Ceramide does not mediate the effect of tumour necrosis factor α on superoxide generation in human neutrophils

Fumi YANAGA* and Steve P. WATSON
Department of Pharmacology, Oxford University, Mansfield Road, Oxford OX1 3QT, U.K.

The effect of tumour necrosis factor α (TNFα) on superoxide generation in human neutrophils was investigated using the Nitro Blue Tetrazolium reduction assay. TNFα stimulated superoxide generation in a time- and concentration-dependent fashion. The maximally effective concentration of TNFα for superoxide generation was 10 nM and maximal response was obtained after 15–20 min. The monoclonal antibody (mAb), utr-1, which was raised against the 75 kDa receptor and behaves as an antagonist, had no effect on superoxide generation, but partially inhibited the response to TNFα. mAb htr-9, which was raised against the 55 kDa receptor and behaves as an agonist, mimicked the effect of TNFα, but with a lower maximal response. As it has been reported that ceramide might act as a second messenger to mediate many of the effects of TNFα, the effects of exogenous sphingomyelinase and the cell-permeable ceramide analogue, C_2-ceramide, on production of superoxide anions, induction of priming in response to formylmethionyl-leucyl-phenylalanine, and cell-shape change were examined. Neither sphingomyelinase nor C_2-ceramide mimicked the effect of TNFα. Ceramide is converted into ceramide 1-phosphate by ceramide kinase and we have measured levels of this metabolite to clarify the effect of TNFα on sphingomyelinase activity in neutrophils. Although exogenous sphingomyelinase increased the amount of ceramide 1-phosphate in a time-dependent manner, and C_2-ceramide was rapidly converted into C_2-ceramide phosphate, TNFα had no effect on the level of ceramide 1-phosphate. These results suggest that TNFα stimulates superoxide generation through both the 55 kDa and 75 kDa receptors, but that ceramide does not act as an intracellular mediator for TNFα in human neutrophils.

INTRODUCTION

Neutrophils are granulocytic white blood cells which function in the killing of micro-organisms and in inflammatory responses [1]. The cytokine tumour necrosis factor α (TNFα) has an important physiological role in the regulation of neutrophils stimulating chemotaxis, adhesion, priming, phagocytosis, shape change, release of inflammatory mediators and generation of superoxide anions [2–6]. Two major types of receptor for TNFα have been identified with approximate molecular masses of 75 kDa and 55 kDa [7,8]. The signalling pathways utilized by these two receptors and their role in the activation of neutrophils by TNFα are not known.

Recently, we and others have shown that TNFα activates sphingomyelinase in the leukaemia cell line HL-60, to generate ceramide, a novel lipid second messenger [9–11]. It has been suggested that ceramide mediates many of the effects of TNFα, including cell growth and differentiation [11,12], programmed cell death (apoptosis) [13], activation of the nuclear factor (NF)-κB [14] and phosphorylation of the epidermal-growth-factor receptor [9]. The role of the sphingomyelinase pathway in the activation of neutrophils by TNFα, however, is not known.

In this study, we have investigated the role of ceramide in the activation of superoxide generation and shape change in neutrophils by TNFα and have examined the subtype of TNF receptor which mediates these responses.

MATERIALS AND METHODS

Materials

[^{32}P]P_i (9000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were from New England Nuclear. Monoclonal anti-(TNFα receptor) antibodies, utr-1 and htr-9, were generously provided by Dr. M. Brockhaus (Roche, Basel, Switzerland). Monoclonal anti-CD32 antibody and monoclonal anti-CD13 antibody were generously provided by Dr. K. Pulsord and Dr. K. Micklan (Dept. of Pathology, John Radcliffe Hospital, Oxford University, Oxford, U.K.). TNFα was a kind gift from Dr. R. Foulkes (Celltech, Slough, Berks, U.K.). The cell-permeable ceramide analogue, C_2-ceramide, was a kind gift from Dr. Y. A. Hannun (Dept. of Medicine and Cell Biology, Duke University, Durham, NC, U.S.A.) Silica-gel 60G plates were purchased from Merck. Type-3 ceramide, sphingomyelinase, cardiolipin, superoxide dismutase, formylmethionyl-leucyl-phenylalanine (fMLP), fatty-acid-free BSA, Nitro Blue Tetrazolium (NBT) and cytochalasin B were from Sigma. Diacylglycerol kinase was from Lipidex. HL-60 cells were generously provided by Dr. C. Madin (Dept. of Pathology, Oxford University, Oxford, U.K.). RPMI 1640 medium was from Gibco.

Isolation of human neutrophils

Human neutrophils were isolated from healthy adult donors as described previously [15]. Briefly, 50 ml of blood was collected into heparin (10 units/ml of blood) and neutrophils were isolated from erythrocytes by sedimentation with 0.6% dextran in normal saline (1 vol. dextran solution: 3 vol. blood) for 30 min at 37 °C. The leucocyte-rich supernatant was collected and centrifuged at 300 *g* for 10 min at room temperature. The pellet was resuspended with 5 ml of 4 °C lysis buffer (0.82% NH_4Cl, 5 mM KCl) to remove erythrocytes and adjusted to pH 7.4 with 4.4% $NaHCO_3$ and left on ice for 5 min. After centrifugation (300 *g*, 5 min, at

Abbreviations used: TNFα, tumour necrosis factor α; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; mAb, monoclonal antibody; NF-κB, nuclear factor-κB; NBT, Nitro Blue Tetrazolium; BSS, balanced salt solution.

* To whom correspondence should be addressed.

4 °C), the pellet was washed in a buffer containing 137 mM NaCl, 5.4 mM KCl, 5.5 mM glucose and 10 mM Hepes, pH 7.4, and resuspended with 4 ml of the same buffer. The cell suspension was layered on 6 ml of Ficoll–Paque and spun at 300 *g* for 40 min at room temperature. The pellet was washed with BSS (balanced salt solution: 137 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose and 10 mM Hepes, pH 7.4) and finally resuspended with BSS containing 0.1 % BSA.

Cell culture

HL-60 cells were maintained in RPMI 1640 medium containing 10 % (v/v) fetal-calf serum, 2 mM glutamine, 1 % (w/v) non-essential amino acids, 1 mM sodium pyruvate, penicillin (100 units/ml) and streptomycin (100 units/ml) at 37 °C under 5 % CO_2 in air as described previously [11].

NBT-reduction assay

To estimate superoxide generation, the NBT-reduction assay was used [16]. Neutrophils (5×10^6 cells/ml) suspended in BSS containing 0.1 % BSA and 1 mg/ml of NBT were incubated with various agonists at 37 °C for the indicated period with or without superoxide dismutase and the reaction was terminated by addition of an equal volume of 0.5 % glutaraldehyde. Differential interference-contrast microscopy was used to score positive cells containing dark deposit formazan (reduced NBT).

Shape-change assay

Neutrophils were incubated with various agonists at 37 °C for 20 min and then were fixed with 0.25 % glutaraldehyde. Cells were examined under 200-fold magnification and scored for shape change [17].

Assay of ceramide 1-phosphate production

Neutrophils and HL-60 cells were labelled with 0.5 mCi/ml of [^{32}P]P_i for 1.5 h at 37 °C and then twice washed with BSS containing 0.1 % BSA. The cells were resuspended in 150 μl of BSS containing 0.1 % BSA and incubated with various agonists at 37 °C. At the end of the incubation 560 μl of chloroform/methanol/HCl (50:100:1, by vol.) was added and phases were separated by adding 190 μl of chloroform and 190 μl of water. The lower organic phase was taken and subjected to mild alkaline hydrolysis to remove glycerophospholipids as described previously [18]. This involved mixing 200 μl of lower organic phase with the same volume of 0.1 M methanolic KOH and incubating for 1 h at 37 °C. After the incubation, 150 μl of 1 M HCl, 2 ml of ether and 1 ml of water were added and the upper phase dried for lipid analysis. The lipids were separated using chloroform/methanol/acetic acid (65:15:4, by vol.) on silica-gel 60G t.l.c. plates. After the autoradiography, the spot corresponding to ceramide 1-phosphate was scraped and counted for radioactivity following addition of scintillant.

Preparation of ceramide 1-phosphate and C_2-ceramide phosphate standards

Ceramide 1-phosphate and C_2-ceramide phosphate standards were prepared enzymically as described [19,20]. Briefly, type-3 ceramide (0.5 mg) and C_2-ceramide (0.5 mg) were solubilized in a solution of 1 mM cardiolipin/7.5 % (w/v) octyl β-glucopyranoside/1 mM di-ethylenetriaminepenta-acetic acid by sonication. The mixture was diluted to 100 μl with a reaction mixture (50 mM sodium phosphate buffer, pH 7.5, 50 mM NaCl, 12.5 mM $MgCl_2$, 1 mM EGTA) containing diacylglycerol kinase (0.4 unit/ml). The reaction was started by addition of 1 mM ATP containing 1 mCi/ml of [γ-^{32}P]ATP and left at 37 °C for 60 min before being stopped with 370 μl of chloroform/methanol/HCl (50:100:1, by vol.). Phases were separated by addition of 130 μl of chloroform and 130 μl of water. The organic phase was dried down and resuspended in chloroform. Samples were resolved by t.l.c. on silica-gel 60G plates using chloroform/methanol/acetic acid (13:3:1, by vol.) [20]. Ceramide 1-phosphate and C_2-ceramide phosphate were identified by autoradiography as single spots.

Data analysis

Results are expressed as mean ± S.E.M. of at least three separate experiments performed in duplicate or triplicate. Statistical significance was evaluated using Student's *t* test.

RESULTS

Effect of TNFα on superoxide generation

TNFα stimulated the generation of superoxide anions in a time- and concentration-dependent manner. The half-maximally and maximally effective concentration of TNFα for generation of superoxide were 0.3 nM and 10 nM respectively (Figure 1a), and the maximal response was obtained after 15–20 min with approx. 60 % of cells scoring positive for NBT reduction (Figure 1b). Superoxide dismutase inhibited NBT reduction induced by TNFα and fMLP over a similar concentration range, confirming that the assay was dependent on generation of superoxide anions. The half-maximally effective and maximally effective concentrations of superoxide dismutase for inhibition of NBT reduction by both stimuli were 25 μg/ml and 200 μg/ml respectively (results not shown).

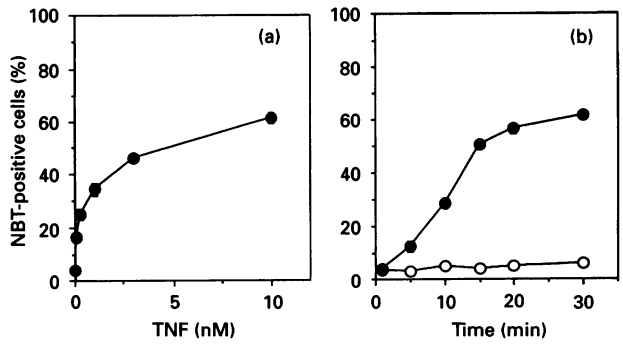

Figure 1 Effect of TNFα on superoxide generation

(**a**) Concentration dependence. Human neutrophils were incubated with various concentrations of TNFα for 20 min. (**b**) Time course. The cells are stimulated with (●) or without (○) 10 nM TNFα for the indicated time. Superoxide generation was measured using the NBT reduction assay as described in the Materials and methods section. The results are the mean ± S.E.M. of three experiments, performed in duplicate.

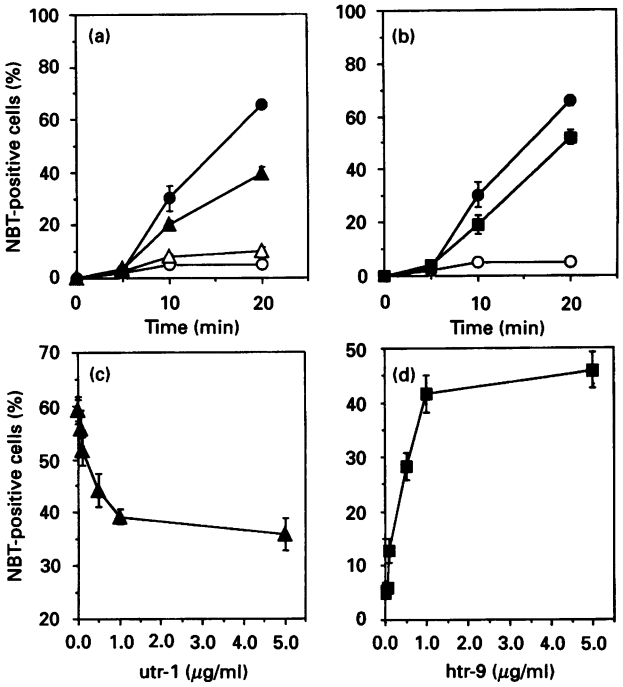

Figure 2 Effect of mAbs utr-1 and htr-9 on superoxide generation

(**a**) and (**b**) show time courses and (**c**) and (**d**) concentration dependence. (**a**) Neutrophils pretreated with (△, ▲) or without (○, ●) mAb utr-1 (5 μg/ml) for 10 min at 37 °C were incubated with (closed symbol) or without (open symbol) 10 nM TNFα for the indicated period. (**b**) The cells were stimulated with 10 nM TNFα (●), 5 μg/ml mAb htr-9 (■), or without stimulant (○). (**c**) Neutrophils pretreated with various concentrations of utr-1 for 10 min at 37 °C were incubated with 10 nM TNFα for 20 min. (**d**) Neutrophils were incubated with various concentrations of htr-9 for 20 min at 37 °C. The results are the mean ± S.E.M. of three experiments, performed in duplicate.

Table 1 Effect of mAbs utr-1 and htr-9 on TNFα-induced superoxide generation

The cells were incubated with or without various stimuli for 20 min at 37 °C in the presence of 1 mg/ml NBT. The results are the mean ± S.E.M. of three experiments, performed in duplicate. Asterisk (*) denotes a significant difference from TNFα (10 nM) by Student's *t* test ($P < 0.01$).

Stimuli	NBT-positive cells (% of total)
Control	6.5 ± 0.8
TNFα (10 nM)	59.4 ± 2.6
TNFα (100 nm)	60.1 ± 2.8
utr-1 (5 μg/ml)	7.3 ± 0.8
TNFα (10 nM) + utr-1 (5 μg/ml)	44.5 ± 3.3*
TNFα (100 nM) + utr-1 (5 μg/ml)	64.5 ± 5.2
htr-9 (5 μg/ml)	46.2 ± 3.7*
TNFα (10 nM) + htr-9 (5 μg/ml)	59.0 ± 3.0

Effect of monoclonal antibodies (mAbs) utr-1 and htr-9 on superoxide generation

Two major types of receptor for TNF with apparent molecular masses of 75 kDa and 55 kDa have been identified [7,8]. To clarify whether one or both of them mediate stimulation of superoxide generation, we used two different mAbs, utr-1 and htr-9.

The mAb utr-1 was raised against the 75 kDa receptor and behaves as an antagonist to TNFα at this receptor [21,22]. Although utr-1 (5 μg/ml) alone had no effect on superoxide generation (Figure 2a), it partially inhibited the effect of TNFα (10 nM) in a concentration-dependent manner with a maximal inhibitory effect of approx. 35% at 5 μg/ml (Figure 2c). This concentration of utr-1 had no effect on the response to fMLP (results not shown). The inhibitory effect of 5 μg/ml utr-1 could be completely overcome by raising the concentration of TNFα to 100 nM, showing that utr-1 behaves as a competitive antagonist (Table 1), consistent with previous reports [21].

The mAb htr-9 was raised against the 55 kDa receptor and behaves as an agonist at this receptor [22,23]. Htr-9 (5 μg/ml) stimulated NBT reduction in a concentration-dependent manner but with an approx. 25% lower maximal response to that induced by TNFα (Figures 2b and 2d). However, when maximally effective concentrations of htr-9 and TNFα were co-administered the response was not significantly different from that induced by TNFα. This demonstrates that htr-9 is not acting as a partial agonist at the 55 kDa receptor and that therefore the generation of superoxide anions induced by TNFα is mediated through both the 55 and 75 kDa receptors.

To confirm the specificity of utr-1 and htr-9, the effect of two mAbs of the same class (IgG1) were examined. One mAb was raised against the external domain of CD32, and the other was raised against the external domain of CD13. Neither mAb induced superoxide generation or altered the response to TNFα (results not shown).

Table 2 Effect of exogenous sphingomyelinase and C_2-ceramide on superoxide generation

Cells pretreated with or without 5 μM cytochalasin B for 10 min were incubated with various stimuli for 10 min and then stimulated with or without 10 nM fMLP for a further 10 min. The results are the mean ± S.E.M. of three experiments, carried out in duplicate.

Cytochalasin B (5 μM) treatment	Stimuli	Stimulation...	NBT-positive cells (% of total) None	fMLP (10 nM)
(−)	Control		3.1 ± 0.7	45.3 ± 3.9
	TNFα (10 nM)		51.3 ± 4.2	77.2 ± 5.4
	Sphingomyelinase (1 munit/ml)		3.2 ± 0.7	48.6 ± 3.2
	C_2-ceramide (30 μM)		2.4 ± 0.4	48.8 ± 5.1
(+)	Control		2.4 ± 0.6	45.4 ± 7.0
	TNFα (10 nM)		5.4 ± 0.5	94.3 ± 1.0
	Sphingomyelinase (1 munit/ml)		2.1 ± 0.4	46.3 ± 4.9
	C_2-ceramide (30 μM)		2.1 ± 0.5	45.7 ± 6.3

Effect of sphingomyelinase and C_2-ceramide on superoxide generation

Recently, TNFα has been shown to activate a sphingomyelinase to generate ceramide and it has been suggested that this has an important role in TNFα-receptor signalling [9–14]. We therefore examined the effect of sphingomyelinase and C_2-ceramide, a cell-permeable analogue of ceramide, on superoxide generation to clarify whether or not ceramide mediates TNFα signalling in human neutrophils.

TNFα induced the formation of superoxide anions measured

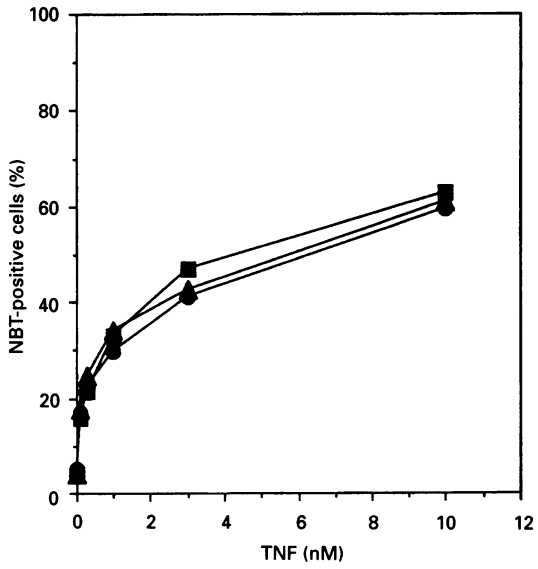

Figure 3 Effect of pretreatment with sphingomyelinase and C_2-ceramide on TNFα concentration dependence for superoxide generation

The cells were pretreated with or without 1 munit/ml sphingomyelinase or 30 μM C_2-ceramide for 10 min and stimulated with various concentrations of TNFα for 20 min. The results are the mean ± S.E.M. of two experiments, performed in duplicate. ●, TNFα, ▲; TNFα + sphingomyelinase, ■; TNFα + C_2-ceramide.

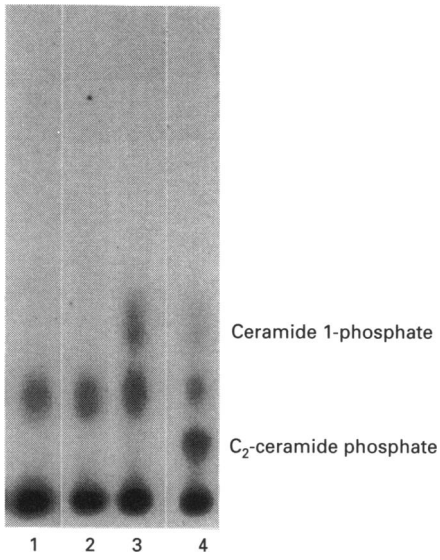

Figure 4 Effect of TNFα, sphingomyelinase, and C_2-ceramide on the production of ceramide 1-phosphate and C_2-ceramide phosphate

The cells were labelled with [^{32}P]P_i, stimulated with agonists for 5 min and lipids extracted as described in the Materials and methods section. Lane 1, control; lane 2, TNFα 10 nM; lane 3, sphingomyelinase 1 munit/ml; lane 4, C_2-ceramide 30 μM. Ceramide 1-phosphate and C_2-ceramide phosphate were indicated using standards prepared as described in the Materials and methods section. The data are representative of five experiments.

Table 3 Effect of TNFα, utr-1, htr-9, sphingomyelinase and C_2-ceramide on cell-shape change

The cells were incubated with or without various stimuli for 20 min at 37 °C and fixed with 0.25% of glutaraldehyde and analysed for shape change as described in the Materials and methods section. For the experiments using utr-1, cells were pretreated with utr-1 for 10 min at 37 °C and then incubated with or without TNFα. The results are the mean ± S.E.M. of three experiments, carried out in duplicate. Asterisk (*) denotes significant difference from TNFα (10 nM) by Student's t test ($P < 0.01$).

Stimuli	Shape-changed cells (% of total)
Control	6.6 ± 0.8
TNFα (10 nM)	87.0 ± 1.6
utr-1 (5 μg/ml)	7.4 ± 0.5
utr-1 (5 μg/ml) + TNFα (10 nM)	64.7 ± 1.4*
htr-9 (5 μg/ml)	64.2 ± 1.3*
Sphingomyelinase (1 munit/ml)	6.0 ± 0.7
C_2-ceramide (30 μM)	5.5 ± 0.6
fMLP (10 nM)	90.0 ± 1.4

using the NBT assay and this was partially additive with fMLP (Table 2). In contrast, neither sphingomyelinase (1 munit/ml) nor C_2-ceramide (30 μM) had a significant effect on superoxide generation or altered the response to fMLP. Furthermore, the concentration-dependence curve of TNFα was not altered by pretreatment with sphingomyelinase (1 munit/ml) or C_2-ceramide (30 μM) (Figure 3). In the presence of cytochalasin B, TNFα only induces priming of the superoxide response to other stimuli e.g. fMLP, and this is exemplified in Table 2. In contrast, neither sphingomyelinase nor C_2-ceramide altered the response to fMLP in the presence of cytochalasin B. These results suggest that sphingomyelinase activation does not mediate the direct or priming effect of TNFα on superoxide generation.

Effect of TNFα, sphingomyelinase and C_2-ceramide on shape change in neutrophils

TNFα (10 nM) and fMLP (10 nM) induced a change in shape of 89.8 ± 0.7% and 90.0 ± 1.4% of cells respectively, during a 20 min incubation (Table 3); in contrast, neither sphingomyelinase nor C_2-ceramide induced a change in cell shape. Thus, sphingomyelinase and C_2-ceramide are also unable to mimic the effect of TNFα on another functional response, cell-shape change. The effect of mAbs utr-1 and htr-9 on shape change in neutrophils was also examined. While utr-1 (5 μg/ml) alone had no effect on shape change, it partially inhibited the effect of TNFα (Table 3). The mAb htr-9, at a concentration that was maximally effective for the generation of superoxide anions (5 μg/ml), mimicked the effect of TNFα (10 nM) although with a lower response (Table 3). These results demonstrate that the two mAbs affect shape change in a similar manner to superoxide generation.

Effect of TNFα, sphingomyelinase and C_2-ceramide on production of ceramide 1-phosphate and C_2-ceramide phosphate

The enzyme sphingomyelinase acts on sphingomyelin to produce ceramide, which is then converted into ceramide 1-phosphate by ceramide kinase [20]. Measurement of [^{32}P]ceramide 1-phosphate can be used to monitor the effect of TNFα on sphingomyelinase activity in neutrophils. Exogenous sphingomyelinase and C_2-ceramide increased the amounts of ceramide 1-phosphate and C_2-ceramide phosphate respectively (Figure 4); the level of these metabolites increased steadily over a 20 min incubation (Figure 5 and not shown). In contrast, TNFα (10 nM) had no effect on

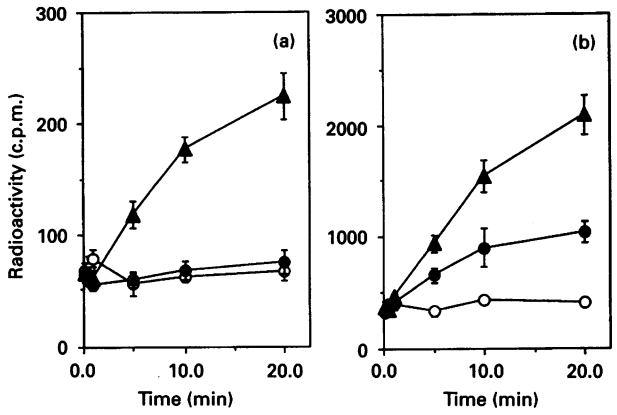

Figure 5 TNFα- and sphingomyelinase-induced formation of ceramide 1-phosphate

Cells (5×10^6 cells/tube), prelabelled with [^{32}P]P_i, were stimulated with 10 nM TNFα or 1 munit/ml sphingomyelinase. (**a**) Neutrophils, (**b**) HL-60 cells. The results are the mean ± S.E.M. of three experiments. ○, control; ●, TNFα 10 nM; ▲, sphingomyelinase 1 munit/ml.

the level of ceramide 1-phosphate in neutrophils over a period of 10 s–20 min (Figures 4 and 5a).

As we and others have previously reported that TNFα stimulates metabolism of [^{3}H]sphingomyelin in HL-60 cells, a human leukaemia cell line, we examined the effect of TNFα on [^{32}P]ceramide 1-phosphate formation in this system. The kinetics of production of ceramide 1-phosphate in HL-60 cells are shown in Figure 5(b). Exogenous sphingomyelinase and TNFα increased the amount of ceramide 1-phosphate over a 20 min period. Thus, TNFα stimulates sphingomyelinase activity in HL-60 cells but not in human neutrophils.

DISCUSSION

In an attempt to clarify whether the 75 kDa or 55 kDa types of receptor mediate the action of TNFα in human neutrophils, the effect on superoxide generation of mAbs raised against these two receptors were examined. The mAb utr-1, which was raised against the 75 kDa receptor and behaves as an antagonist, had no effect on its own but partially inhibited the number of NBT-positive cells induced by TNFα on superoxide generation. The mAb htr-9, which was raised against the 55 kDa receptor and behaves as an agonist, mimicked the effect of TNFα but with a lower maximal effect. These results suggest that TNFα stimulates superoxide generation in human neutrophils through both types of receptor. It is unclear, however, whether these results reflect 'cross-talk' between the signalling pathways linked to the 55 and 75 kDa receptors or the presence of two populations of neutrophils which express either the 55 or 75 kDa receptor types. Recently, it has been reported that the 55 kDa receptor mediates stimulation of phospholipase A_2 and sphingomyelinase, induction of NF-kB, accumulation of c-fos, synthesis of prostaglandin E_2 and the inhibition of cell growth by TNFα. In contrast, the 75 kDa receptor has been reported to mediate the proliferation of thymocytes and of a cytotoxic T-cell line by TNFα [24–29].

TNFα has been shown to stimulate sphingomyelinase to generate ceramide in several cell types and it has been suggested that ceramide may be a second messenger mediating the effect of TNFα. We have therefore compared the action of exogenous sphingomyelinase and C_2-ceramide, a cell-permeable analogue of ceramide, with TNFα on the generation of superoxide, the induction of priming in the presence of cytochalasin B, and shape change in human neutrophils. We have also examined the effect of TNFα on sphingomyelin hydrolysis in this cell type. Neither sphingomyelinase nor C_2-ceramide mimicked or mediated the effect of TNFα on any of these responses. Further, TNFα had no effect on sphingomyelin hydrolysis in human neutrophils, although it did stimulate this pathway in HL-60 cells. The ability of TNFα to induce activation of sphingomyelinase in HL-60 cells through the 55 kDa receptor [11,25], in contrast with the results in neutrophils, suggests either that this receptor is able to couple with different effector systems in different cell types or, perhaps more likely, that activation of sphingomyelinase is a 'downstream' event from the primary response to receptor activation. This is analogous to the regulation of phospholipase D which can occur downstream from the activation of phospholipase C in some cell types [30].

The levels of [^{32}P]ceramide 1-phosphate in HL-60 cells are approx. 10-fold higher than those in neutrophils despite the use of a similar cell number in both experiments. The explanation for this difference is not known but may indicate a lower expression of ceramide kinase (i.e. the enzyme that converts ceramide into ceramide 1-phosphate) in neutrophils. This possibility is consistent with the apparent unimportance of the sphingomyelinase pathway in TNFα-induced responses in neutrophils, as suggested by the lack of effect of C_2-ceramide or sphingomyelinase on TNFα-induced responses. It is possible therefore that several of the enzymes involved in the sphingomyelin pathway will be absent, or present in low levels, in neutrophils.

Thus, in conclusion, sphingomyelin metabolism does not mediate the activation of TNFα in neutrophils and is therefore not a universal second messenger for this cytokine.

This work was supported by the ARC. We are grateful to Dr. Y. Shimizu, Yamanouchi, Oxford, U.K. for advice on the NBT assay and preparation of neutrophils. We are grateful to Dr. R. Foulkes, Celltech for kindly supplying TNFα, Dr. M. Brockhaus, Roche for supplying mAbs utr-1 and htr-9, Dr K. Pulsord and Dr K. Micklan for supplying mAbs raised against CD13 and CD32, and Dr. Y. A. Hanuun for supplying C_2-ceramide. S.P.W. is a Royal Society University Research Fellow.

REFERENCES

1 Lehrer, R. I. (1988) Annu. Intern. Med. **109**, 127–142
2 Klebnoff, S. J., Vadas, M. A., Harlan, J. M., Sparks, L. H., Gamble, J. R., Agosti, J. M. and Waltersdroph, A. M. (1986) J. Immunol. **136**, 4220–4225
3 Ming, J. I. W., Bersani, L. and Mantovani, A. (1987) J. Immunol. **138**, 1469–1474
4 Gamble, J. R., Harlan, J. M., Klebnoff, S. J. and Vadas, M. A. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 8667–8671
5 Nathan, C. F. (1987) J. Clin. Invest. **80**, 1550–1560
6 Shimizu, Y., Daniels, R. H., Elmore, M. A., Finnen, M. J., Hill, M. E. and Lackie, J. M. (1993) Biochem. Pharmacol. **45**, 1743–1751
7 Loetscher, H., Pan, Y. E., Lahm, H. W., Gentz, R., Brockhous, M., Tauchi, H. and Lesslauer, W. (1990) Cell **61**, 351–359
8 Schall, T., Lewis, M., Koller, K. J., Lee, A., Rice, G. C., Wong, G. H. W., Gatanaga, T., Granger, G. A., Leutz, R., Rabb, H., Kohr, W. J. and Goeddel, D. V. (1990) Cell **61**, 361–370
9 Dressler, K. A., Mathias, S. and Kolesnick, R. N. (1992) Science **255**, 1715–1717
10 Kim, M. Y., Linardic, C., Obeid, L. and Hannun, A. Y. (1991) J. Biol. Chem. **266**, 484–489
11 Yanaga, F. and Watson, S. P. (1992) FEBS Lett. **314**, 297–300
12 Olivera, A., Buckley, N. E. and Spiegel, S. (1992) J. Biol. Chem. **267**, 26121–26127
13 Obeid, L. M., Linardic, C. M., Karolarck, L. A. and Hannun, Y. A. (1993) Science **259**, 1769–1771
14 Schutze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K. and Kronke, M. (1992) Cell **71**, 765–776
15 Korchak, H. M., Vosshal, L. B., Zagon, G., Ljubich, P., Rich, A. M. and Weissman, G. (1988) J. Biol. Chem. **263**, 11090–11097
16 Park, B. H., Fikring, S. M. and Smithwick, E. M. (1968) Lancet **2**, 532–534
17 Shields, J. M. and Haston, W. S. (1985) J. Cell Sci. **74**, 75–93

18 Hubscher, G., Hawthorne, J. N. and Kemp, P. (1960) J. Lipid Res. **1**, 433–438
19 Schneider, E. G. and Kennedy, E. (1973) J. Biol. Chem. **248**, 3739–3741
20 Dressler, K. A. and Kolesnick, R. N. (1990) J. Biol. Chem. **265**, 14917–14921
21 Shalaby, M. R., Sundan, A., Loetscher, H., Brockhous, M., Lasslauer, W. and Espevik, T. (1990) J. Exp. Med. **172**, 1517–1520
22 Hohmann, H., Brockhous, M., Baeuerle, P. A., Remy, R., Kolbeck, R. and von Loon, A. P. G. M. (1990) J. Biol. Chem. **265**, 22409–22417
23 Espevick, T., Brokhaus, M., Loetscher, H., Nostad, U. and Shalaby, R. (1990) J. Exp. Med. **171**, 415–426
24 Brockhous, M., Schoenfeld, H. J., Schlaeger, E. J., Hunziker, W., Lesslauer, W. and Loetscher, H. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 3127–3131
25 Wiegman, K., Schutze, S., Kampen, E., Himmler, A., Machleidt, T. and Kronke, M. (1992) J. Biol. Chem. **267**, 17997–18001
26 Tartaglia, L. A., Weber, R. F., Figari, I. S., Reynolds, C., Palladino, M. A. and Goeddel, D. V. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 9292–9296
27 Engelman, H., Holtmann, H., Brakebusch, C., Avni, Y. S., Sarov, I., Nophar, Y., Hadas, E., Leitner, O. and Wallach, D. (1990) J. Biol. Chem. **265**, 14497–14504
28 Thoma, B., Grell, M., Pfizenmaier, K. and Scheurich, P. (1990) J. Exp. Med. **172**, 1019–1023
29 Kruppa, G., Thoma, B., Machleidt, T., Wiegmann, K. and Kronke, M. (1992) J. Immunol. **148**, 3152–3157
30 Billah, M. M. and Anthes, J. C. (1990) Biochem. J. **269**, 281–291

Received 18 June 1993/5 October 1993; accepted 1 November 1993