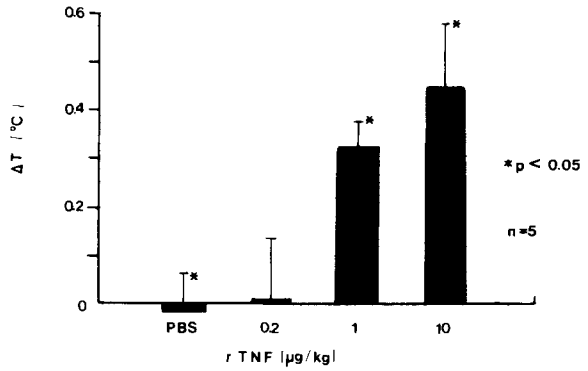

FIGURE 3. Mean peak fever (±SEM) after 40 min in C3H/HeJ mice injected with materials indicated. Significance was reached between the PBS control and the rTNFα as shown using analysis of variance.

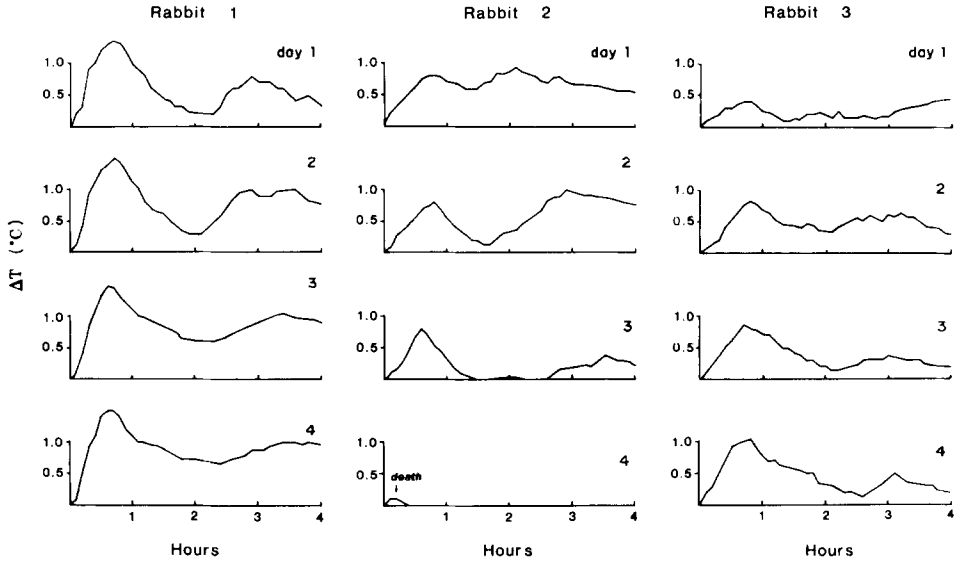

FIGURE 4. Febrile responses to four daily injections of rTNFα (10 μg/kg) in three rabbits.

plasma was pooled and stored for 24 h at 4°C. This plasma was injected into fresh rabbits, and as shown in Fig. 5, produced a monophasic fever. The pyrogenicity of the circulating plasma was destroyed by heating at 70°C for 30 min. The heat lability of the plasma factor is similar to that of rIL-1, as shown in Fig. 1*A*. On the other hand, rTNFα, heated in the same water bath as the rIL-1, retained its ability to induce the first fever peak (see Fig. 2). The data from these experiments support the concept that the second fever peak after rTNFα is due to the production of IL-1 in vivo.

rTNFα and rIL-1 Induce Hypothalamic PGE_2 In Vitro. The rapid rise in body temperature that characterizes the febrile response to either rTNFα or rIL-1 suggests a direct hypothalamic stimulation, most likely mediated, in part, by an increase in PGE_2 synthesis. A single intravenous injection of ibuprofen (10 mg/kg) given immediately before rTNFα blocked the febrile response, as pre-

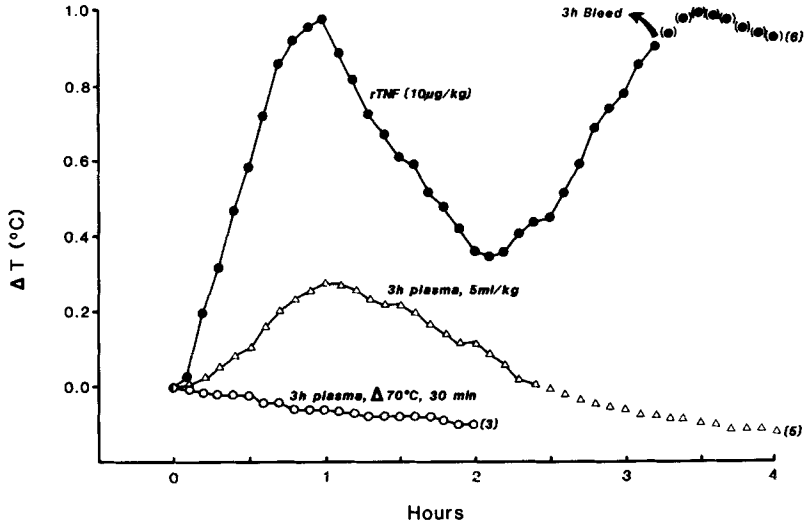

FIGURE 5. Mean fevers in six rabbits injected with materials indicated. After 3 h, three rabbits were bled via the central ear artery. The plasma was pooled, stored at 4°C and warmed to 37°C before administration the next day. This plasma was infused intravenously over 2 min.

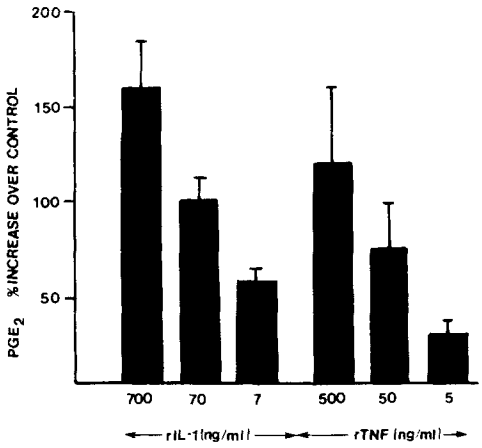

FIGURE 6. Mean percent increase of PGE_2 as measured in supernantants of stimulated rabbit hypothalamic minces. Unstimulated cells produce 30–60 pg/mg of tissue.

viously shown for IL-1 (32). Rabbit hypothalamic minces were incubated with rTNFα or rIL-1 for 60 min, and the level of PGE_2 was assayed in the supernatant media. As reported previously (12, 21, 28), one onehundredth of the amount of IL-1 necessary to produce 0.6°C fever in rabbits approximately doubled the PGE_2 detected in the supernatant of hypothalamic minces after 60 min. As shown in Fig. 6, the specific activities of rTNFα and rIL-1 were similar in terms of PGE_2 production in three separate experiments.

Production of IL-1 In Vitro from Human MNC Incubated with rTNFα. To elucidate the probable mechanism by which intravenous injection of rTNFα in rabbits induces the production of a circulating endogenous pyrogen (IL-1 or

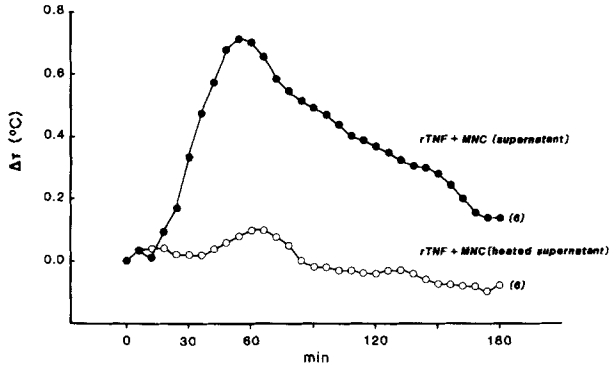

FIGURE 7. Mean fevers in rabbits injected with supernatants derived from human MNC stimulated with rTNFα (50 ng/ml). Supernatants from the MNC of two human subjects were pooled; half was heated (70°C for 30 min) before injection. Rabbits were injected with supernatants derived from 10^6 monocytes/kg. The numbers in parentheses indicate the number of rabbits used to assay the supernatants.

TABLE I
Neutralization of Pyrogenic Activity in MNC Supernatants by Anti-IL-1

MNC supernatants* plus:	Mean temperature peak (°C)§
Normal rabbit serum‡	0.64 ± 0.08
Anti-human IL-1‡	0.17 ± 0.03

* 24-h MNC supernatants were generated in 25-ml flasks containing 5 × 10^6 MNC/ml (5 ml/flask) in the presence of 100 ng/ml rTNFα. For these experiments, MNC were isolated from two human subjects. Rabbits were injected with the supernatant derived from 10^6 monocytes/kg.
‡ Anti-IL-1 (15) or normal rabbit serum was mixed at 1% (vol/vol) with the MNC supernatants, incubated overnight at 4°C and centrifuged (10,000 *g*) for 2 min.
§ Number of rabbits used to assay supernatants.

TNF), human MNC were stimulated in vitro with various concentrations of rTNFα, and the supernatant media were assayed for endogenous pyrogen activity in rabbits. As shown in Fig. 7, the supernatant medium from human MNC incubated for 24 h with rTNFα induced a monophasic fever when injected into rabbits. The amount of rTNFα added to the MNC (50 ng/ml) was below the rabbit pyrogen threshold (see Fig. 1*B*). After the incubations, the supernatant media were tested for TNFα activity using an ELISA, and there was no increased TNFα in the supernatant over the amount that was added exogenously (data not shown). The pyrogenic activity in these supernatants was destroyed by heating at 70°C for 30 min, suggesting that this was likely due to IL-1 and not monocyte-derived TNF/cachectin. As shown in Table I, the monophasic fever–inducing activity in the stimulated MNC supernatants was IL-1, since it was neutralized by anti-human monocyte IL-1. This antibody did not recognize human rTNF or naturally-derived cachectin, as determined by western blot analysis (Fig. 8). Anti-IL-1 had no effect on rTNFα in the cytotoxicity assay (data not shown).

The dose response of rTNFα induction of IL-1 is shown in Fig. 9. At

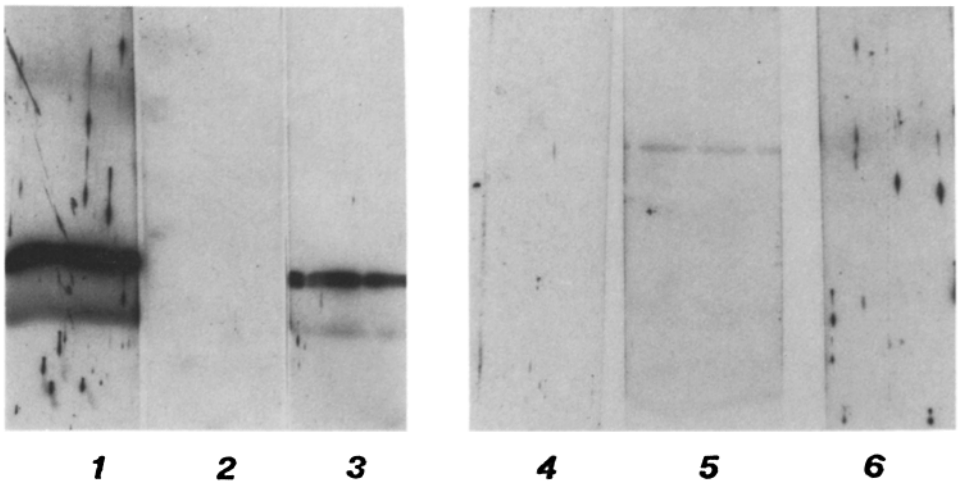

FIGURE 8. Western blot analysis of anti-IL-1. Lanes *1–3* each contain 1 μg purified natural cachectin (37). Lane *1* was developed with anticachectin (1:200); lane *2* with anti-IL-1 (1:100) (15); and lane *3* with anti-human rTNFα (1:200). Lanes *4–6* each contain 10 μg human rIL-1. Lane *4* was developed with anticachectin; lane *5* with anti-human IL-1; and lane 6 with anti-rTNFα.

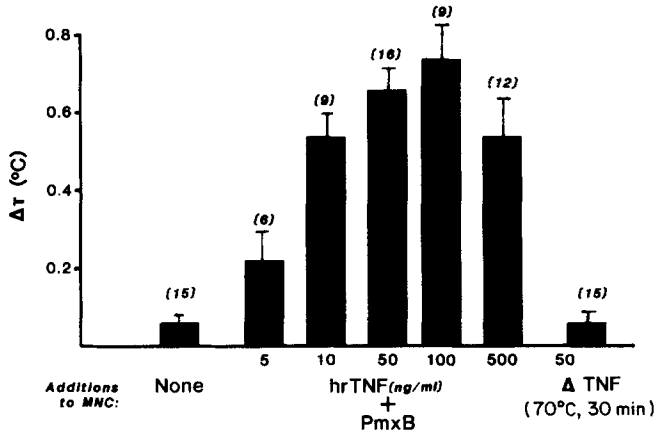

FIGURE 9. Mean peak fever ± SEM in rabbits injected with materials indicated under the abscissa. All MNC incubations were in the presence of 12.5 μg/ml polymyxin B. The numbers in parentheses indicate the number of rabbits used to assay the pyrogenic activity in the MNC supernatants.

concentrations of 10–500 ng/ml, rTNF induced IL-1 (as measured by peak fever in rabbits). The amount of rTNFα that induced IL-1 was optimal between 50 and 100 ng/ml, whereas concentrations of 500 ng/ml induced less IL-1 (although not significantly different from 100 ng/ml, $p > 0.05$). Heating rTNFα for 30 min at 70°C destroyed its ability to induce IL-1 in vitro; this finding confirms the observation that heat-treated rTNFα does not induce a second fever peak in rabbits (see Fig. 2).

As expected, supernatants of human MNC stimulated with rTNFα contained IL-1, as measured by augmentation of T cell proliferation in response to PHA (Table II). The IL-1-inducing property of rTNFα was sensitive to heat and

TABLE II
Production of IL-1 by Human MNC In Vitro

Additions to MNC	[^{3}H]TdR incorporation (cpm)*			
	Donor 1	Donor 2	Donor 3	Donor 4
None (MNC control)	6,763	4,711	5,096	4,793
rTNFα (ng/ml)				
1	35,049	10,212	13,542	ND
10	48,924	20,118	8,366	ND
100	24,864	9,426	7,254	10,471
100; trypsinized	ND	ND	ND	4,470
Trypsin only	ND	ND	ND	6,086
Endotoxin (10 ng/ml)	125,864	33,824	64,357	ND

* Mean counts per minute of [^{3}H]TdR incorporation. SD of triplicate wells were 5–10% of the mean. MNC supernatants were diluted 1:20 and 1:200 in the thymocyte assay. Only 1:20 data are shown.

trypsinization. However, the amount of IL-1 detected in the T cell assay was less than that assayed in endotoxin-stimulated MNC supernatants. This may have been due to interference of TNFα in the IL-1 assay, although incubating rIL-1 with rTNFα in the thymocyte assay resulted in only slight inhibition of the IL-1 activity (data not shown). It is also possible that rTNFα stimulated the production of inhibitory substances from the mononuclear cells. Although detection of these IL-1-inhibiting substances in the rabbit pyrogen assay is unlikely, several investigators (33) have shown that stimulated monocytes release substances that inhibit T cell proliferation in this assay.

Production of IL-1 from human MNC by rTNFα was enhanced by coincubation with rIFN-γ. As depicted in Fig. 10, rIFN-γ (1 ng/ml) enhanced IL-1 production by rTNFα at 0.25 ng/ml.

rTNFα has no intrinsic proliferation-enhancing activity except at high concentrations (500 ng/ml; $p < 0.05$) (Fig. 11). This activity may reflect the induction of IL-1 from contaminating murine thymic epithelial cells or macrophages, since it was neutralized with anti–mouse IL-1. In situ production of IL-1 during the T cell assay has been observed with other substances, such as the polypeptide toxin elaborated by certain strains of toxic shock syndrome–associated *Staphylococcus aureus* (34).

Discussion

These studies show that rTNFα causes fever because it is intrinsically pyrogenic and is capable of increasing hypothalamic PGE_2 synthesis. Following an intravenous injection of 1 μg/kg, rabbits develop monophasic fevers that reach peak elevations after 45–55 min and then return to baseline levels. The only other well-defined substance of leukocyte origin that produces this rapid increase in core temperature is IL-1. Because it is a product of stimulated leukocytes, TNF/cachectin must now be considered an endogenous pyrogen. In Phase I clinical trials, rTNFα injected into humans is pyrogenic (S. Sherwin and S. Saks, unpublished observations). Mice passively immunized with anticachectin antibodies survive a lethal dose of endotoxin but still develop fever (35). In these mice

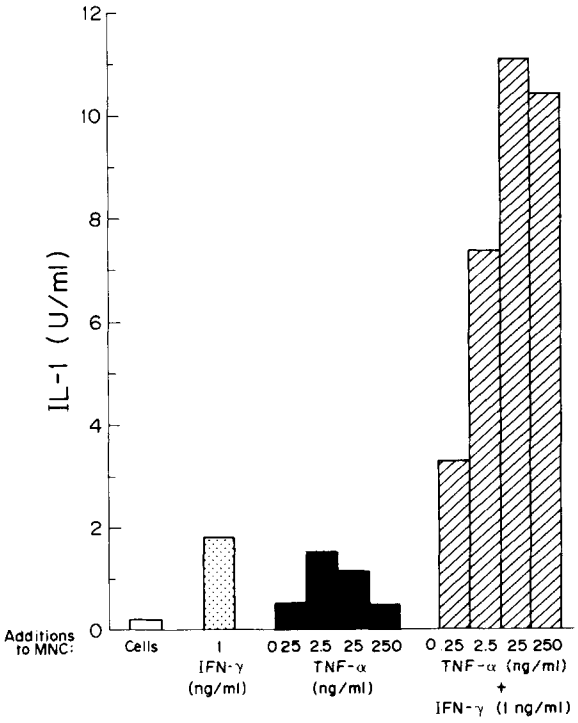

FIGURE 10. IL-1 production by rTNFα and enhancement by rIFN-γ. MNC supernatants were incubated in the presence of 10% FCS for 48 h with materials indicated under the abscissa and diluted 1:8 in the thymocyte assay.

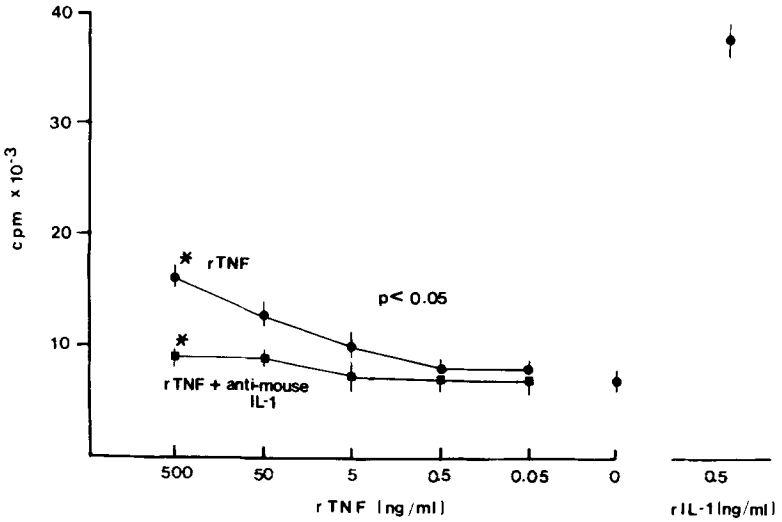

FIGURE 11. Comparison of rTNFα and rIL-1 on thymocyte proliferation to PHA. Anti-mouse IL-1 (1:2,000 dilution, vol/vol) was added to some wells with the rTNFα. Anti-mouse IL-1 was raised in goats to IL-1 purified from P388D cell line (kind gift of Dr. Morris Sheetz, Eli Lilly and Co., Indianapolis, IN). With these thymocytes, concentrations of rTNFα at 500 ng/ml induced significant IL-1-like activity ($p < 0.05$, Student's t-test.).

it is likely that both cachectin and IL-1 are produced, but IL-1 is not neutralized by the anticachectin antibody. Thus, it is IL-1 that probably mediates the fever in these passively immunized mice. It appears that rTNFα and rIL-1 have approximately the same specific activities in terms of their ability to induce rapid-onset fevers after intravenous injection in mice and rabbits. At present, there are no studies of rIL-1 injected into human subjects, and the sensitivity of humans to IL-1 remains to be studied. Although there is another endogenous pyrogen, IFN-α (21, 22), the amount of IFN-α required to produce fever in rabbits or mice is 100-fold greater than rIL-1 or rTNFα. However, IFNs have known species specificities which may explain this dose-relationship in nonhuman species.

From clinical and laboratory data, three leukocyte products can now be identified as being endogenous pyrogens, IL-1, IFN-α and TNF/cachectin. What remains to be established is whether any or one or all three of these substances mediates the febrile response to a particular disease. In patients with infectious diseases in which endotoxin plays a pathogenic role, it is likely that all are produced. Extensive laboratory evidence has established that endotoxin stimulates TNF/cachectin production (36, 37), IL-1 (13), and IFN production (38, 39). However, it may be that in some viral diseases, i.e., cytomegalic disease and hepatitis B, IFN predominates as the primary endogenous pyrogen, since many components of the acute-phase response are not induced by IFN and are frequently absent in various viral diseases. Likewise, TNF/cachectin may be the mediator of fever in certain parasitic diseases. Considerable clinical and laboratory investigation remain before the role of each or combinations of these endogenous pyrogens can be established in a comprehensive explanation for the pathogenesis of fever.

The recently reported amino acid sequences of human TNF-α (24), cachectin (23) and IL-1 (16, 40) indicate no obvious sequence homologies. Two forms of IL-1 have been identified (16, 17), which correspond to the two isoelectric points, pI 7 and 5. These two forms were originally described as endogenous pyrogens in 1974 (18), but their physical relationship at that time was not clear. At the amino acid level, these two forms share little homology; however, at the carboxyl end of each form, 38% absolute and 78% conserved sequence homology exist (41), and it seems likely that the active site for the biological properties which the two IL-1 forms share resides in these sequences. Nevertheless, when the shared carboxyl segments are matched with TNFα or lymphotoxin (TNFβ), no striking sequence homology can be observed. Despite their independent structure, TNF/cachectin and IL-1 both produce fever, stimulate neutrophils (11, 42), induce synovial cell collagenase production (43), decrease lipoprotein lipase (37, 44), and are cytotoxic for tumor cells (45, 46).

Using rTNFα in these studies, as has been shown (37) using purified natural cachectin, TNF/cachectin has no IL-1 activity on T cells. This may explain the findings of Damais et al. (47), who reported that muramyl dipeptide polyalanine/lysine stimulated macrophages to release a pyrogenic substance that is not active on lymphocytes. Induction of TNF/cachectin would explain the activity responsible for the fever-producing property of these supernatants.

At higher doses, rTNFα induces a biphasic fever. Using passive transfer of

plasma taken during the rise of the second fever peak, a circulating substance produced a monophasic fever when injected into new rabbits. This circulating pyrogenic substance is probably not residual rTNFα, since the pharmacokinetics of rTNF and natural cachectin indicate that >95% of the injected material is cleared from the circulation in 3 h (48). The experiments presented in the present study suggest that the second fever peak represents IL-1 induced by rTNFα in vivo. To support this observation, rTNFα was shown to induce IL-1 production from human MNC in vitro. Although the rabbit pyrogen assay was used to test the IL-1 content of the stimulated MNC supernatants, it can no longer be assumed that brisk monophasic fevers in rabbits is a valid assay for the presence of IL-1. As a result of these studies, it is now clear that either rTNFα or rIL-1 induce indistinguishable fevers within 60 min of intravenous injection. Thus, the endogenous pyrogen assay in rabbits requires conditions for specificity. At present, these seem to be (*a*) the ability of heating at 70°C for 30 min to destroy the fever-inducing property of IL-1 but not rTNFα, and (*b*) the use of an anti–human IL-1, which neutralizes IL-1's pyrogenic property but does not recognize TNF/cachectin. Using these two conditions, it seems clear that the pyrogenic moiety in rTNFα-stimulated MNC supernatants is indeed IL-1 and not more TNF. The use of the T cell assay was also important in demonstrating that rTNFα induces IL-1, since TNF/cachectin is not active on T cells.

The biphasic fever curve seen after administration of rTNFα suggests that rTNFα has two separate effects: the induction of PGE_2 and the induction of IL-1. The initial fever peak (PGE_2-mediated) has a much lower threshold (<1 μg/kg) than the secondary fever peak (IL-1-mediated) in which the dose of rTNFα must be between 5 and 10 μg/kg in order to evoke the delayed fever (Fig. 1*B*). In addition, the primary response is not abolished by prior heating of rTNFα at 70°C for 30 min, whereas the secondary response is completely eliminated by this treatment. However, based on the cytotoxicity assay, the heat-treated rTNFα still retains 20% of its original activity. The loss of the secondary fever response probably reflects this decrease in potency. Currently, we have no evidence for the presence of two distinct receptors mediating these two effects for rTNFα, but is seems unlikely that a single receptor would have two different thresholds, each mediating a separate response such as the production of PGE_2 and IL-1.

Previous work (49) has shown that colony-stimulating factors induce IL-1 production. In addition, there are partially characterized lymphokines, secreted in response to mitogen or antigen, which induce monocytes to produce IL-1 (50–52). The present studies raise the question of whether one of these lymphokines is lymphotoxin (TNFβ), since this molecule is closely related to TNF/cachectin (24).

These studies shed light on the long-unexplained finding (53–55) that during the second fever peak in rabbits given intravenous injections of influenza virus, the plasma contained a substance which, when injected into new rabbits, induced another biphasic fever. It had been established that the pyrogenic moiety circulating during the second fever peak was not due to remaining virus but rather to a host product. It was also shown that fever-inducing factor(s) circulating during the plasma-induced second fever peak contained characteristic endogenous pyrogen (54), and it now seems certain that this pyrogen was IL-1. However,

using large amounts of purified rabbit IL-1, only monophasic fevers have been observed (P. Murphy, personal communication). Thus, the concept that IL-1 induces more IL-1 in vivo has been difficult to substantiate even using in vitro production methods (55). Considering the data of the present report, it seems possible that the circulating factor detected by passive transfer of plasma from febrile rabbits may have been to due to TNF/cachectin rather than IL-1.

Summary

Recombinant human tumor necrosis factor (rTNFα) injected intravenously into rabbits produces a rapid-onset, monophasic fever indistinguishable from the fever produced by rIL-1. On a weight basis (1 μg/kg) rTNFα and rIL-1 produce the same amount of fever and induce comparable levels of PGE_2 in rabbit hypothalamic cells in vitro; like IL-1, TNF fever is blocked by drugs that inhibit cyclooxygenase. At higher doses (10 μg/kg) rTNFα produces biphasic fevers. The first fever reaches peak elevation 45–55 min after bolus injection and likely represents a direct action on the thermoregulatory center. During the second fever peak (3 h later), a circulating endogenous pyrogen can be shown present using passive transfer of plasma into fresh rabbits. This likely represents the in vivo induction of IL-1. In vitro, rTNFα induces the release of IL-1 activity from human mononuclear cells with maximal production observed at 50–100 ng/ml of rTNFα. In addition, rTNFα and rIFN-γ have a synergistic effect on IL-1 production. The biological activity of rTNFα could be distinguished from IL-1 in three ways: (*a*) the monophasic pyrogenic activity of rIL-1 was destroyed at 70°C, whereas rTNFα remained active; (*b*) anti-IL-1 neutralized IL-1 but did recognize rTNFα or natural cachectin nor neutralize its cytotoxic effect; and (*c*) unlike IL-1, rTNFα was not active in the mitogen-stimulated T cell proliferation assay. The possibility that endotoxin was responsible for rTNFα fever and/or the induction of IL-1 was ruled-out in several studies: (*a*) rTNFα produced fever in the endotoxin-resistant C3H/HeJ mice; (*b*) the IL-1-inducing property of rTNFα was destroyed either by heat (70°C) or trypsinization, and was unaffected by polymyxin B; (*c*) pyrogenic tolerance to daily injections of rTNFα did not occur; (*d*) levels of endotoxin, as determined in the *Limulus* amebocyte lysate, were below the minimum rabbit pyrogen dose; and (*e*) these levels of endotoxin were confirmed by gas chromatography/mass spectrometry analysis for the presence of β-hydroxymyristic acid. Although rTNFα is not active in T cell proliferation assays, it may mimic IL-1 in a T cell assay, since high concentrations of rTNFα induced IL-1 from epithelial or macrophagic cells in the thymocyte preparations. These studies show that TNF (cachectin) is another endogenous pyrogen which, like IL-1 and IFN-α, directly stimulate hypothalamic PGE_2 synthesis. In addition, rTNFα is an endogenous inducer of IL-1. Together, these results support the concept that the febrile response to infection is a fundamental event in host defense and the induction of fever by endogenously produced molecules is not imparted to a single substance.

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