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# The selective augmentation by recombinant human tumour necrosis factor-alpha of neutrophil responses to pathogenic *Escherichia coli*

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## SUMMARY

Endotoxin release may amplify the neutrophil (PMN) responses to bacterial infection through the release of monocyte-derived tumour necrosis factor (TNF). The present study was designed to assess the effect of recombinant human TNF-α (rhTNF-α) on the *in vitro* response of human PMN to two defined strains of pathogenic *Escherichia coli*. In the absence of rhTNF-α, a P-fimbriate strain caused significant release of the PMN secondary granule marker vitamin B12-binding protein (B12 BP), and a low level of release of leukotriene B4 ($LTB_4$). Type 1-fimbriate strain 504, however, stimulated the release of the primary granule marker myeloperoxidase (MPO) and PMN chemiluminescence (CL), in addition to B12 BP and $LTB_4$ release. Following rhTNF-α ($10^{-9}$ M) pretreatment, the release of $LTB_4$ by PMN stimulated with the P-fimbriate strain was synergistically augmented, while B12 BP and MPO release were additively increased. In contrast, rhTNF-α did not significantly affect any of the responses by the type 1-fimbriate strain. These results suggest selectivity in the priming of PMN by rhTNF-α and confirm the independence of PMN responses to phagocytic stimuli.

Successful microbial colonization and infection is controlled by the expression of a variety of virulence factors (Harber, Topley & Asscher, 1986). In particular, the colonization of the urinary tract, which precedes acute *E. coli* infection, is dependent upon the expression of proteinacious adhesins (P fimbriae) of a type which bind specifically to the carbohydrate structure of the P blood group antigen expressed on the uroepithelium (Kallenius *et al.*, 1980). In contrast, it is the expression of type 1 fimbriae (with a specificity for cell-surface mannoside residues) that is significantly correlated with the formation of scars in an animal model of chronic infection. Those bacteria expressing P fimbriae, although initiating a neutrophil (PMN) infiltrate of similar magnitude, do not cause significant scar formation (Topley *et al.*, 1989). The scarring in this model is entirely dependent on PMN activation (Slotki & Asscher, 1982; Harber *et al.*, 1986). In addition, the *in vitro* activation of the human PMN respiratory burst (Svanborg Edén *et al.*, 1984; Topley *et al.*, 1989), comprehensive degranulation (Steadman *et al.*, 1988) and phagocytosis (Blumenstock & Jann, 1982) in response to *E. coli* are dependent on type 1 fimbrial expression. The release and metabolism of arachidonic acid to leukotrienes, however, is initiated by a mechanism involving the cytotoxin α-haemolysin secreted from haemolytic strains of *E. coli* (Scheffer *et al.*, 1985) and by a mechanism of cell-surface contact which is independent of type 1 or P fimbriae (Steadman, Knowlden & Williams, 1989).

The interaction of PMN with cytokines such as TNF may be an important mechanism *in vivo* for augmenting the inflammatory reponse (Movat *et al.*, 1987). The release of endotoxin at sites of infection may represent a mechanism by which the PMN/bacteria interaction is amplified through the release of TNF from adjacent stimulated mononuclear phagocytes. The present study was designed to assess the *in vitro* action of recombinant human TNF-α (rhTNF-α) on the PMN responses to *E. coli* in an attempt to understand the events which may occur during PMN activation following *E. coli* infection *in vivo*.

Two uropathogenic strains of *E. coli* (from our own collection of isolates) were subcultured at least three times overnight, harvested by centrifugation (2000 *g* for 15 min), washed twice in phosphate-buffered saline (PBS), pH 7·3, and resuspended to an optical density (OD) of 1·0 at 560 nm ($5 \times 10^8$ CFU/ml). The strains were characterized by the mannose-sensitive haemagglutination of guinea-pig or human erythrocytes, a specific latex agglutination test for P fimbriae (PF test; CC Laboratories, Market Harborough, Leics) and by their haemolytic potential against an equal volume of sheep erythrocyte suspension (10% v/v; Tissue Culture Services Ltd, Botolph Claydon, Bucks) at 37° for 60 min in a Kreb's Ringer phosphate buffer containing 11 mM glucose, 0·54 mM $Ca^{2+}$ and 1·2 mM $Mg^{2+}$ (KRPG). The absorbance at 415 nm of the supernatants after centrifugation at 11,000 *g* for 1 min was measured as a percentage of the lysis of erythrocytes in water. Strain 504 (06:K

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not known) grown in NB 2 was haemolytic and expressed only type 1 (mannose-sensitive) fimbriae. The non-haemolytic strain SC (01:K1) grown in NB 2 expressed only P fimbriae.

Normal human leucocytes were isolated from citrated peripheral blood by dextran sedimentation and rendered plasma-free and platelet-poor by washing three times with PBS. Neutrophils (PMN) were purified by density gradient centrifugation at 400 *g* for 35 min at 23° on cushions of Ficoll–Hypaque (Pharmacia, Milton Keynes, Bucks) and erythrocytes lysed in 0·2% w/v NaCl. The PMN were returned to isotonicity and washed in PBS. The preparations were > 98% PMN (Steadman *et al.*, 1988).

PMN were incubated with concentrations of rhTNF-$\alpha$ (a kind gift from Dr G. R. Adolf, Ernst-Boehringer Institute, Vienna, Austria) in KRPG for up to 60 min at 37°, immediately centrifuged at 2000 *g* for 20 seconds, resuspended and placed on ice. Cells were incubated on ice for 30 min with 50 $\mu$l of monoclonal anti-CR3 (anti-CD11b; Serotec, Oxford, Oxon) at 1/2000 dilution, washed with PBS/BSA (2% w/v), then incubated on ice with 50 $\mu$l of rabbit anti-mouse- FITC 2nd antibody (Serotec) at 1/30 dilution for 30 min before washing three times in PBS/BSA. PMN were then fixed in an equal volume of 4% (v/v) paraformaldehyde (TAAB Laboratories Equipment Ltd, Reading, Berks). Increases above initial fluorescence were recorded by fluorescence-activated cell sorting (FACS) on a FACS 440 (Becton-Dickinson, Oxford, Oxon).

Following preincubation at $1 \times 10^7$/ml with $10^{-9}$ M rhTNF-$\alpha$ in KRPG, or in KRPG alone, for 20 min, PMN were further incubated at 37° for times up to 60 min alone or with 100 $\mu$l of the *E. coli* strains at a bacteria/cell ratio of 100:1. Incubations were ended by centrifugation at 11,000 *g* for 1 min and the supernatants taken to assay for leukotriene B4 (LTB$_4$), myeloperoxidase (MPO), vitamin B12 binding protein (B12 BP) or lactate dehydrogenase (LDH) (Steadman *et al.*, 1988). In all experiments there was a low level of B12 BP release from the secondary lysosomal (secretory) granules as a consequence of the manipulation and centrifugation of the cells. LDH release was always less than 5% in control cells and there was no LTB$_4$ synthesis.

One-hundred microlitres of supernatant from the cell stimulation or a standard dilution of authentic LTB$_4$ were assayed in duplicate using a specific radioimmunoassay (RIA) for LTB$_4$ (Rokach *et al.*, 1984). Free LTB4 was separated from antibody bound by adsorption to dextran-coated charcoal (Steadman *et al.*, 1989).

Samples containing immunoreactive LTB$_4$ identified in the RIA were separated by RP-HPLC in methanol:water:acetic acid (65:35:0·1), pH 5·6, on a Nucleosil C18 5 $\mu$ reversed-phase column (25·4 cm × 4·6 mm) (Hichrom Ltd, Reading, Berks) (Steadman *et al.*, 1989).

For luminol-dependent chemiluminescence (CL) analysis, $5 \times 10^5$ PMN were incubated with 2 mM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) in 400 $\mu$l KRPG buffer (alone or containing rhTNF-$\alpha$) at 37° for 20 min, prior to addition of 100 $\mu$l of each bacterial suspension ($5 \times 10^7$ CFU). CL readings were taken in a Lumac Biocounter (Lumac BV, Landgraaf, The Netherlands) at precise 2-min intervals and peak levels were compared to those of unstimulated incubations (Harber & Topley, 1986).

rhTNF-$\alpha$ caused a dose-dependent increase in CR3 expression that was optimum at $10^{-9}$ M and was time-dependent, reaching a maximum by 20 min. This paralleled the release of B12 BP from PMN secondary granules. $10^{-9}$ M rhTNF-$\alpha$ caused a small rise in CL, which peaked [184 ± 91 relative light units (rlu); mean ± SD, $n = 3$] at 30 min. There was no LTB$_4$, MPO or LDH release, however, in response to any dose of rhTNF-$\alpha$ used.

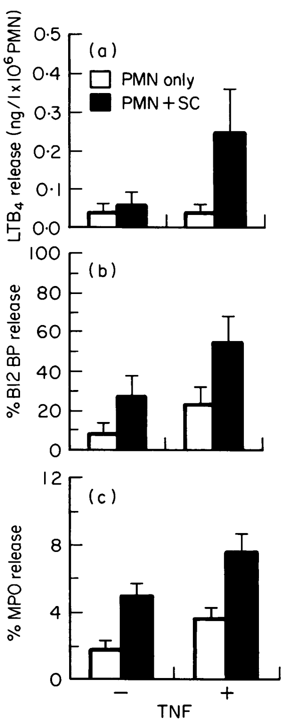

**Figure 1.** The release of (a) LTB$_4$ (b) B12 BP and (c) MPO from $1 \times 10^6$ PMN following incubation for 60 min at 37° with *E. coli* strain SC at a bacteria/PMN ratio of 100:1. PMN were pre-incubated in KRPG alone or KRPG containing $10^{-9}$ M rhTNF-$\alpha$ for 20 min at 37°. The data represents the mean ± SD of three experiments, each using PMN from a different donor.

Following pre-incubation with rhTNF-$\alpha$, the interaction of PMN with the P-fimbriate *E. coli* strain (SC) resulted in a significant ($P < 0·02$; Wilcoxon Rank Sum) and synergistic augmentation of the release of LTB$_4$. This augmentation was maximal following pretreatment with $10^{-9}$ M rhTNF-$\alpha$ for 20 min. In addition there was an additive effect ($P < 0·05$; Wilcoxon Rank Sum) on PMN CL and the release of B12 BP and MPO (Fig. 1). There was no change in LDH release, however, in response to this strain.

In contrast, rhTNF-$\alpha$ pretreatment did not significantly affect the response of PMN to type 1-fimbriate strain 504 (Fig. 2) nor the LTB$_4$ generation from PMN in response to a haemolytic culture supernatant (0·31 ± 0·17, compared to 0·28 ± 0·11 ng/$1 \times 10^6$ in the presence of $10^{-9}$ M rhTNF-$\alpha$; mean ± SD, $n = 3$). This supernatant did not stimulate PMN CL, MPO, B12 BP or LDH release nor were these responses affected by rhTNF$\alpha$ pretreatment.

Neutrophil infiltration into the renal parenchyma occurs in response to *E. coli* strains expressing a variety of virulence markers. The initiation of tissue damage leading to extensive parenchymal scarring, however, depends on the expression of a

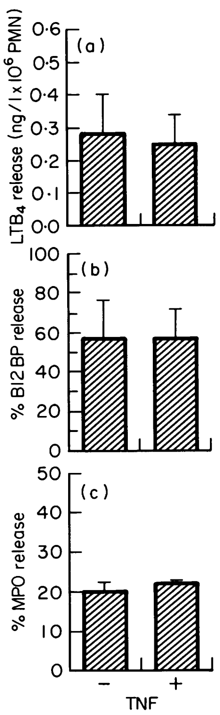

**Figure 2.** The release of (a) LTB$_4$, (b) B12 BP and (c) MPO from 1 × 10$^6$ PMN following incubation for 60 min at 37° with *E. coli* strain 504 at a bacteria/cell ratio of 100:1. PMN were preincubated in KRPG alone or KRPG containing $10^{-9}$ M rh TNF-α for 20 min at 37°. The data represent the mean ± SD in the same three experiments as Fig. 1.

defined set of virulence markers, in particular type 1 fimbriae (Harber *et al.*, 1986; Steadman *et al.*, 1988; Topley *et al.*, 1989). In the present study, the degree of PMN secondary granule release and lipo-oxygenase activation following incubation with the P-fimbriate strain SC was selectively raised to the same level as that of the more 'virulent', type 1-fimbriate strain 504 after rhTNF-α pretreatment. The release of the primary granule marker, MPO, was also increased in response to strain SC, following rhTNF-α pretreatment: but to levels which were < 30% of those reached in response to strain 504. Furthermore, type 1 fimbriae-dependent responses remained unchanged.

These results suggest that the secretion of TNF by mononuclear phagocytes stimulated by endotoxin released during infection *in vivo* may be important in controlling the subsequent neutrophil responses to invading pathogens. Future studies in animal models of renal scarring using antibodies to TNF may highlight the degree of cytokine involvement in the inflammatory response to strains of *E. coli* expressing a range of different virulence characteristics.

In addition, the results support the view that the neutrophil does not respond in an identical manner to all particulate stimuli (Williams *et al.*, 1986; Topley *et al.*, 1987). The primary granule enzyme release is activated by few stimuli and is not significantly increased either in response to direct activation or priming by rhTNF-α. In addition, while the selective release of LTB$_4$ from PMN may be synergistically increased in response to *E. coli* following rhTNF-α priming, the release of LTB$_4$ in response to α-haemolysin is unaffected. Thus the different inflammatory pathways within the PMN are independently controlled and selectively primed by rhTNF-α.

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