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Zymosan-induced leukotriene B_4 generation by human neutrophils is augmented by rhTNF-α but not chemotactic peptide

M. PETERSEN, R. STEADMAN, M. B. HALLETT,* N. MATTHEWS† & J. D. WILLIAMS *Institute of Nephrology and Departments of * Surgery and † Medical Microbiology, University of Wales College of Medicine, Cardiff*

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SUMMARY

Tumour necrosis factor (TNF) is a mediator of inflammation that has been shown to enhance neutrophil responses to soluble and particulate stimuli. The release of leukotriene B_4 (LTB_4) by human neutrophils stimulated by unopsonized zymosan was measured in the presence or absence of recombinant human TNF-α (rhTNF-α) preincubation. There was a threefold increase in the LTB_4 response at an optimal TNF concentration of 10^{-9} M and an optimal preincubation time of 10–20 min. A similar time and dose dependency was observed for CR3 receptor expression and for the release of the secondary lysosomal granule marker, vitamin B12-binding protein. In contrast, the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (FMLP), although stimulating an increase in both CR3 receptor number and in particle phagocytosis, failed to induce an increase in LTB_4 release in response to zymosan. In addition, the present study demonstrated that, unlike FMLP, the exocytotic mechanism for secondary granule release by rhTNF-α functioned in the absence of a rise in cytosolic free calcium. Furthermore, it was independent of changes in cyclic nucleotide concentrations and did not require an intact cytoskeleton. Thus the capacity of rhTNF-α to amplify the neutrophil response to zymosan through the CR3 receptor appears to be related to the amplification of post-membrane events as well as to an increase in the number of functionally active receptors.

INTRODUCTION

The infusion of recombinant human TNF-α (rhTNF-α) results in neutrophilia, margination and extravasation of neutrophils (PMN) into the perivascular space (Remick, Larrick & Kunkel, 1986). *In vitro* incubation of rhTNF-α with PMN results in their enhanced adherence to cultured endothelial cells (Gamble *et al.*, 1985), a response which is dependent on an increased expression of the CD11/CD18 family of adhesion glycoproteins. Furthermore, the degranulation response of human PMN to both soluble and particulate ligands may be enhanced by preincubation with rhTNF-α (Klebanoff *et al.*, 1986; Berkow *et al.*, 1987; Ferrante *et al.*, 1988). Incubation of PMN with macrophage-conditioned medium containing TNF activity results in the release of the secondary granule marker, vitamin B12-binding protein (B12 BP), an effect that is inhibited by antibodies to TNF (Berger, Wetzler & Wallis, 1988). This degranulation response may explain the increase in CD11/CD18 glycoprotein expression through the transfer to the plasma membrane of secondary granule-associated stores (Todd *et al.*, 1984; Arnaout *et al.*, 1984; Jones *et al.*, 1988). The functional capacity of one member of this glycoprotein family, CD11b/CD18, complement receptor type 3 (CR3), has been confirmed in studies where the phagocytosis of unopsonized zymosan was increased following rhTNF-α preincubation (Klebanoff *et al.*, 1986), a finding consistent with the observation that unopsonized zymosan has been shown to bind to the CR3 receptor on PMN (Ross *et al.*, 1987). The intracellular mechanism of action of TNF has not been established. It has been implied, however, that the priming effect of TNF may be similar to the action of the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (FMLP; Tsujimoto *et al.*, 1986; Berger *et al.*, 1988). The activation of neutrophils by FMLP, however, involves a rise in intracellular calcium, a mechanism which does apper to be a feature of rhTNF-α priming (Watson, Lewis & Westwick, 1988; Richter, Andersson & Olsson, 1989). In contrast, both rhTNF-α (Berkow *et al.*, 1987) and FMLP (Bengtsson *et al.*, 1988) stimulate a rapid increase in actin polymerization in neutrophils, implicating a role for the cytoskeleton in their priming action.

The present study identifies functional differences between the rhTNF-α and FMLP priming of zymosan-stimulated PMN leukotriene B_4 (LTB_4) release under conditions that increase the expression of CR3 and the phagocytosis of zymosan.

Correspondence: Dr J. D. Williams, Institute of Nephrology, University of Wales College of Medicine, Kidney Research Unit Foundation, Cardiff Royal Infirmary, Newport Road, Cardiff CF2 1S2, U.K.

MATERIALS AND METHODS

All chemicals were Analar grade from BDH Chemicals, Poole, Dorset unless otherwise indicated.

Recombinant human TNF (rhTNF-α) was kindly supplied by Dr G. R. Adolf, Ernst Boehringer Institute, Vienna, Austria, 100 U/ng, and stored at 1 μg/ml in phosphate-buffered saline (PBS) at −70°. It was 95% pure by SDS–PAGE and had no detectable endotoxin contamination measured in the Limulus assay. A23187, FMLP, cytochalasin B and ionomycin were obtained from Sigma (Poole, Dorset), stored at −70° in dimethylsulphoxide and then diluted in buffer before use.

Cell preparation

This was carried out according to the method of Boyüm (1968). Normal human leucocytes were purified from citrated peripheral blood after dextran (Macrodex, Norfolk) sedimentation of erythrocytes, rendered platelet poor by centrifugation at 200 *g* for 10 min, and washed twice with cold, sterile PBS, pH 7·3 (Oxoid, Basingstoke, Hants). Mononuclear cells and PMN were separated on Ficoll–Paque (Pharmacia, Milton Keynes, Bucks) by centrifugation at 400 *g* for 35 min at 20°. PMN were treated with hypotonic saline to lyse contaminating erythrocytes, resuspended after washing in PBS and counted in a Coulter counter (Coulter Electronics Ltd, Luton, Beds).

Cytocentrifuge (Shandon, Runcorn, Cheshire) preparations of PMN, stained with Neat stain (Guest Medical, Sevenoaks, Kent) were >95% pure by morphology and essentially platelet free.

Preparation of cell monolayers

The procedure followed has been described previously (Williams *et al.*, 1986). PMN were resuspended at 3×10^6/ml in RPMI-1640 (Gibco, Paisley, Renfrewshire) containing 0·2% w/v bovine serum albumin (BSA; Miles, ICN Biomedicals Ltd, High Wycombe, Bucks) (RPMI-BSA). One millilitre was layered onto 35-mm plastic culture dishes (Nunc, Gibco, Uxbridge, Middlesex) and PMN allowed to adhere in a 5% CO_2 incubator at 37° for 40 min. After three washes with 1 ml Tyrodes, containing 136·7 mM NaCl, 12 mM $NaHCO_3$, 5 mM $MgCl_2$, 5 mM glucose, 1·36 mM $CaCl_2$, 0·27 mM KCl, 0·4 mM NaH_2PO_4 and 0·1% w/v gelatin (Difco, East Molesey, Surrey), pH 7·3 (TG), non-adherent cells were counted to allow quantification of adherent cells, which was routinely greater than 50%.

Zymosan preparation

Zymosan A (Sigma) particles were boiled for 20–30 min in 0·9% w/v sodium chloride, washed and counted, and finally resuspended at a concentration of $1{\cdot}4 \times 10^8$ particles/ml.

Phagocytosis

Cell monolayers prepared as above were treated with 10^{-9} M rhTNF-α or 10^{-7} M FMLP in TG for 10 min at 37°, the supernatant was decanted and zymosan ($1{\cdot}4 \times 10^8$ particles/ml) added and incubated for a further 15 min. The zymosan was washed off with PBS and the cells air dried, fixed and stained with Neat stain. This allows a clear distinction between ingested and adherent particles. The percentage of cells containing two or more particles was assessed by counting 200 cells/plate using a light microscope at ×400 magnification.

Leukotriene generation

Cell monolayers were incubated in TG buffer alone or containing doses of rhTNF-α or FMLP. The supernatant was decanted and zymosan (1 ml containing $1{\cdot}4 \times 10^8$ particles in TG) added for a further 25 min incubation. In parallel experiments, PMN monolayers were preincubated with 10^{-9} M rhTNF-α for 10 min then treated for a further 10 min with doses of A23187 instead of zymosan. Supernatants were decanted, spun at 11,000 *g* for 30 seconds and either assayed directly for LTB_4 or stored at −20°.

LTB_4 assay

Supernatants from stimulated cells were assayed by radioimmunoassay (RIA) in a total volume of 300 μl in Tris Isogel buffer containing 10 mM Tris-HCl, 0·9% NaCl w/v, 0·01% w/v NaN_3, 0·1% w/v gelatin, pH 7·4. Synthetic LTB_4 (kindly supplied by Dr B. Spur, Institute Henri Beaufour, Paris, France), diluted to standard concentrations ranging from 10 to 0·04 ng/ml (100 μl) or 100 μl of sample, was mixed with 100 μl of specific anti-LTB_4 antibody at 1/4000 dilution (Merck Frosst, Dorval, Canada) and 100 μl of [^{3}H]LTB_4 [(5,6,8,9,11,12,14,15) n[^{3}H]LTB_4, 210 Ci/mmol; Amersham, Amersham, Bucks]) in 3·5-ml polypropylene tubes (Luckham, Burgess Hill, Sussex), and incubated overnight at 37°. Antibody-bound [^{3}H]LTB_4 was separated from free tracer by addition of 200 μl of cold dextran (T70; Pharmacia) coated charcoal (Norit SX-1; BDH), both 1% w/v in Tris Isogel, and centrifuged at 2500 *g* for 10 min at 4°. Supernatants were decanted, mixed with 3·5 ml scintillant (Optiphase-MP, LKB Pharmacia, Milton Keynes, Bucks) and counted for 2 min in a β-counter (LKB). Fifty per cent LTB_4 binding occurred at approximately 0·4 ng/ml and LTB_4 was measured over the linear part of the standard curve from 0·1 to 4·0 ng/ml. LTB_4 was further authenticated by comparing its retention time on reverse-phase high-pressure liquid chromatography (RP-HPLC) with that of synthetic standard (Williams, Czop & Austen, 1984).

Secondary granule release

The method of Gottlieb *et al.* (1965) was used. Briefly, 5×10^5 cells were suspended in 1 ml Krebs buffer containing 12·7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 120 mM NaCl, 4·8 mM KCl, 1·2 mM $MgSO_4$, 0·7 mM $CaCl_2$ and 11 mM dextrose, pH 7·4. Various concentrations of rhTNF-α were added and the cells incubated at 37° for the times shown. The cells were separated from the supernatant by centrifugation at 11,000 *g* for 15 seconds. Duplicate aliquots of 100 μl of supernatants were mixed with 250 μl [^{57}Co]cyanocobalamin (15 μCi/μg; Amersham) (30,000 c.p.m.). After 30 min incubation at room temperature, unbound tracer was removed by the addition of 1 ml ice-cold charcoal (Norit SX-1; 5% w/v in water containing 1% w/v BSA), incubated for 10 min at room temperature, and then centrifuged for 12 min at 4000 r.p.m. at 4°. One millilitre of supernatant was counted in a gamma counter (Kontron, Watford, Herts) for 1 min. A water blank and a sample of sonicated cells were included to give non-specific binding and total vitamin B12-binding protein (B12 BP) release, respectively, thus allowing the percentage release to be calculated. A small background release of B12 BP at time 0 was observed due to manipulation of the cells (Steadman *et al.*, 1988).

CR3 expression

PMN were suspended in PBS containing 2% w/v BSA at $2{\cdot}5 \times 10^6$/ml and treated with the soluble ligand for various times at 37°. The cells were spun down at 11,000 *g* for 15 seconds, resuspended in 50 μl PBS/BSA and put on ice immediately. The

cells were incubated with 50 μl of a 1/2000 dilution of monoclonal antibody (mAb) to CD11b (CR3; Serotec, Oxford, Oxon) on ice for 30 min. After three washes with 500 μl PBS/BSA, 50 μl of FITC rabbit anti-mouse antibody (Miles) diluted 1/30 (v/v) in PBS/BSA were added to the cell suspension, incubated for 30 min and washed again. The cells were finally suspended in 100 μl PBS/BSA, with 100 μl of 4% (w/v) paraformaldehyde (TAAB, Reading, Berks) in water, pH 7·2, added as a fixative, and stored at 4°. Fluorescence was analysed using a fluorescence-activated cell sorter (FACS 440; Becton-Dickinson, Oxford, Oxon), and the data expressed as the percentage of cells demonstrating an increase in fluorescence above background.

Lactate dehydrogenase assay

Duplicate samples (100 μl) were incubated with 200 μl NADH (Sigma) (2·5 mg/ml in PBS) and 700 μl PBS at room temperature. Ten microlitres sodium pyruvate (10 mg/ml in PBS) were added to each tube to start the reaction and the absorbance at 340 nm read against a reagent blank at 2-min intervals for 12 min on a spectrophotometer. Lactate dehydrogenase activity was calculated from the linear fall in absorbance, corresponding to consumption of NADH, and the results expressed as a percentage of the sample change in absorbance compared to that of sonicated cells over the same time period.

Calcium influx

PMN (1×10^6) were suspended in 500 μl Krebs containing 0·1 μCi [^{45}Ca]Cl_2 (25·48 mCi/mg; Amersham) and were treated with buffer alone, with 10^{-9} M rhTNF-α or with 5 μM A23187, for 5 min at 20°. After centrifugation at 11,000 *g* for 15 seconds, cells were washed with cold PBS and treated with 500 μl of 1% Triton X-100 v/v in PBS for 5 min. Scintillant (3 ml) was added and the cell-associated [45Ca] counted in a β-counter.

Cytosolic free calcium

PMN in suspension at 1×10^7/ml in PBS were loaded for 25 min at room temperature with 1 μM Fura-2 acetoxy methylester (Molecular Probes, Eugene, OR). The cells were washed and resuspended in 2 ml Krebs buffer containing 120 mM NaCl, 4·8 mM KCl, 1·2 mM $MgSO_4$, 1·1 mM KH_2PO_4, 1·3 mM $CaCl_2$, 0·1% BSA and 25 mM HEPES, adjusted to pH 7·4 with NaOH, in a quartz cuvette, maintained at 37° and stirred in the light path of a dual wavelength fluorescence apparatus (Spex Fluorolog 3), set at an emission wavelength of 505 nm. Fluorescence excitation at 340 nm and 380 nm was monitored during the addition of 10^{-9} M rhTNF-α, 10^{-7} M FMLP and 2×10^{-6} M ionomycin. From the 340/380 ratio, free Ca^{2+} concentrations were calculated, assuming a K_d of 186 nM, as previously described (Al-Mohanna & Hallet, 1988).

Cyclic nucleotide assays

Cyclic AMP was measured in 1×10^6 PMN incubated in suspension with or without 10^{-9} M rhTNF-α, or 10^{-3} M sodium fluoride for various times in 500 μl Krebs buffer, at 37°. The cell incubation mixture was frozen rapidly, extracted with 500 μl of 0·7 M perchloric acid (PCA) and neutralized with potassium carbonate. The supernatant was immediately assayed by RIA (New England Nuclear Dupont Ltd, Stevenage, Herts).

Cyclic GMP was measured in 1×10^7 PMN incubated in suspension in 500 μl of Hanks' balanced salt solution (Gibco) alone, with 10^{-9}M rhTNF-α or with 10^{-6} M sodium nitroprusside (SNP), for 1 min at 37°. The extraction method of Coffey was used (Coffey, Davis & Djeu, 1988). Briefly, the reaction was stopped by addition of cold PCA, cGMP was purified by sequential chromatography on neutral alumina (Merck, BDH) followed by Dowex AGI-X8 (Biorad, Watford, Herts) and eluted with 5 M formic acid. The samples were dried down under vacuum, reconstituted in RIA buffer (New England Nuclear), and assayed by RIA the same day.

Cytochalasin B

PMN were preincubated with 2 μg/ml cytochalasin B for 5 min at 37° before the addition of the stimulus.

Statistics

The data were analysed using the non-parametric Mann-Whitney one-tailed test.

RESULTS

Phagocytosis

Preincubation of PMN monolayers with rhTNF-α for 10 min resulted in an increase in the phagocytosis of unopsonized zymosan particles from a mean of 38±10% in control incubations to a mean of 52±10% ($P=0.025$, $n=6$) following incubation with 10^{-9} M rhTNF-α and to 58±10% following incubation with 10^{-7} M FMLP ($P=0.025$, $n=6$).

LTB_4 release

There was a synergistic augmentation by rhTNF-α of the immunoreactive LTB_4 released by PMN monolayers stimulated with a wide range of particle numbers of zymosan (0·18–1.4×10^8) (Fig. 1). At the highest concentration, the addition of TNF at 10^{-9} M resulted in an increase from 0·64±0·13 ng/10^6 PMN to 2·23±0·88 ng/10^6 PMN (mean±SEM) ($P<0.01$, $n=6$). This augmentation was significant by 10^{-11} M rhTNF-α ($P=0.01$, $n=6$) and optimal at 10^{-10} M rhTNF-α (Fig. 2a), while the optimum preincubation time was 10 min (Fig. 2b). The LTB_4 release at time = 0 represents the LTB_4 generated by zymosan alone for 25 min, without any preincubation with rhTNF-α. Following RP-HPLC separation of supernatants containing 9·27 ng immunoreactive LTB_4, and RIA of each 1-ml fraction, a peak of immunoreactive LTB_4 containing 5·66 ng (61%) of the

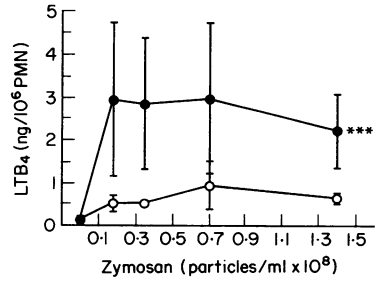

Figure 1. Zymosan-induced release of LTB_4 augmentation by TNF. PMN incubated with (●) or without (○) 10^{-9} M TNF for 10 min. *** $P<0.01$ ($n=6$). Data represents mean±SEM of three separate experiments, each with PMN from a different donor.

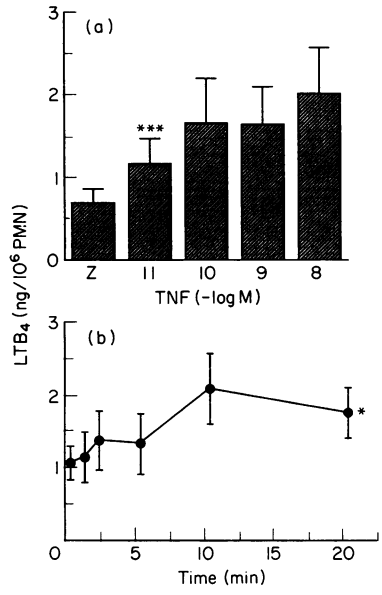

Figure 2. Effect of rhTNF-α concentration and preincubation time on LTB_4 release from zymosan-treated PMN. (a) Cell monolayers were incubated with doses of rhTNF-α for 10 min at 37° then treated with $1{\cdot}4 \times 10^8$ particles/ml zymosan for a further 25 min. *** $P = 0{\cdot}01$ compared to zymosan alone. (b) Cell monolayers were incubated with 10^{-9} M rhTNF-α for various times at 37°, then treated with zymosan as described under (a). * $P = 0{\cdot}05$ compared to zymosan alone. Data represents mean ± SEM of three separate experiments, each with PMN from a different donor.

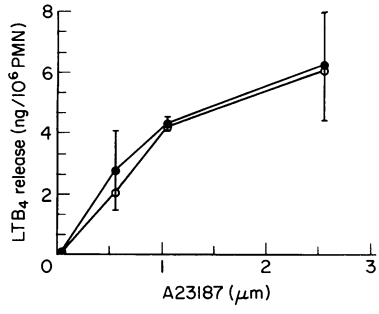

Figure 3. Release of LTB_4 from PMN monolayers treated with (●) or without (○) 10^{-9} M rhTNF-α for 10 min, then incubated for a further 10 min with doses of A23187. Data represents mean ± SEM of three separate experiments, each with PMN from a different donor.

applied material eluted at 24 min. This peak co-eluted with both authentic standard and tritiated LTB_4. In parallel experiments, preincubation of PMN with doses of FMLP from 10^{-9} M to 10^{-7} M did not augment zymosan-induced LTB_4 release. Zymosan alone gave $0{\cdot}27 \pm 0{\cdot}03$ ng $LTB_4/10^6$ PMN, while preincubation with FMLP gave a mean maximum of $0{\cdot}34 \pm 0{\cdot}07$ ng/10^6 PMN (mean ± SEM, $n = 3$). No release of LTB_4 was detected from PMN treated with FMLP or rhTNF-α alone.

In a separate series of experiments ($n = 3$), there was no augmentation by 10^{-9} M rhTNF-α of A23187-stimulated LTB_4 release from either PMN monolayers (Fig. 3) or PMN in suspension (data not shown).

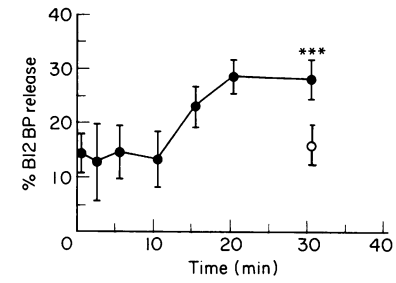

Figure 4. Time-dependent release of B12 BP from PMN following incubation with 10^{-9} M rhTNF-α at 37°. *** $P = 0{\cdot}01$ compared to untreated controls (○). Data represents mean ± SEM of three separate experiments, each with PMN from a different donor.

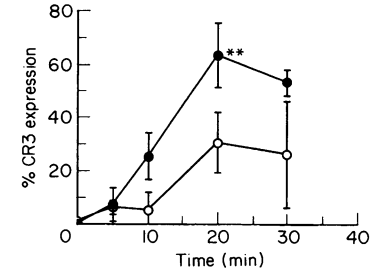

Figure 5. Time-course of CR3 receptor expression. PMN were incubated with (●) or without (○) 10^{-9} M rhTNF-α at 37°. ** $P = 0{\cdot}025$ compared to untreated controls. Data represents mean ± SEM of three separate experiments, each with PMN from a different donor.

B12 BP release

The secondary granule marker B12 BP was released in a time- and dose-dependent manner following treatment of PMN with rhTNF-α at 37°. By 30 min of incubation there was a mean $28 \pm 4\%$ release of B12 BP at a rhTNF-α concentration of 10^{-9} M, compared to a mean of $16 \pm 4\%$ for control cells without rhTNF-α ($P < 0{\cdot}01$, $n = 6$) (Fig. 4). There was no significant release of lactate dehydrogenase from PMN treated with rhTNF-α when compared to control cells in the same experiment.

CR3 expression

rhTNF-α (10^{-9} M) induced a time-dependent increase in CR3 expression by PMN incubated at 37°. Maximal expression was seen by 20 min, when there was a mean twofold rise over control values ($P = 0{\cdot}025$, $n = 6$) (Fig. 5). Incubation of PMN with 10^{-7} M FMLP for 20 min resulted in a fivefold increase in CR3 expression to 32% from 7% in control cells.

Cytoplasmic calcium

Incubation of PMN with rhTNF-α failed to induce [^{45}Ca] uptake greater than that of untreated cells (rhTNF-α 462 ± 110 c.p.m., controls 446 ± 100 c.p.m.; mean ± SD, $n = 3$). This was confirmed by measuring the cytosolic free Ca^{2+} directly with Fura-2. The resting Ca^{2+} concentration was 85 ± 5 nM ($n = 3$) and after the addition of 10^{-9} M rhTNF-α the concentration was

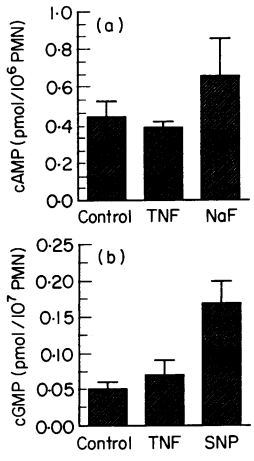

Figure 6. (a) Generation of cAMP by PMN. Cells were incubated in medium alone or containing 10^{-9} M rhTNF-α or 10^{-2} sodium fluoride for 5 min at 37°. (b) Generation of cGMP by PMN. Cells were incubated in medium alone or containing 10^{-9} M rhTNF-α or 10^{-3} M sodium nitroprusside for 60 seconds at 37°. Data represents mean ± SEM of three separate experiments, each with PMN from a different donor.

Table 1. Effect of cytochalasin B on rhTNF-α-treated PMN

	% CR3	% B12 BP	Zymosan % phagocytosis
Control	10±0·6	9±4	23±12
Cytochalasin B 2 μg/ml)	7±0·4	11±4	13±8
10^{-9} M rhTNF-α	24±9	21±6	58±12
rhTNF-α and cytochalasin B	21±1	18±0·5	33±10

Results expressed as mean ± SD, $n=3$.

unchanged at 85±7 nM ($n=3$). The absence of a change in cytoplasmic Ca^{2+} also eliminated the possibility of Ca^{2+} release from an intracellular store by this agent. In contrast, the addition of 10^{-7} M FMLP promoted a transient rise in intracellular Ca^{2+}, to 875 nM.

Cyclic nucleotides

The effect of rhTNF-α on cyclic nucleotide concentrations was measured over periods of 15 seconds to 5 min. There was no significant change in cyclic AMP concentrations in PMN treated with 10^{-9} M rhTNF-α for 5 min under conditions where NaF (10^{-3} M) gave a fivefold increase (Fig. 6a). In addition, no change in cyclic GMP occurred under conditions where 10^{-3} M SNP gave a twofold increase (Fig. 6b).

Cytochalasin B

The addition of 2 μg/ml cytochalasin prior to the incubation of PMN with 10^{-9} M rhTNF-α did not affect the subsequent threefold rise in CR3 expression or the near doubling of the B12 BP. At this concentration of cytochalasin B the number of cells phagocytosing two or more zymosan particles was reduced by 44% both in the presence or absence of rhTNF-α. Cytochalasin B had no effect on the unstimulated CR3 expression or B12 BP release (Table 1).

DISCUSSION

Human PMN preincubated with either rhTNF-α or FMLP showed a significant increase in the phagocytosis of unopsonized zymosan. The release of LTB_4 by human PMN in response to unopsonized zymosan, however, was increased only following rhTNF-α pretreatment.

Zymosan phagocytosis is reported to occur through the CR3 receptor, and CR3 expression has been linked to secondary granule exocytosis. Both rhTNF-α and FMLP induced secondary granule release and increased CR3 expression in the present study (Gamble *et al.*, 1985; Berger *et al.*, 1988).

Since preincubation with FMLP did not alter the zymosan-generated LTB_4 response, these results suggest that rhTNF-α may augment neutrophil activation through a post-membrane mechanism unrelated to increased CR3 expression. Macrophage activation in response to rhTNF-α is accompanied by the generation of prostaglandin E_2 (PGE_2) (Lehmann, Benninghoff & Droge, 1988), implying that the activation of the cell is associated with the mobilization of arachidonic acid from membrane phospholipids. It has been reported, however (Berkow & Dodson, 1988), and unpublished observations from our own laboratory confirm, that there is no arachidonic acid release in response to rhTNF-α from PMN prelabelled with [^{3}H]arachidonic acid. Previous studies have reported priming by rhTNF-α of the calcium ionophore-stimulated 5-lipo-oxygenase pathway of human PMN (Roubin *et al.*, 1987; Meyer *et al.*, 1988). Using more specific methods for LTB_4 analysis (by RP-HPLC in combination with a highly sensitive specific RIA), however, no synergy between rhTNF-α and A23187 could be measured for LTB_4 generation, either from PMN monolayers or PMN in suspension. The present study demonstrates, for the first time, the priming of the 5-lipo-oxygenase pathway in response to a phagocytic stimulus under conditions where the ionophore-stimulated release was not enhanced. Taken together, these results indicate that rhTNF-α acts to augment intracellular events at a level between the CR3 receptor and the 5-lipo-oxygenase enzyme.

It has been suggested previously that calcium influx may play a pivotal role in the priming of neutrophils by rhTNF-α (Tsujimoto *et al.*, 1986; Berger *et al.*, 1988), and that this mechanism is similar to that of cell activation by FMLP. In the present study, however, no influx of [^{45}Ca] into PMN was detectable following incubation with rhTNF-α. Furthermore, in support of recent reports (Watson *et al.*, 1988; Richter *et al.*, 1989), intracellular calcium concentrations measured with Fura-2 did not alter following rhTNF-α addition, although in the same experiments the addition of FMLP caused a marked increase.

Studies with macrophages incubated with rhTNF-α have suggested an association between cell activation and the rise of intracellular cyclic AMP, indicating a role for cyclic nucleotides in the activation of the cell (Lehmann *et al.*, 1988). In the present study the preincubation of PMN with rhTNF-α did not result in changes in either cyclic AMP or cyclic GMP concentrations.

Thus a similarity between the mechanisms of FMLP stimulation and rhTNF-α priming (Berger *et al.*, 1988) is not supported by the results presented in this paper. For stimuli such as FMLP, receptor–response coupling involves activation of phospholipase C, via a pertussis toxin-sensitive G protein, generating inositol triphosphate and diacyglycerol, which in turn mobilize intracellular calcium and activate protein kinase C, respectively (Verghese, Smith & Snyderman, 1985). The lack of any calcium mobilization by rhTNF-α together with an insensitivity to pertussis toxin (Berkow & Dodson, 1988; Meurer & MacIntyre, 1988) indicates a different mode of action for the TNF activation of PMN.

The cytoskeleton has been implicated in the activation of PMN by rhTNF-α through the polymerization of actin filaments (Berkow *et al.*, 1987). In the present study the preincubation of PMN with cytochalasin B, although resulting in a decrease in the phagocytosis of zymosan, did not influence either the release of B12 BP or the increased expression of the CR3 receptor in response to rhTNF-α, suggesting that the increase in CR3 expression produced by TNF, while paralleled by the release of secondary granule contents, was not dependent on an intact cytoskeleton.

Recent studies have indicated that a degree of protein phosphorylation is required for exocytosis (Gomperts, Barrowman & Cockcroft, 1986; Howell, Kramer & Gomperts, 1988) and is a pre-requisite for Ca^{2+} and GTP to participate in the exocytotic effector mechanism. Protein phosphorylation in neutrophils has been demonstrated in response to rhTNF-α (Berkow & Dodson, 1988). The present study, however, demonstrates that, following rhTNF-α stimulation, the exocytotic mechanism functions in the absence of a rise in intracellular calcium, is independent of a rise in cyclic nucleotides and does not require an intact cytoskeleton. The failure of FMLP to 'prime' the zymosan-induced LTB_4 synthesis in PMN suggests functional differences in post-receptor events between CR3 receptors expressed in response to FMLP stimulation and those expressed in response to rhTNF-α.

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