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RESEARCH REPORT

Endotoxic Activities of Tumor Necrosis Factor Independent of IL1 Secretion by Macrophages/Monocytes

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ABSTRACT

Recombinant tumor necrosis factor (TNF) had hypothermic activity in vivo. Intravenous injection of TNF resulted in a hypothermic reaction of mice within 3 to 6 hours. This reaction was not the result of interleukin 1 (IL1) release from macrophages/monocytes. Peritoneal exudate cell cultures from endotoxin low responder mice or human peripheral mononuclear leukocyte cultures did not generate IL1 activity in the supernatant after exposure to TNF. The addition of interferon-γ (IFN-γ) or preexposure to IFN-γ and then stimulation with TNF did also not result in IL1 secretion. No IL1 inhibitor was generated and TNF did not interfere with the IL1 test systems. Therefore, we conclude that the hypothermic activity of TNF is not mediated via the induction of IL1 production by mononuclear phagocytes.

INTRODUCTION

TNF has been demonstrated as the mediator responsible for the tumor necrosis phenomenon which occurs after injection of endotoxin into tumor bearing hosts (1). The cellular source of TNF are activated macrophages which produce TNF after stimulation with bacterial lipopolysaccharide (LPS) (2). With the availability of recombinant TNF (3) a wide spectrum of biological activities for TNF has been described besides its tumor cytotoxic effect. It also became obvious that TNF shares a number of biological functions with interleukin 1 (IL1) (4) and that it mediates endotoxin effects (5). The question was asked whether the endotoxic effects of TNF were a consequence of IL1 induction or whether they were direct TNF effects.

Induction of fever in rabbits serves as a quantitative determination of minute amounts of endotoxin. The mechanism of this very sensitive fever test includes the production of prostaglandins of the E series via induction of endogenous IL1 (4). In contrast to rabbits, mice and rats react with hypothermia upon endotoxin injection when they are kept at room temperature (6). This endotoxin-induced hypothermia is probably not due to changes of the thermoregulation in the hypothalamus but rather a toxic effect on the blood vessels. In this study we demonstrate the induction of hypothermia in mice upon TNF injection similar to

the endotoxin-induced hypothermia. We investigated whether this endotoxin-like function of TNF is a direct effect of TNF or whether it is mediated via IL1 production by macrophages.

MATERIALS AND METHODS

Mice

Male C3H/He or C3H/HeJ mice 4-8 weeks of age were either obtained from Zentralinstitut für Versuchstierkunde, Hannover, F.R.G. or from Bomholtgard Ltd., Ry, Denmark or from the Jackson Laboratories, Bar Harbor, Maine, U.S.A.

Reagents

Purified recombinant human TNF was generously supplied by the BASF AG, Ludwigshafen, F.R.G. The preparation contained less than 1.3ng endotoxin per mg protein. Recombinant human IFN-γ was supplied by Dr. Carl Thomae GmbH, Biberach, F.R.G. Highly purified recombinant human IL2 from *E. coli* was obtained from Cetus Corporation, Emeryville, California, U.S.A. For standard purposes human IL1β was semipurified as described recently (7). Lipopolysaccharide preparations were either derived from *S. montevideo* SH94 prepared according to the phenol water extraction method followed by the phenol-chloroform petrol ether procedure (8) or from *S. typh.* 0901 (Difco, FRG). Glutaraldehyde-fixed *Staphylococus aureus* cells (Pansorbin, Calbiochem, Behring Diagnostics, LaJolla, California) were used as 0.1% (w/v) suspension.

Temperature Determination

Rectal temperature were measured using an electronic temperature probe (Haake DT-10, Karlsruhe, F.R.G.). Groups of five mice were kept in cages without making special arrangements to keep the animals warm during the experiment.

Culture Medium

The culture medium used was RPMI 1640 (Gibco) with 10% heat inactivated fetal calf serum (Seromed, Biochrom KG, Berlin, F.R.G.). The special batch of FCS used was previously tested for lack of any IL1 inducing activity as described (9).

IL1 Generating Cultures

Peritoneal exudate cells ($2x10^6$/ml) from either C3H/He or C3H/HeJ mice were obtained 18 h after injection of 1ml PBS i.p. They were cultured with the indicated agents in culture medium for 24 hours. Human mononuclear cells were prepared from buffy coats (ACD stabilizer) of normal blood units by Ficoll-Hypaque density gradient centrifugation (10). The cells were incubated at $3x10^6$ cells/ml at 37°C for 1.5 h in culture flasks (Falcon, Becton Dickinson) and nonadherent cells were removed by washing the cells 3 times with 37°C culture medium. Flasks with the adherent cells attached were placed on ice for 20 minutes and adherent cells recovered by vigorously pipetting with ice cold culture medium. The adherent cells were immediately seeded and cultured at $3x10^5$/ml in microtiter plates (Falcon) in the presence of reagents as indicated. Alternatively, they were incubated at $1x10^6$/ml in 50ml polypropylen tubes (Falcon) at an angle of 45° for 24 h in the presence or absence of IFN-γ (100U/ml) or TNF as indicated. Thereafter they were washed 3 times and seeded at $3x10^5$/ml in microtiter plates (Falcon) and incubated for further 24 h in the presence of LPS (*S. typh.*). After the indicated times supernatants from the macrophage/ monocyte cultures were collected for IL1 determination.

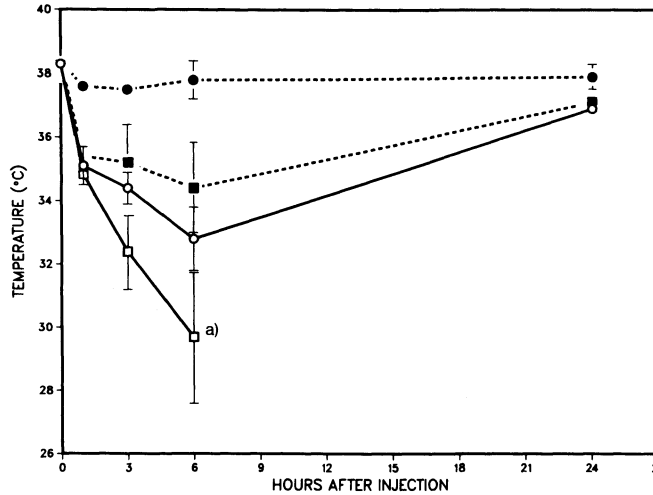

FIGURE 1. Hypothermia in Mice after Endotoxin or TNF Injection.
C3H/He (open symbols) or C3H/HeJ (solid symbols) mice were injected intravenously with either 100µg of LPS from S.montevideo (circles) or with 40µg of TNF (squares) in 50µl of PBS. The rectal temperature was measured before the injection and at 1,3,6 and 24 h after injection and expressed as mean ± S.D. of groups of 5 to 7 mice. a) C3H/He mice injected with TNF died after 6 hours.

IL1 Assay Systems

Thymocytes ($0.5\text{-}1x10^6$) from C3H/HeJ mice were cultured in a volume of 0.1ml in the presence of PHA/M (50µg/ml) (Serva, München, F.R.G.) with serial dilutions of the supernatants for 72 h. For Fig.2B instead of PHA recombinant IL2 was used as costimulator (7). Proliferation was determined by a 4h pulse at the end of the culture period with 1.0µCi of methyl-(^{3}H)-thymidine (^{3}(H)-TdR, specific activity 50 Ci/mmol, Amersham International Ltd., Amersham U.K.) unless otherwise stated. IL1 titers are given as the final dilutions of the supernatants which cause cpm 2.5-fold higher than background. Units of semipurified human IL1β correspond to the dilution which causes 1/2 maximal proliferation (Fig.2).

RESULTS

TNF-Induced Hypothermia

Injection of purified lipopolysaccharide (LPS) into LPS-sensitive mice leads to hypothermia when the animals are kept at room temperature. This reaction is a typical endotoxin effect and can easily be measured. Values of body temperature of C3H/He mice at different times after i.v. application of LPS are shown in Fig.1. Within 6 hours the temperature dropped by 3 to 5°C and reclined thereafter. A similar drop in temperature was measured after application of recombinant TNF (Fig.1). This hypothermic effect after TNF injection was not due to contaminating LPS, since concentrations of LPS equivalent to the contamination were unable to induce such a reaction (data not shown). Also, a significant hypothermic reaction was induced with TNF in LPS-low responder C3H/HeJ mice in which LPS itself did not induce hypothermia. This indicated that the hypothermic reaction after application of the TNF preparation was not mediated by LPS. It has been shown in a separate study that the mediation of typical endotoxin effects -including hypothermia - by TNF in vivo is a dose dependent, important function of TNF (11).

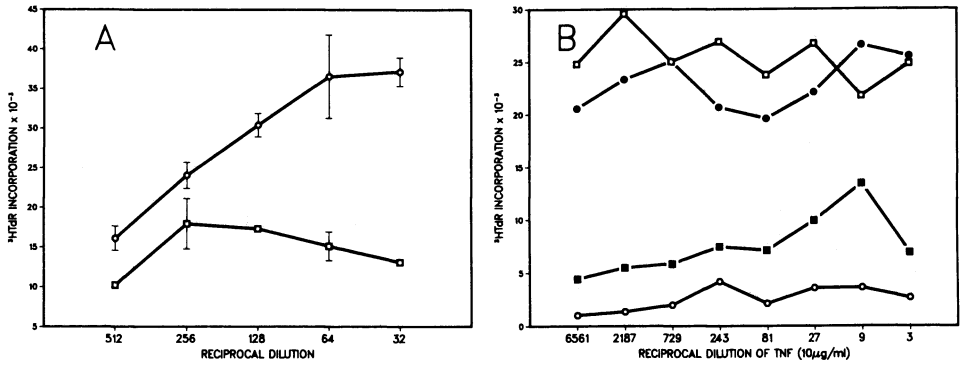

FIGURE 2. Lack of IL1-Activity by TNF

A) Thymocytes ($5x10^5$) from C3H/HeJ mice were cultured in the presence of a suboptimal concentration of mitogen and different dilutions of either IL1 (6U/ml) (O) or TNF (10ng/ml) (□) in 0.2ml volume. ^{3}H TdR uptake was measured in a 16h pulse.

B) Thymocytes ($5x10^5$) from C3H/HeJ mice were cultured in the presence of IL2 (25U/ml) without IL1 (O), with 0.013U/ml (■), 0.93U/ml (●), 9U/ml (□) of IL1, and different dilutions of TNF (10µg/ml). ^{3}H TdR uptake was measured in a 6h pulse.

IL1 Serum Levels after TNF Injection

Since IL1 is a well known mediator of LPS-induced fever in rabbits, LPS-induced hypothermia in mice could also be mediated by endogenous production of IL1. Therefore, the possibility was tested whether TNF injection induced IL1 production in the animals. No significant IL1 activity was detected in sera of either LPS-sensitive or LPS-insensitive mice collected at different times (1h, 3h, 6h and 24h) after TNF (40µg) application. Only in a few LPS sensitive animals little IL1 was detected 6 h after LPS (200µg) injection. TNF activity was still detectable in serum 6h after injection of TNF (data not shown). This observation agreed very well with our earlier findings and data from other laboratories, which showed that serum levels of interleukin 1 can only be measured after LPS injection in mice which have a highly activated mononuclear phagocyte system (R. Urbaschek et al., unpublished results).

Influence of TNF on IL1 Test Systems

The observation that no IL1 was measurable in the sera of the TNF injected animals even though TNF activity was still present indicated that TNF had no IL1 effect in the thymocyte costimulator assay system. To test whether TNF had any effect in the IL1 assay or whether it could mask IL1 activity TNF was titrated into the thymocyte costimulator assay. TNF did not substitute for IL1 in this classical IL1 test system (Fig.2A). Also, in a second test system for IL1 activity in which proliferation of murine thymocytes was induced by simultaneous addition of IL2 plus IL1 (7), TNF was unable to substitute or modulate IL1 activity (Fig.2B).

Failure of TNF to Induce IL1 Production in vitro

To further examine whether TNF could induce IL1 activity in macrophages, IL1 generation in vitro was tested. Murine peritoneal exudate cells (PEC) were cultured in different concentrations of recombinant TNF and the supernatants were tested for IL1 activity. Low amounts of IL1 activity were generated in supernatants of PEC from LPS-sensitive mice after 24 hours (Table 1A). PEC from LPS-insensitive C3H/HeJ mice, however, did not release IL1 into the supernatant upon

TNF exposure. However, macrophages of C3H/HeJ mice were able to produce IL1 when stimulated with Staph. aureus as shown in Table 1B. TNF completely lost the cytotoxic activity after heating at 100°C for 10 minutes (data not shown). This heated material still induced the same titer of IL1 activity in the C3H/He macrophage cultures as the untreated TNF. This indicated that the IL1 activity was not induced by biologically active TNF but rather by a heat resistant contamination of the TNF preparation.

Human and mouse TNF are not completely homologous (12). In order to test whether a possible IL1 induction might be a species-specific activity of TNF, enriched human peripheral blood adherent cells (mostly monocytes) were exposed to graded amounts of recombinant human TNF in the presence or absence of LPS. TNF by itself did not induce IL1 production in these cultures (Table 2). This was not different in the presence of IFN-γ which is known to enhance LPS-induced IL1 secretion by human monocytes (13). Finally, TNF 10µg -3pg/ml did not influence the titer of IL1 which was induced by stimulation with LPS. These data showed that neither IL1 secretion itself nor any detectable inhibitory activity was induced by TNF.

Monocytes from different human donors secrete widely differing amounts of IL1 upon stimulation with a given dose of LPS. Nevertheless, enriched human monocytes represent a sensitive system for monitoring IL1 production (9). Table 3 shows that LPS-stimulated enriched monocytes released higher titers of IL1 than homologous mononuclear cells containing comparable amounts of adherent cells. IFN-γ can further enhance IL1 titers. The data described above obtained with IFN-γ containing monocyte-enriched cultures do not exclude the possibility that TNF might influence monocyte IL1 secretion via an indirect route implicating lymphocytes. Therefore, the effect of TNF on the IL1 secretion by mononuclear cells was tested. Again, TNF 10µg -3pg/ml did neither induce any IL1 secretion by itself, nor did it affect IL1 production by mononuclear leucocytes when these cells were stimulated with LPS (1pg to 10ng/ml) (data not shown).

Peripheral human monocytes, when kept in culture for 24 hrs in the absence of stimulants, lose their sensitivity to LPS as measured by induction of IL1. It has been shown that this process can be prevented or delayed by IFN-γ (13,14). The possibility was tested whether TNF might interfere with this in vitro differenti-

TABLE 1

IL1 Induction in Murine Peritoneal Exudate Cell Cultures

stimulus[a]	IL1 (titer)[b] C3H/He cells	C3H/HeJ cells
A) LPS 10µg	24	<4
TNF 10µg	6	<4
1µg	4	<4
0.1µg	<4	<4
none	<4	<4
B) LPS 50µg	64	<4
TNF 10µg	32	<4
inactivated TNF 10µg	32	<4
Staph. aureus	>512	256
none	16	<4

[a] Peritoneal exudate cells ($2x10^6$/ml) of either C3H/He or C3H/HeJ mice were cultured in the presence of either LPS (S. montevideo SH94, 10 or 50µg/ml), TNF (0.1-10µg/ml as indicated), heat inactivated TNF which was kept at 100°C for 10 minutes (10µg/ ml), Staph. aureus cells (Pansorbin 0.1% (w/v)) or no stimulus.
[b] IL1 activity in the supernatant was determined in the thymocyte costimulator assay.

TABLE 2

Influence of TNF on the IL1 Activity Generated in Monocyte Cultures

TNF/ml	IL1 (titer) Addition to the cultures[a] 0	IFN-γ	LPS
10 µg	<4	<4	64
1 µg	<4	<4	64
0.1 µg	<4	<4	32
10 ng	<4	<4	64
1 ng	<4	<4	64
0.1 ng	<4	<4	64
0.03 ng	<4	<4	64
0.003ng	<4	<4	64
none	<4	<4	64

[a] IL1 activity in supernatants of adherent human peripheral mononuclear leucocyte cultures ($3x10^5$/ml) was determined in the thymocyte costimulator assay after 24 h. The cultures contained the indicated amounts of TNF in the presence or absence of either IFN-γ (300 U/ml) or LPS from S.typh. (100ng/ml).

ation process in a similar way as IFN-γ. Table 4 shows that this was not the case. Preincubation for 24 h at 37°C of enriched monocytes led to nearly complete abrogation of IL1 production during a subsequent 24 hrs LPS-stimulation period (column B). Preincubation at 4°C had no such effect, i.e. left IL1 inducibility intact. Preincubation at 37°C in the presence of IFN-γ was also able to sustain optimal IL1 inducibility. In contrast, TNF 10µg -0.1ng/ml could not sustain IL1 inducibility, nor did it adversely affect IL1 production by responsive monocytes. Monocyte viability was not influenced by incubation at 37°C and there was no IL1 inhibitor detectable in supernatants of cultured cells (data not shown).

DISCUSSION

TNF is a typical mediator of endotoxin effects (5,11). It induces a number of symptoms seen in animals with bacterial infections or other metabolic disorders. The identity of TNF with cachectin and the knowledge of cachectin function shed some light on the molecular mechanism of the metabolic derangement seen in cachectic animals (15). Cachectin is a mediator responsible for weight loss and

TABLE 3

IL1 Activity Generated in Different Culture Systems

cells[a]	IL1 (titer) LPS (ng/ml) 100	1	0.1	0.01	0.001	0
MNL	8	4	4	<4	<4	<4
adherent cells	32	16	16	4	<4	<4
adherent cells + IFN-γ	32	32	16	16	8	<4

[a] Peripheral human mononuclear leucocytes ($2x10^6$/ml) containing 15% monocytes or the adherent cell fraction ($3x10^5$/ml) containing 90-95% monocytes were cultured with or without human IFN-γ (300 U/ml) and the indicated amount of LPS from S.typh. Numbers given in the Table represent titers of IL1 measured in 24 hrs supernatants by the thymocyte costimulator assay.

TABLE 4

Influence of TNF preculture of human monocytes on the IL1 activity generated by LPS

Addition to the cultures[a]	IL1 (titer) after preculture A none	B 24h 37°C	C 24h 4°C
TNF 10 µg/ml	256	4	256
1 µg/ml	256	4	256
100 ng/ml	256	4	256
10 ng/ml	256	4	256
1 ng/ml	256	4	256
0.1 ng/ml	256	4	256
γ-IFN 100 U/ml	256	256	256
0	256	4	256

[a] Peripheral human adherent mononuclear leucocytes ($3x10^5$/ml) were cultured with the indicated amount of TNF or human IFN-γ (100U/ ml). The cultures were stimulated with LPS from S.typh. (10ng/ml) either immediately at the onset of cultures (A), after 24h of preculture at 37°C (B), or after 24h of preculture at 4°C (C). IL1 activity was determined in the supernatants 24h after LPS stimulation in the thymocyte costimulator assay.

wasting of the body's energy reserves apparently due to reduced lipoprotein lipase production and function (16). Passive immunization with antibodies to TNF can shift the LD50 of bacterial endotoxin in mice to higher doses of LPS, thus indicating the involvement of TNF/cachectin in the lethal effects of endotoxin (17). Also, the typical pattern of plasma enzyme levels which is seen after endotoxin application can be obtained with TNF injections (11). Since TNF is not the only mediator released in an organism after endotoxin exposure it is important to investigate whether all endotoxin effects can be mediated by one single mediator like TNF. Alternatively, several different mediators could be induced which together are responsible for the endotoxic reaction or different mediators could be elicited with identical or similar functions.

The hypothermic reaction of mice after endotoxin application is a typical in vivo effect of LPS which can easily be measured. TNF injection of mice also induced hypothermia (Fig.1). This hypothermic reaction was not the result of contaminating LPS in the TNF preparation since the hypothermia was also induced by TNF in LPS low responder C3H/HeJ animals. Mediation of the fever reaction of LPS in most species is supposedly due to induction of IL1-release in macrophages (4). Purified recombinant IL1 is pyrogenic in rabbits and qualifies as endogenous pyrogen (4). The same has been described for TNF (18). Because of the overlapping effects of IL1 and TNF it seemed important to us to determine whether these TNF effects were direct effects of TNF or whether they were mediated via the endogenous induction of IL1 production. The observation that TNF did not interfere with IL1 test systems neither on the level of IL2 production nor on the level of IL2 receptor induction in thymocytes made it possible to measure IL1 without having to remove TNF from the test samples.

We did not detect IL1 activity in the serum of animals 1 h to 24 h after they had received large amounts of TNF and showed a strong hypothermic reaction. PEC cultures from LPS responder mice stimulated with graded amounts of TNF produced low amounts of IL1 activity, especially when background levels of IL1 activity were already seen without stimulation. These observations support the notion that TNF might induce a factor(s) which synergizes with IL1 in the costimulator assay (19). But also a biologically inactive, heat inactivated TNF preparation was still able to induce the same IL1 response. IL1 production was not induced when cultures containing peritoneal macrophages of LPS-low responder C3H/HeJ mice were stimulated with the same amounts of TNF. Addition of indomethacin to block prostaglandin production that is known to inhibit IL1 production did also not lead to higher IL1 activity (data not shown).

In culture systems with human cells no IL1 production was induced by TNF under a variety of culture conditions. Special care was taken to use reagents i.e. fetal calf serum in these systems which were tested previously to show that they were functionally LPS-free and did not induce any detectable background production (9). Cultures of enriched monocytes could not be induced by TNF in a wide dose range to produce IL1. Such cultures of enriched monocytes respond with measurable IL1 production to as little as 1pg LPS/ml, depending on the sensitivity of the individual donor (9). The sensitivity for LPS can be further increased by IFN-γ. Still, even in the presence of IFN-γ, TNF could not induce IL1 secretion. On the other hand, TNF did not induce any IL1 inhibitor, as well, nor did it interfere with LPS-induced IL1 production in any sense.

Although unpurified mononuclear cells are usually less effective in generating IL1 than equal amounts of purified monocytes, it could not be excluded that TNF might stimulate IL1 secretion indirectly by acting on lymphocytes. The lymphocytes might secrete an IL-1 inducing activity in response to TNF. This possibility can, however, be dismissed, since unpurified mononuclear cells were also not induced by TNF to produce IL1.

IFN-γ is known to sustain the IL1-inducibility by LPS in monocyte cultures after preculture at 37°C. TNF had no comparable effect on modulation of IL1 secretion. Thus, under a variety of stimulation protocols TNF did neither enhance nor reduce the IL1 activity generated by monocytes within 24 h.

All our experimental evidence points to a direct IL1-independent activity of TNF when applied in vivo. Our observations, therefore, support the data of Dinarello et al. who showed a direct pyrogenic effect of TNF in rabbits (18). Different findings have been reported to the question whether TNF induces IL1 production in monocyte/macrophage cultures (19,20,21). The different results by Hoffmann (19) can be explained by the fact that in these studies cell-associated IL1 activity and not released IL1 was measured.

The activation of murine thymocytes for IL2 production and proliferation seems to be the specific action of IL1 and not shared by TNF inspite of the wide overlap of the biological activities of the two macrophage products. According to the presented data TNF does not induce stimulation for IL1 secretion by monocytes/macrophages. Therefore, the effects seen after endotoxin application in vivo seem to be the sum and the direct collaborative action of several mediators like TNF and IL1. These mediators are most likely induced at the same time and not sequentially by each other.

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