Relationships between tumour necrosis factor, eicosanoids and platelet-activating factor as mediators of endotoxin-induced shock in mice

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1 The toxicity of intravenous recombinant human tumour necrosis factor (rhTNF), a TNF fragment (TNF_{114–130}), endotoxin and combinations of rhTNF or TNF_{114–130} were tested in mice. Neither rhTNF nor TNF_{114–130} was lethal alone, but when combined with a non-lethal dose of endotoxin, rhTNF provoked dose-dependent mortality, as did higher doses of endotoxin alone.

2 Both the toxicity and the vasopermeability changes induced by endotoxin alone were blocked by the platelet-activating factor (PAF) antagonist BN52021, indomethacin or the dual cyclo-oxygenase/lipoxygenase inhibitor BW755C.

3 The lethality of the combined low dose endotoxin/rhTNF challenge was unaffected by pretreatment with BN52021, indomethacin or BW755C, or by treatment at 6 h intervals with BN52021 or BW755C.

4 The results of these studies suggest that TNF, a putative, early mediator of septic or endotoxin shock, cannot by itself mimic all of the effects of bacterial endotoxin in the model used in this study. Apparently, TNF works synergistically with other mediators whose release is stimulated by endotoxin.

5 The results also suggest that the mechanism of shock production by the rhTNF/endotoxin combination in mice is not dependent on the early stimulation of eicosanoid or PAF synthesis by rhTNF.

Introduction

Among the endogenous mediators of shock and lethality induced by i.v. injection of bacterial endotoxin, tumour necrosis factor (TNF), eicosanoids and platelet-activating factor (PAF) have been the focus of considerable recent attention. Pretreatment of experimental animals with TNF antibodies (Tracey *et al.*, 1987) or antagonists of thromboxane A₂ (Olanoff *et al.*, 1985), peptido-leukotrienes (Hagmann & Keppler, 1982) or PAF (Terashita *et al.*, 1985; Handley *et al.*, 1988) can block many of the pathophysiological sequelae of i.v. endotoxin or live *Escheria coli* injection. Further, i.v. injection of TNF (Tracey *et al.*, 1986; Kettelhut *et al.*, 1987; Satomi *et al.*, 1988), PAF (Lanara *et al.*, 1982; Myers *et al.*, 1983a) or thromboxane agonists (Myers *et al.*, 1983b) can, under some circumstances, produce shock and death.

Results of early studies of i.v. recombinant human TNF (rhTNF) suggested that this macrophage-derived cytokine was a primary mediator of endotoxin shock, and that it alone was capable of producing all of the major pathophysiology associated with endotoxaemia (Tracey *et al.*, 1986). As TNF can stimulate the release of lipid mediators such as PAF and eicosanoids from some cell types (Roubin *et al.*, 1987; Sun & Hsueh, 1988), the hypothesis has been developed that endotoxin-induced shock and lethality is largely mediated by the initial release of TNF from macrophages, followed by release of PAF and eicosanoids from various tissues, leading to the associated platelet activation, bronchoconstriction, hypotension, increased vascular permeability and eventually death. In fact, some studies have demonstrated that rhTNF induces shock and death in mice and rats which can be prevented by prior administration of cyclo-oxygenase inhibitors (Kettelhut *et al.*, 1987; Satomi *et al.*, 1988).

In contrast to studies which show a clear shock-producing effect of rhTNF alone, we have been unable to produce shock by i.v. injection of even large doses of rhTNF in either rats or mice (A.K. Myers & R.M. Price, unpublished results). Others have published similar findings (Rothstein & Schreiber, 1988), and suggest that a possible factor enhancing rhTNF toxicity

in some studies is contamination by endotoxin, although this cannot account for TNF toxicity in all studies. However, under circumstances in which rhTNF displays no toxicity, simultaneous administration of low dose endotoxin leads to death in mice (Rothstein & Schreiber, 1988).

In fact, many recent studies demonstrate synergistic relationships between TNF and bacterial lipopolysaccharide (Rothstein & Schreiber, 1988; Sun & Hsueh, 1988) or other cytokines such as interleukin-1 (Ruggiero & Baglioni, 1987; Waage & Espevik, 1988). Based on these and our own observations, we hypothesized that TNF alone is only minimally toxic in healthy animals and that interaction with other mediators is an essential component of its role in endotoxaemia. In the present study, we investigated the lethality in mice of i.v. rhTNF and a TNF fragment, alone and in combination with bacterial endotoxin, and compared the efficacy of various drugs in blocking the effects of endotoxin and TNF.

Methods

Animals

Female CD-1 mice weighing 18 to 20 g were purchased from Charles River Breeding Laboratories (Wilmington, MA, U.S.A.) and assigned randomly to the various groups below. Mice were anaesthetized by sodium amylal injection (100 mg kg⁻¹, i.p.) before challenge (below) with rhTNF, endotoxin or related compounds via a surgically exposed jugular vein. After completion of experiments, animals were killed by an overdose of pentobarbitone (i.p.).

Toxicity studies

Challenges consisted of i.v. endotoxin (*E. coli* serotype 0127:B8, Sigma), rhTNF (Cetus Corp., Emeryville, CA, U.S.A.), the TNF fragment, TNF_{114–130} (Pennisula Laboratories, Belmont, CA, U.S.A.) or a solution containing both rhTNF and lipopolysaccharide (LPS). Biological activity of our rhTNF samples was periodically confirmed in an L929 cell cytotoxicity assay (data not shown), with no loss of activity observed over the period in which these experiments were

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performed. Doses of the challenges are indicated below. In the mortality studies, each group consisted of at least 12 mice except where indicated below. Survival of animals was followed for 7 days in the studies presented in Figure 1. In later studies, based on these results, the observation period was limited to 48 h. Because death only rarely occurred after 24 h, 24 h mortality was compared between groups by the Chi-square statistic.

Vasopermeability studies

In further experiments, haematocrit measurements were made just before and 15 min after endotoxin challenge. Blood for the basal haematocrit was collected from a small incision in a rear foot pad; the final haematocrit sample was obtained from freely flowing blood from an incision in the exposed jugular vein. The latter method is necessary for collection of blood from mice in the shock state. The change in haematocrit was compared between various experimental and control groups by Student's *t* test for independent means. Each group consisted of at least 9 mice. Haematocrit was also determined in preliminary studies before and at various intervals after rhTNF and endotoxin challenge. However, at no time after this challenge was a consistent, large rise in haematocrit observed, so the experiment was not pursued.

Drug treatments

Some groups of mice in the experiments below were treated with the PAF antagonist (3-(1,1-dimethylethyl) hexahydro-1,4,7b-trihydroxy-8-methyl-9H-1,7 α (epoxymethano)-1H, 6 α H-cyclopenta [c] furo (2,3b) [3',2':3,4) cyclopenta-(1,2-d]furan-5,9,12(4H)-trione) (BN 52021) (Braquet *et al.*, 1985), the cyclo-oxygenase inhibitor indomethacin (Sigma Chemical Co., St. Louis, MO, U.S.A.), or the combination cyclo-oxygenase/lipoxygenase inhibitor (3-amino-1-[*m*-(trifluoromethyl)-phenyl]-2-pyrazoline) (BW 755C) (Higgs *et al.*, 1979) 30 to 45 min before challenge with rhTNF or endotoxin. Doses for these drugs were 40 mg kg⁻¹, 10 mg kg⁻¹ and 40 mg kg⁻¹, respectively, and were based on preliminary dose-finding studies in which they were tested against endotoxin lethality. All three drugs were dissolved in dimethyl sulphoxide (DMSO) at a concentration such that the i.p. injection volume was 2.5 μ l g⁻¹ body weight. Controls for these experimental groups received i.p. DMSO, 2.5 μ l g⁻¹ body weight. Each specific drug treatment group had a corresponding control group. In an additional experiment, the effects of repeated doses of BW 755C, BN 52021 or the DMSO vehicle were tested, with the first injection 30 to 45 min before challenge, and subsequent treatments at 6 h intervals for one day.

Results

Toxicity of rhTNF, TNF₁₁₄₋₁₃₀ and endotoxin

Among the tested compounds, only endotoxin produced mortality by itself (Figure 1). The response was dose-dependent, with an LD₅₀ of approximately 25 mg kg⁻¹. In contrast, neither rhTNF nor the synthetic TNF fragment, TNF₁₁₄₋₁₃₀, produced mortality at doses of up to 0.4 and 5.0 mg kg⁻¹, respectively. Mice injected with these compounds recovered normally from anaesthesia and appeared healthy for the entire 7 day observation period.

The addition of a dose of endotoxin (0.24 mg kg⁻¹), which by itself was well below the endotoxin dose-mortality curve, had a profound potentiating effect on rhTNF toxicity. This combination of rhTNF with a constant, low dose of endotoxin produced death with an LD₅₀ of approximately 0.15 mg kg⁻¹ rhTNF combined with 0.24 mg kg⁻¹ endotoxin (Figure 1). Unlike rhTNF, TNF₁₁₄₋₁₃₀, 5 mg kg⁻¹, was not toxic in combination with this dose of endotoxin.

Figure 1 Lethality of i.v. endotoxin (○), recombinant human tumour necrosis factor (TNF) (●), TNF₁₁₄₋₁₃₀ (□), and low dose endotoxin (0.24 mg kg⁻¹) in combination with TNF (■) or TNF₁₁₄₋₁₃₀ in mice (△). Neither TNF nor TNF₁₁₄₋₁₃₀ produced any mortality in the absence of endotoxin; the TNF fragment, at the one dose tested, did not provoke death in combination with low dose endotoxin. Each data point represents mortality in a group of at least 12 mice.

A notable difference was observed in time of death in these comparative studies. With the approximate LD₈₀ for the combination challenge, 0.4 mg kg⁻¹ rhTNF/0.24 mg kg⁻¹ endotoxin, 88% of the deaths occurred four or more hours after challenge. In contrast, 82% of deaths provoked by the approximate LD₈₀ of endotoxin (40 mg kg⁻¹) occurred in less than four hours after challenge.

Effects of drugs on endotoxin-induced mortality and haematocrit changes

When mice were pretreated with doses of BN 52021, indomethacin or BW 755C as stated in the Methods, mortality induced by an approximate LD₈₀ of endotoxin was significantly inhibited (Figure 2), with both the PAF antagonist and the joint cyclo-oxygenase/lipoxygenase inhibitor providing 100% protection. Similarly, the rise in haematocrit following endotoxin LD₈₀ administration was significantly attenuated by each of the three drugs (Figure 3).

Effects of drugs on the toxicity of a combination of rhTNF and low dose endotoxin

The rhTNF/low dose endotoxin combination (0.4 mg kg⁻¹ rhTNF/0.24 mg kg⁻¹ endotoxin) which resulted in approximately 80% mortality (Figure 1), was equiactive in the pre-

Figure 2 Effects of the PAF antagonist BN 52021, the cyclo-oxygenase inhibitor indomethacin, and the dual cyclo-oxygenase/lipoxygenase inhibitor BW 755C on 24 h mortality induced by an approximate LD₈₀ challenge of i.v. endotoxin. Each drug treatment group and corresponding control group consisted of at least 12 mice. Open columns, control groups; shaded columns, drug pretreated groups. **P* < 0.05; ***P* < 0.01 compared to the control group.

Figure 3 Rise in haematocrit (Hct) 15 min following an approximate LD_{50} endotoxin challenge in groups of mice pretreated with BN 52021, indomethacin, BW 755C or vehicle. Each treatment and the corresponding control group consisted of 9 mice. Open columns, control groups; shaded columns, drug pretreated groups. * $P < 0.05$ compared to the control group.

sence or absence of BN 52021, indomethacin or BW 755C pretreatment (Figure 4). Repeated administration of BW 755C or BN 52021, before challenge and at 6 h intervals after challenge, did not improve survival after rhTNF/low dose endotoxin (Table 1). The rhTNF/low dose endotoxin combination produced only a variable and modest rise in haematocrit compared to the lethal doses of endotoxin alone (data not shown). Based on this lack of a substantial rise in haematocrit, effects of indomethacin, BW 755C and BN 52021 were not examined.

Discussion

Under some circumstances, i.v. TNF can be highly toxic, producing lethal shock in both mice (Satomi *et al.*, 1988; Waage & Espevik, 1988) and rats (Tracey *et al.*, 1986; Kettelhut *et al.*, 1987). In our laboratories, and in others (Rothstein & Schreiber, 1988), rhTNF alone has failed to provoke lethality. Rothstein & Schreiber (1988) attributed the discrepancy to

Figure 4 Effects of BN 52021, indomethacin and BW 755C on 24 h mortality induced by an approximate LD_{50} challenge of combined i.v. recombinant human tumour necrosis factor (0.4 mg kg^{-1}) and low dose endotoxin (0.24 mg kg^{-1}). Each drug treatment group and corresponding control group consisted of at least 12 mice. Open columns, control groups; shaded columns, drug pretreated groups.

Table 1 Effects of BN52021 and BW755C given in repeated doses at 6 h intervals, on mortality induced by combined recombinant human tumour necrosis factor (0.4 mg kg^{-1}) and low dose endotoxin (0.24 mg kg^{-1}).

Treatment	Number of animals	Mortality (%)	
		12 h	24 h
Control	7	14	86
BN52021	6	33	67
BW755C	6	33	100

possible contamination of some rhTNF preparations by bacterial endotoxin, and showed that death could be produced by rhTNF when given simultaneously with low doses of endotoxin. Our studies confirm their experimental result. They failed to observe any lethality of rhTNF, even at $40 \mu\text{g}$ i.v. per mouse, but addition of a non-lethal dose of endotoxin resulted in dose-dependent death following rhTNF exposure. Interestingly, Waage & Espevik (1988), who found TNF to be toxic to mice at high doses, also observed great potentiation of TNF toxicity by interleukin-1 (IL-1), another cytokine which might be released by macrophages during sepsis. Waage *et al.* (1989) also correlated septic deaths in humans with increased release of IL-1 and IL-6.

Conflicts concerning the toxicity of TNF might also be attributed to a variety of other factors, including species and strain differences, condition of animals and differences in TNF preparations besides endotoxin level. It is also possible that the previous exposure of rodents to immunological challenges such as viral or bacterial infection or tumours might duplicate the priming effect of endotoxin. In addition, most studies of TNF toxicity in rodents, including the present one, have utilized rhTNF, which is more readily available than murine TNF. The experiments of Waage & Espevik (1988) involved murine recombinant TNF, and thus the one study of murine TNF-induced death in rodents suggests that it alone can produce lethal shock. Whether this result is specific to particular experimental conditions, as is apparently the case for rhTNF, is an important issue which should be addressed in further experiments and has obvious implications with respect to the current study.

The questions regarding TNF toxicity are also important from the standpoint of the role of TNF as a primary mediator of endotoxin-induced shock, capable of mimicking all of the major effects of endotoxin. Besides the apparently low toxicity of rhTNF alone in these experiments, other aspects of our studies suggest that TNF is probably not by itself a central, early mediator of endotoxin shock and demonstrate substantial differences between the pathophysiological effects of the two substances. First, rhTNF, even in combination with low dose endotoxin, was not capable of producing the rapid death which follows high dose endotoxin injection. Most of the mortality was expressed 4 or more hours after i.v. rhTNF/endotoxin challenge, whereas high dose endotoxin was lethal in most animals before this time. Second, rhTNF/low dose endotoxin-induced death was not blocked by previous treatment with indomethacin, BW 755C or the PAF antagonist, in contrast to the efficacy of these drugs against the effects of endotoxin. Apparently, endotoxin, directly or indirectly, stimulates the production of lipid mediators, which participate in its toxicity. Whether or not the rhTNF/endotoxin combination stimulates a similar, early production of PAF and eicosanoids, the toxicity of this combination is not dependent on such an event. The rapid, large elevation in haematocrit which follows endotoxin challenge was also mediated by PAF and eicosanoids, but a similar large rise in haematocrit was not seen with rhTNF/endotoxin. Further, Kiener *et al.* (1988) found that both toxic and non-toxic forms of Lipid A (the biologically active part of the structure of endotoxin) elicited similar increases in TNF secretion in mice. However, such increases in serum TNF levels were not uniformly associated with toxicity, leading the authors to suggest that 'although it is essential, TNF alone is not necessarily sufficient to induce septic shock in mice'.

In addition to the issue regarding the precise nature of the role of TNF in endotoxin shock, the question of mediation of the effects of TNF by PAF and eicosanoids is also of interest. Under those circumstances in which rhTNF alone produces lethal shock, its actions are probably mediated in part by lipid mediators, since some lipoxxygenase and cyclo-oxygenase inhibitors are protective (Kettelhut *et al.*, 1987; Satomi *et al.*, 1988). In our studies, rhTNF-induced death is not dependent on an early stimulation of lipid mediator production as occurs following endotoxin administration. Because it is quite pos-

sible that the BN 52021, BW 755C and indomethacin pretreatments had lost efficacy by the time of death, we also tested the effects of repeated dosing at 6 h intervals with two of these drugs which were most efficacious against endotoxin. Neither BN 52021 nor BW 755C displayed any protective action with repeated dosing against the combination of rhTNF and endotoxin. The main purpose of our experiments was to provide a basis for comparing the acute toxicity of rhTNF and endotoxin, and our results demonstrate a clear divergence in mice between the toxicity of endotoxin as opposed to rhTNF, the time of death following the challenge and the effects of the pretreatments.

The synthetic TNF fragment, TNF₁₁₄₋₁₃₀ was included in these studies because we wished to determine whether this fragment, which is commercially available and represents a highly conserved region of TNF, could produce effects similar to rhTNF and could thus be used to model TNF effects. Its failure to cause any obvious pathophysiology, alone or in combination with endotoxin, suggests that at least *in vivo*, a larger or different portion of the peptide is required for TNF activity.

References

- BRAQUET, P., ETIENNE, A., TOUVAY, S., BOURGAIN, R.H., LEFORT, J. & VARGAFTIG, B.B. (1985). Involvement of platelet-activating factor in respiratory anaphylaxis, demonstrated by PAF-acether inhibitor BN52021. *Lancet*, **i**, 1501.
- HAGMANN, W. & KEPPLER, D. (1988). Leukotriene antagonists prevent endotoxin lethality. *Naturwissenschaften*, **69S**, 594-595.
- HANDLEY, D.A., VAN VALEN R.G., MELDEN, M.K., HOULIHAN, W.J. & SAUNDERS, R.N. (1988). Biological effects of the orally active platelet activating factor receptor antagonist SDZ 64-413. *J. Pharmacol. Exp. Ther.*, **247**, 617-623.
- HIGGS, O.A., FLOWER, R.J. & VANE, J.R. (1979). A new approach to anti-inflammatory drugs. *Biochem. Pharmacol.*, **28**, 1959-1961.
- KIENER, P.A., MAREK, F., RODGERS, G., LIN, P.-F., WARR, G. & DESIDERIO, J. (1988). Induction of tumor necrosis factor, IFN- γ , and acute lethality in mice by toxic and non-toxic forms of lipid A. *J. Immunol.*, **141**, 870-874.
- KETTELHUT, I.C., FIER, W. & GOLDBERG, A.L. (1987). The toxic effects of tumor necrosis factor *in vivo* and their prevention by cyclooxygenase inhibitors. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 4273-4277.
- LANARA, E., VAKIRTZI-LEMONIAS, C., KRITIKOU, L. & DEMOPOULOS, C.A. (1982). Response of mice and mouse platelets to acetyl glyceryl ether phosphorylcholine. *Biochem. Biophys. Res. Commun.*, **109**, 1148-1156.
- MYERS, A., RAMEY, E. & RAMWELL, P. (1983a). Glucocorticoid protection against Paf-acether toxicity in mice. *Br. J. Pharmacol.*, **79**, 595-598.
- MYERS, A., PENHOS, J., RAMEY, E. & RAMWELL, P. (1983b). Thromboxane agonism and antagonism in a mouse sudden death model. *J. Pharmacol. Exp. Ther.*, **224**, 369-372.
- OLANOFF, L.S., COOK, J.A., ELLER, T., KNAPP, D.R., & HALUSHKA, P.V. (1985). Protective effects of trans-13-APT, a thromboxane receptor antagonist, in endotoxemia. *J. Cardiovasc. Pharmacol.*, **7**, 114-120.
- ROTHSTEIN, J.L. & SCHREIBER, H. (1988). Synergy between tumor necrosis factor and bacterial products causes hemorrhagic necrosis and lethal shock in normal mice. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 607-611.
- Based on these results, we propose that rhTNF toxicity is minimal in healthy, uncompromised rodents. The role of TNF in endotoxin-induced shock, while obviously important, probably involves synergism with other mediators, including interleukin 1, which is known to act synergistically with TNF in some systems (Ruggiero & Baglioni, 1987; Waage & Espevik, 1988). The pathophysiology of high dose endotoxin exposure apparently differs from that of rhTNF in terms of rapidity of onset and degree of dependence on early production of lipid mediators. We suggest that high dose, i.v. endotoxin-induced shock might differ from other models of sepsis in this regard, in that the cardiopulmonary alterations and lethality can be provoked in a time frame which might not allow the full production and interaction of all of the many mediators of sepsis, but instead relies heavily on a particular class of mediators, the eicosanoids and PAF.
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- ROUBIN, R., ELSAS, P.P., FIER, W. & DESSEIN, A.J. (1987). Recombinant human tumor necrosis factor (rTNF) enhances leukotriene biosynthesis in neutrophils and eosinophils stimulated with the Ca^{2+} ionophore A23187. *Clin. Exp. Immunol.*, **70**, 484-490.
- RUGGIERO, V. & BAGLIONI, C. (1987). Synergistic anti-proliferative activity of interleukin 1 and tumor necrosis factor. *J. Immunol.*, **138**, 661-663.
- SATOMI, N., SAKURAI, A., HARANAKA, R. & HARANAKA, K. (1988). Preventive effects of several chemicals against lethality of recombinant human tumor necrosis factor. *J. Biol. Resp. Modifiers*, **7**, 54-64.
- SUN, X.-M. & HSUEH, W. (1988). Bowel necrosis induced by tumor necrosis factor in rats is mediated by platelet-activating factor. *J. Clin. Invest.*, **81**, 1328-1331.
- TERASHITA, Z.-I., IMURA, Y., NISHIKAWA, K. & SUMIDA, S. (1985). Is platelet activating factor (PAF) a mediator of endotoxin shock? *Eur. J. Pharmacol.*, **109**, 257-261.
- TRACEY, K.J., BEUTLER, B., LOWRY, S.F., MERRYWEATHER, J., WOLPE, S., MILSARK, I.W., HARIRI, R.J., FAHEY, T.J., III, ZENTELLA, A., ALBERT, J.D., SHIRES, G.T. & CERAMI, A. (1986). Shock and tissue injury induced by recombinant human cachectin. *Science*, **234**, 470-474.
- TRACEY, K.J., FONG, Y., HESSE, D.G., MANOGUE, K.R., LEE, A.T., KUO, G.C., LOWRY, S.F. & CERAMI, A. (1987). Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteremia. *Nature*, **330**, 662-664.
- WAAGE, A. & ESPEVIK, T. (1988). Interleukin 1 potentiates the lethal effect of tumor necrosis factor α /cachectin in mice. *J. Exp. Med.*, **167**, 1987-1992.
- WAAGE, A., BRANDTZAEG, P., HALSTENSEN, A., KIERULL, P. & ESPEVIK, T. (1989). The complex pattern of cytokines in serum from patients with meningococcal septic shock. Association between interleukin 6, interleukin 1 and fatal outcome. *J. Exp. Med.*, **169**, 333-338.

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