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Platelet-activating factor and endotoxin induce tumour necrosis factor gene expression in rat intestine and liver

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SUMMARY

We have shown previously that endotoxin, tumour necrosis factor (TNF) and platelet-activating factor (PAF) are important in the pathogenesis of bowel injury, and that endotoxin and TNF induce PAF formation in bowel tissue. In the present study we investigated the effects of endotoxin and PAF on TNF gene expression. Adult rats were injected with endotoxin (2 mg/kg), PAF (1 μg/kg) or endotoxin plus PAF, and were killed after 30 min. Endotoxin had little systemic effect. PAF induced transient hypotension and mild bowel injury. Endotoxin plus PAF caused profound shock, severe haemoconcentration, leukopenia and intestinal necrosis. Sham-operated rats had barely detectable TNF mRNA in the liver or intestine. Endotoxin or PAF induced a marked increase in TNF mRNA, especially in the distal ileum and in the liver, but much less in the jejunum. Endotoxin plus PAF did not further increase TNF mRNA, probably due to development of tissue injury. Serum TNF levels in animals treated with endotoxin, PAF and endotoxin plus PAF were elevated. Endotoxin induces TNF gene expression probably via both PAF-dependent and PAF-independent pathways, since TNF mRNA formation was only partially blocked by PAF antagonist.

INTRODUCTION

Tumour necrosis factor-α (TNF), an inflammatory peptide mediator that plays a pivotal role in endotoxin shock (reviewed in refs 1 and 2), has been shown to induce gastrointestinal injury in animals.[3] *In vitro* experiments have shown that the major producer of TNF is the mononuclear phagocyte.[1,2] Transcription and translation of this cytokine are markedly increased following stimulation with lipopolysaccharide (LPS).[4] Recent studies have suggested that, *in vivo*, the splanchnic organs, including the liver, are the major source of TNF.[5]

Platelet-activating factor (PAF-acether; PAF) is an endogenous phospholipid mediator of endotoxin shock (reviewed in refs 6 and 7). We have previously demonstrated that systemic administration of endotoxin (LPS)[8] or PAF[9] induces intestinal injury in the rat, and the effects of these two agents are synergistic.[9] More recently, we showed that LPS and TNF synergize to induce intestinal injury in rats[10] and mice.[11]

It is intriguing that the intestine would be the target organ of injury in response to agents with widespread effects, such as LPS, PAF and TNF. It is possible that high local production of inflammatory mediators in the gut facilitates the selective injury in this organ. We have previously shown that intestinal tissue is capable of producing PAF in response to LPS[8] and TNF.[10] It has been shown that the liver can produce TNF following LPS injection[5] or ischaemia/reperfusion injury.[12] However, it remains elusive whether the gut tissue can produce TNF during injury. Since local production of this endogenous cytokine may be important in the pathogenesis of intestinal injury, we deemed it important to investigate: (1) whether LPS induces TNF gene expression in the intestine and liver; (2) whether PAF induces TNF mRNA formation in the intestine and liver and causes TNF production; and (3) whether the LPS effect depends on endogenous PAF production.

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Abbreviations: Hct, haematocrit; LPS, lipopolysaccharide; NEC, necrotizing enterocolitis; PAF, platelet-activating factor; TNF, tumour necrosis factor-α.

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MATERIALS AND METHODS

Lipopolysaccharides

LPS (from *Salmonella typhosa*) and PAF (1-*O*-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine) were purchased from Sigma (St Louis, MO). PAF was prepared fresh in albumin/saline (5 mg/ml) solution.

A murine (mu)-TNF cDNA-containing pUC9 plasmid (a gift from Dr B. Beutler, University of Texas, Dallas, TX) was transformed into J109 *Escherichia coli* cells. The TNF cDNA was inserted into the pUC9 plasmid at the *Pst*1 and *Bam*H1 restriction sites. However, since the insert contains a portion of the 3′-untranslated region of the TTATTTATT consensus sequence that is also present in interleukin-1 (IL-1) and other cytokines,[13] *Pst*1 and *Eco*R1 were used to cut the insert off the plasmid. This is because *Eco*R1 cleaves off this portion of

consensus sequence, which might result in cross-hybridization with other cytokine transcripts.

Adult male Sprague–Dawley rats (approximately 200 g) were used. The carotid artery and the jugular vein were catheterized for continuous blood pressure recording and drug injection. The rats were divided into five groups: (1) sham-operated; (2) 1 μg/kg PAF; (3) 2 mg/kg LPS; (4) LPS (2 mg/kg) followed immediately by PAF (1 μg/kg); and (5) WEB 2086 (1 mg/kg; a PAF antagonist; a gift from Dr H. Heuer, Boehringer Ingelheim, Mainz, Germany) 30 min before LPS (2 mg/kg). Low doses of PAF (1 μg/kg) and LPS (2 mg/kg) and a short experimental period (30 min) were used to avoid the development of shock and extensive intestinal necrosis, which may result in mRNA degradation. Thirty minutes after PAF injection, blood samples were collected for white blood cells (WBC), haematocrit (Hct) and TNF assay. The small intestine was divided into four portions, and a 10-cm segment was taken from each portion. After rinsing the lumen with diethyl-pyrocarbonate (DEPC)–water, the tissue was immediately frozen in liquid nitrogen. A piece of liver (1 g) was also frozen and stored. The remainder of the small bowel was fixed in formalin and processed for histological examination.

Isolation of mRNA and Northern hybridization

Total RNA was extracted with 4 M guanidine isothiocyanate by a single-step method.[14] Preliminary experiments using total RNA from approximately 50 intestinal samples of control and LPS (2 or 20 mg/kg)-treated rats for Northern hybridization failed to detect TNF transcripts. Thus, poly(A)$^+$ RNA was prepared from total RNA by column chromatography on oligo-deoxythymidine cellulose (Gibco BRL, Life Technologies Inc., Gaitherburg, MD). Ten micrograms of glyoxal-denatured poly(A)$^+$ mRNA was fractionized in 1% agarose gel, transferred to a nylon membrane, and hybridized with [^{32}P]-labelled mu-TNF cDNA probe.[15] The mu-TNF-α cDNA (1 kb *Eco*R1/*Pst*1 fragment) was labelled with α-[^{32}P]-dCTP (Amersham, Arlington Heights, IL) to a specific activity of 10^9 c.p.m./μg DNA using a random priming method.[16] The blots were exposed for autoradiography for 3–7 days at −70°. A [^{32}P]-labelled β-actin probe was used as a negative control in a second hybridization of the same Northern blot after removing the TNF cDNA probe from the nylon membrane. The transcripts were quantified by a Bio-Rad Imaging GS densitometer, model 620, and analysed by computer software 1-D Analyst II (Bio-Rad, Richmond, CA).

Serum TNF assays

Serum TNF levels were assayed by using a TNF-sensitive 1591 RE 3.5 cell line cloned from a UV-induced fibrosarcoma, as previously described.[17] The cells were maintained in minimum essential medium containing 10% fetal bovine serum. The cells were detached from the flask, washed, counted, and plated on 96-well plates (5×10^3 cells/well) to which 100 μl of serially diluted serum samples had been previously added. (The highest concentration was 1 : 10 dilution, and the assay was done in duplicate.) After a 48-hr incubation at 37°, 7·5% CO_2, cytotoxicity was assessed by impaired uptake of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Due to the lack of a rat TNF standard, TNF quantity is expressed as the percentage of cell killing.

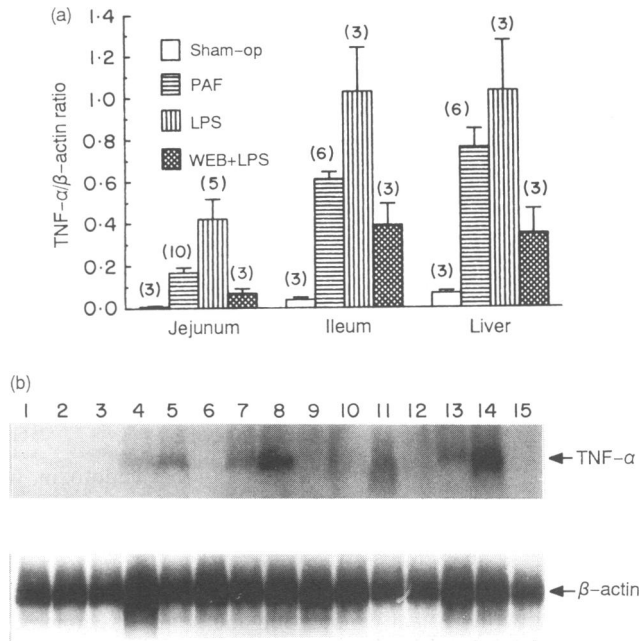

Figure 1. (a) Increase of TNF mRNA in jejunum, ileum and liver 30 min following injection of PAF (1 μg/kg), LPS (2 mg/kg) and WEB 2086 (1 mg/kg) plus LPS. (WEB: WEB 2086, a PAF antagonist). TNF transcripts were analysed by Northern blots, which were subsequently quantified by densitometry. Numbers in the parentheses are numbers of experiments. (b) A typical Northern blot: lanes 1–3, sham-operated; lanes 4–6, LPS (2 mg/kg); lanes 7–9, PAF (1 μg/kg); lanes 10–12, LPS + PAF; lanes 13–15, WEB 2086 + LPS; lanes 1, 4, 7, 10 and 13, distal ileum; lanes 2, 5, 8, 11 and 14, liver; lanes 3, 6, 9, 12 and 15, jejunum.

RESULTS

Preliminary experiments using total RNA from 50 control rats and rats injected with 2 or 20 mg/kg LPS (intravenously) at 15, 30 and 60 min failed to show any significant amount of TNF transcripts. Thus, poly(A)$^+$ mRNA was prepared in all subsequent experiments and presented in the figures.

Control rats had a very low (barely discernable) level of TNF mRNA in the tissues examined (Fig. 1). In contrast, 30 min following LPS (2 mg/kg) or PAF, there was a marked increase in TNF mRNA in the distal ileum (Fig. 1; $P < 0{\cdot}05$, which gradually diminished at 60 min (data not shown). This PAF- or LPS-induced response, i.e. TNF mRNA production, was less marked in the proximal ileum (data not shown), and was lowest in the proximal and distal jejunum (Fig. 1). Combined treatment of LPS (2 mg/kg) and PAF did not further increase TNF mRNA level in the bowel (Fig. 1b). A marked variation in the amount of TNF mRNA (in some animals undetectable) was observed in this group (data not shown). This was probably due to the extensive and severe necrosis of the small intestine (see Table 1). The liver is a rich source of TNF; TNF mRNA was markedly elevated in response to LPS or TNF (Fig. 1; $P < 0{\cdot}05$). The response of serum TNF protein levels was similar to that of the tissue TNF mRNA contents (Fig. 2). Sham-operated rats did not have detectable serum TNF. Administration of either LPS or PAF caused an elevated serum TNF level, as shown in Fig. 2. Combined

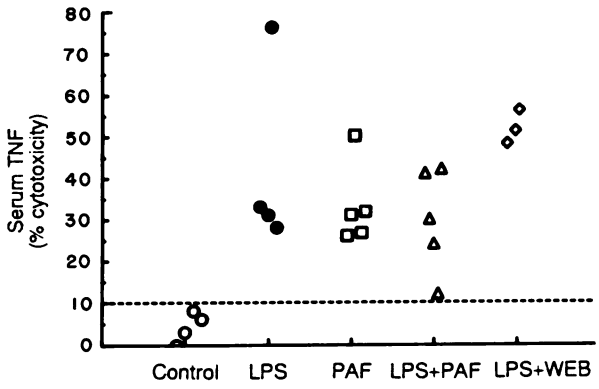

Figure 2. Serum TNF (expressed as percentage cytotoxicity) following various treatments (30 min). (TNF concentration = 0 U/ml when cytotoxicity < 10%, according to a standard curve constructed with recombinant human TNF.)

treatment of LPS and PAF did not result in TNF levels higher than those induced by either agent alone.

The LPS-induced TNF production was not affected by pretreatment with WEB 2086 (Fig. 2). However, TNF mRNA production in response to LPS was partially inhibited by PAF antagonist (Fig. 1).

The effects of PAF, LPS, and PAF plus LPS on blood pressure, WBC and Hct are shown on Figs 3 and 4. LPS alone did not cause any significant changes in the systemic blood pressure or Hct. Administration of PAF caused transient hypotension. PAF combined with LPS induced profound shock throughout the entire 30-min experimental period (Fig. 3). These two groups also manifested marked haemoconcentration ($P < 0{\cdot}001$ compared to the control; Fig. 4a). Injection of LPS (2 mg/kg) or LPS plus PAF resulted in leukopenia ($P < 0{\cdot}01$ and $P < 0{\cdot}001$ respectively, compared with control; Fig. 4b). The LPS effect on WBC was not blocked by PAF antagonist.

PAF induced widespread congestion of the small intestine in all animals. Eight of the 10 rats in this group also developed mild focal (< 10% of the entire length) necrosis, frequently in the distal ileum. Combined treatment with LPS and PAF caused extensive (often > 50% of the length), severe necrosis of the small bowel, also more frequently in the ileum. Confirmation of necrosis and assessment of its severity was performed by microscopic examination (Table 1). No changes in blood pressure, Hct or WBC was observed in sham-operated animals; nor was there any intestinal injury.

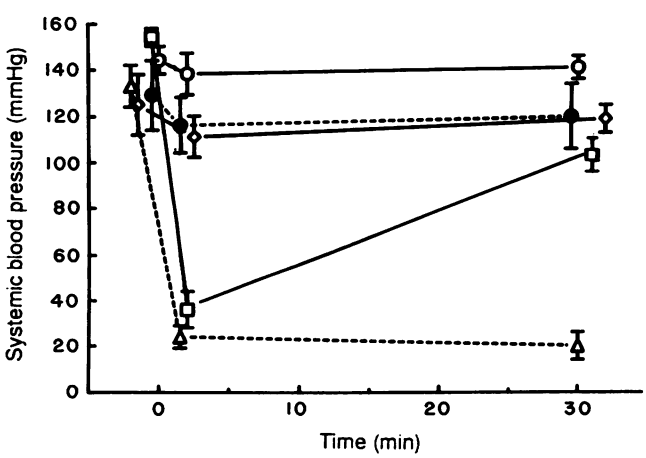

Figure 3. Change in arterial blood pressure. (○), sham-operated ($n = 7$); (●), LPS (2 mg/kg; $n = 4$); (□), PAF (1 μg/kg; $n = 10$); (△), LPS + PAF ($n = 5$); (◇), WEB 2086 (1 mg/kg) + LPS ($n = 3$).

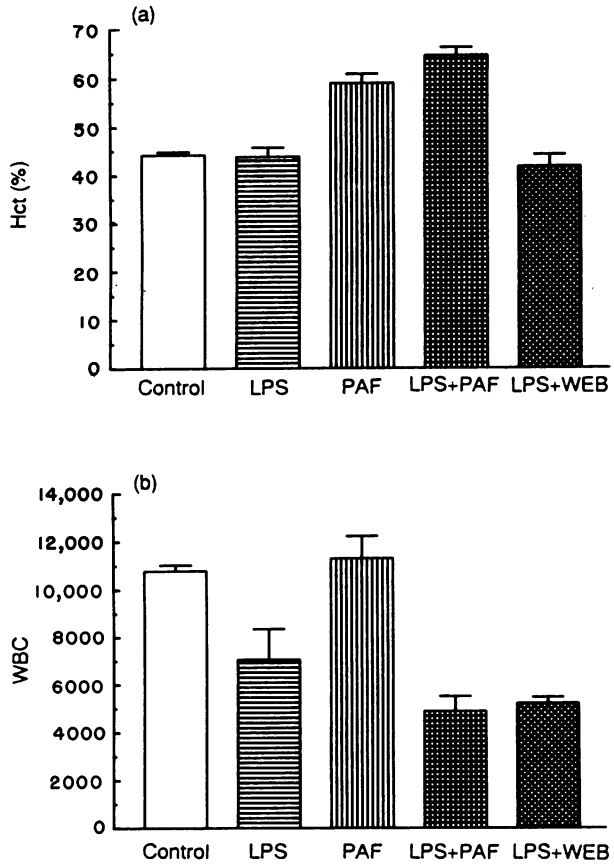

Figure 4. (a) Changes in haematocrit (30 min), and (b) WBC count (30 min) following various treatments. (See Fig. 3 legend for number of animals and dosage.)

Table 1. Histological changes of the small intestine

Treatment	No. of animals	No. with necrosis		
		Minimal*	Mild†	Moderate‡
Saline	7	1	0	0
LPS (2 mg/kg)	4	1	2	0
PAF (1 μg/kg)	5	0	4	1
LPS + PAF	5	0	0	5
WEB + LPS	3	1	1	0

* Focal detachment of epithelial cells from tip of villi.

† Loss of epithelial cells or ischeamic necrosis confined to top third of villi.

‡ Necrosis involving more than one-third of villi, but not extending beyond mucosa.

DISCUSSION

TNF, a cytokine produced predominantly by the mononuclear phagocytes,[1,2] has many proinflammatory actions, such as activation of polymorphonuclear leucocytes (PMN)[18] and endothelial cells,[19] induction of endothelial and leucocyte adhesive molecules,[20] transendothelial neutrophil migration,[21] production of other cytokines[2] and lipid mediators such as prostaglandins, thromboxane,[22] leukotrienes[23] and PAF.[24,25] TNF is also a pivotal mediator of endotoxin shock[1-3,26] and gastrointestinal injury.[3] Our previous study showed that TNF and LPS synergize to induce bowel necrosis.[11] Furthermore, human patients with necrotizing enterocolitis (NEC) have elevated serum TNF levels.[27] Thus, it is likely that TNF plays a role in the pathophysiology of intestinal injury during shock, sepsis or NEC.

It is reported that the splanchnic bed, including liver, is a major source of TNF production.[5] However, it is not clear whether the intestine is a major contributor of this cytokine. TNF gene expression and protein production is enhanced by a variety of agents, among which LPS is by far the most potent *in vitro*[1,2] and *in vivo*.[28] Recent reports also indicate that PAF, a potent inflammatory mediator, induces TNF production in monocytes,[29] and treatment with a PAF antagonist reduces LPS-induced TNF production in macrophages.[30] However, the effect of PAF on TNF production *in vivo* is unclear. Treatment with a PAF antagonist has been reported to either have no effect[30,31] or decrease[32] the LPS-induced elevation of serum TNF. PAF induces shock[6,7] and bowel injury.[9] In the present study, we showed that PAF, at a dosage causing transient hypotension and mild bowel injury, also induces TNF gene expression in the intestine and liver, and increases circulating TNF levels.

TNF is not stored within cells and tissues. Thus, the TNF produced appears to be synthesized *de novo*. However, a large amount of TNF mRNA was detected in cultured macrophages,[33] especially in macrophages pretreated with dexamethasone. It has been reported that although no circulating TNF is detected, TNF mRNA is also present in abundance in many organs, including the liver and intestines.[34] These results are discordant with our observation that TNF mRNA is barely detectable in unstimulated rats, and only after stimulation with LPS or PAF is tissue TNF gene expressed. Interestingly, TNF expression was confined to the distal portion of the ileum, whereas the jejunum contained little TNF mRNA. The pathophysiological significance of this preferential localization is unclear. It is known that in animals injected with LPS and/or PAF, as well as in human NEC patients, necrosis is frequently observed in the ileum, and less so in jejunum. Since injury of the small intestine, especially ileum, develops in the absence of sustained hypotension, it is possible that local production of inflammatory mediators such as TNF may play an important role. We have shown, by *in situ* hybridization, that TNF transcripts markedly increase in Paneth cells, lamina propria eosinophils and infiltrating macrophages, of the ileum of NEC patients.[35] Normal individuals, in contrast, have a minimal amount of TNF mRNA detected only in Paneth cells. These findings support a role of TNF in the pathogenesis of NEC.

Our previous study has shown that LPS induces PAF formation in the intestine.[8] The present study suggests that PAF induced TNF gene expression in this organ. Thus, it is possible that the effect of LPS on TNF mRNA formation is via PAF formation. The observations that PAF antagonist had no effect on TNF release and only partially blocked TNF mRNA production indicate that LPS induces TNF formation via both PAF-dependent and PAF-independent pathways. The present study also shows that LPS and PAF synergized to produce profound shock, haemoconcentration and severe bowel necrosis. However, the expression of TNF gene was not further enhanced in these animals. This was probably due to the tissue destruction brought about by the combination of the two agents, which in turn caused mRNA degradation. Alternatively, the increase in TNF production was not due to an increase in transcription, but was dependent on a post-transcriptional event. Regulation of TNF production *in vitro* is known to be predominantly post-transcriptional.[2] A final possibility is that the synergistic effect of LPS and PAF is unrelated to TNF production. We have shown that LPS and TNF induce intestinal PAF production,[8,10] and LPS and PAF induce intestinal TNF transcription and production. This positive feedback mechanism of mediator regulation may provide the prime impetus for development of intestinal injury in septic shock.

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