Modulation of transmembrane signalling in HL-60 granulocytes by tumour necrosis factor- α

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Differentiated HL-60 granulocytes were used to study the mechanism by which tumour necrosis factor- α (TNF) enhances responses to *N*-formyl-methionyl-leucylphenylalanine (FMLP). Cultivation of differentiated HL-60 cells with 100 units of TNF/ml for 24 h resulted in a 3-fold increase in superoxide release and 4-fold increase in prostaglandin E_2 production on stimulation with 1 μ M-FMLP. On the other hand, cultivation with TNF failed to increase phorbol diester stimulation of superoxide release. Formyl-peptide-receptor expression determined on isolated membranes from cells cultivated with TNF (TNF-M) was increased by 50 % compared with membranes from control cells (NM). Similarly, FMLP binding to intact HL-60 cells was increased by cultivation with TNF. Guanine-nucleotide-binding proteins (G-protein) levels were not different between TNF-M and NM, as determined by pertussis-toxin-catalysed ADP-ribosylation and by immunoblotting with antisera recognizing α_2 subunit. Binding of guanosine 5'-[γ -thio]triphosphate and GTP hydrolysis stimulated by FMLP were enhanced by about 50 % in TNF-M. The efficiency of G-protein activation by formyl-peptide receptors did not differ between TNF-M and NM. TNF regulates expression of formyl-peptide receptors independently of G-protein levels. The regulation of receptor expression is one mechanism by which TNF enhances cell responses to formylated peptides.

INTRODUCTION

Tumour necrosis factor- α (TNF) is a M_r -17000 glycoprotein described originally as a factor which produced haemorrhagic necrosis of tumours. Subsequent delineation of the actions of this cytokine suggests that it plays a prominent role in the regulation of inflammatory responses. TNF induces a 'primed' state in neutrophils, characterized by increased neutrophil adherence [1,2], increased antibody-dependent tumour-cell cytotoxicity [3], and an enhanced oxidative burst [4–9]. TNF also enhances neutrophil expression of complement receptors and the adhesive protein, Mac-1 [10,11]. A role for TNF in inflammatory responses *in vivo* is supported by increased serum levels of TNF in children with Gram-negative septicemia and following infusion of endotoxin to normal individuals [12,13]. In addition, administration of anti-TNF antibodies prevented fatal bacterial septicemia in animal models [14,15], whereas treatment with TNF protected against fatal *Legionella pneumophila* infection in mice [16].

Neutrophil responses to bacterial infections are thought to be stimulated by formyl peptides, such as *N*-formylmethionyl-leucyl-phenylalanine (FMLP) [17]. Formyl-peptide receptors are one of a family of receptors coupled to intracellular effector enzymes by guanine-nucleotide-binding proteins (G-proteins). Atkinson *et al.* [4] reported that short-term exposure of neutrophils to TNF altered the dissociation constants of formyl-peptide receptors without changing the total number of receptors sites. They suggested that the functional alterations produced by TNF were related to the altered receptor affinities.

To examine the mechanism by which TNF primes the respiratory burst, we studied the effect of exposure of HL-60 granulocytes to TNF. Differentiated HL-60 cells acquire many of the features of neutrophils, including receptors for formyl

peptides, the ability to undergo directed migration to chemo-attractants, and the ability to produce superoxide in response to FMLP [18–20]. The results demonstrate that TNF enhances transmembrane signalling by regulating the expression of formyl-peptide receptors independently of G-protein levels.

MATERIALS AND METHODS

Reagents

FMLP was obtained from Sigma (St. Louis, MO, U.S.A.). GDP, GTP and guanosine 5'-[γ -thio]triphosphate (GTP[S]) were obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Recombinant human TNF (specific activity 5.2×10^7 units/mg) was a gift from Dr. Christine Czarnecki (Genentech, South San Francisco, CA, U.S.A.). FMLP-[3 H]P (55–60 Ci/mol), GTP-[35 S] (1200–1300 Ci/mol), [γ - 32 P]GTP and [32 P]NAD $^+$ were from DuPont–New England Nuclear (Boston, MA, U.S.A.). Pertussis toxin was from List Biological Laboratories (Campbell, CA, U.S.A.).

Cell culture and membrane preparation

HL-60 cells were grown in suspension culture in RPMI 1640 supplemented with 10 % (v/v) horse serum, 1 % (w/v) non-essential amino acids, 2 mM-L-glutamine, 50 units of penicillin/ml and 50 μ g of streptomycin/ml in a humidified atmosphere at 37 °C in 8 % CO $_2$. To induce myeloid differentiation, cells were seeded at a density of 10^6 cells/ml and were cultivated for 5 days in medium containing 1.25 % (v/v) dimethyl sulphoxide. HL-60 cells membranes were prepared as previously described, except that centrifugation through Percoll gradients was omitted [21].

Abbreviations used: FMLP, *N*-formylmethionyl-leucyl-phenylalanine; G-proteins, guanine-nucleotide-binding proteins; GTP[S], guanosine 5'-[γ -thio]triphosphate; NM, plasma membranes prepared from normal differentiated HL-60 cells; PGE $_2$, prostaglandin E_2 ; PMA, phorbol myristate acetate; TNF, tumour necrosis factor- α ; TNF-M, plasma membranes prepared from HL-60 cells cultivated with TNF.

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FMLP binding

FMLP binding was determined in a reaction mixture (100 μ l) containing 20 mM-Tris, 1 mM-EDTA, 5 mM-MgCl₂ (unless otherwise stated) and FML[³H]P. The reaction was initiated by addition of 15–25 μ g of membrane protein to each tube. The reaction was terminated after 30 min at 25 °C by rapid filtration through GF/C Whatman filters, followed by washing with 4 $\times$ 2.5 ml of ice-cold buffer containing 50 mM-triethanolamine/HCl, 5 mM-MgCl₂ and 1 mM-EDTA. Filters were dried and counted for radioactivity in a liquid-scintillation spectrometer. Membrane protein was determined as described by Bradford [22], with bovine IgG as the standard. Specific binding was calculated as fmol of FMLP/mg of membrane protein by subtracting the amount of FML[³H]P bound in the presence of 10 μ M-FMLP from the total FML[³H]P bound.

Binding parameters were estimated by using a non-linear least-squares curve-fitting procedure (SCTFIT). The variance function used by SCTFIT to assign weights to data points is $\text{var}(y) = a_0 + a_1 y^{a_2}$, where y is the amount of radioligand bound to the membrane. The values for a_0 , a_1 and a_2 used (2.09×10^{-6} , 1.58×10^{-3} and 1.53 respectively) were obtained from analysing 130 triplicate determinations of FML[³H]P binding to HL-60-cell membranes [23]. Testing for statistically significant resolution of two receptor subtypes was performed by comparing the residual variance of the fit obtained when the data were analysed in accordance with a model for a single class or two classes of binding sites, using a partial F-test [24].

Binding of FML[³H]P to intact HL-60 cells was performed at 4 °C in a total volume of 120 μ l. HL-60 cells at a concentration of 10^6 /ml were incubated for 24 h in the absence or presence of 100 units of TNF/ml. The cells were washed free of medium in ice-cold Ca²⁺-free Hanks' balanced salts solution (HBSS), then resuspended in ice-cold HBSS with 1.3 mM-Ca²⁺ at a concentration of 10^7 /ml. Portions (100 μ l) of cells were added to duplicate tubes containing 20 μ l of FML[³H]P at various concentrations (2–45 nM) and incubated at 4 °C for 30 min. Binding was measured by filtration through glass-fibre filters (GF/C) and quantified by liquid-scintillation spectrometry.

GTP[S] binding

GTP[S] binding was determined in a reaction mixture (100 μ l) containing 50 mM-triethanolamine/HCl, pH 7.4, 1 mM-dithiothreitol, 1 mM-EDTA, 5 mM-MgCl₂, 150 mM-NaCl, 0.5 μ M-GDP and GTP[³⁵S] (0.02–0.04 μ Ci per tube). The reaction was initiated by addition of 2–10 μ g of membrane protein to each tube. The reaction was terminated after 20 min at 30 °C by rapid filtration through Whatman GF/C filters, followed by washes with 4 $\times$ 2.5 ml of ice-cold buffer containing 50 mM-triethanolamine/HCl and 5 mM-MgCl₂. Filters were dried and counted for radioactivity in a liquid-scintillation spectrometer. Specific binding was calculated as fmol of GTP[S] bound/mg of membrane protein by subtracting GTP[³⁵S] bound in the presence of 10 μ M-GTP[S] from the total GTP[³⁵S] bound.

GTPase assay

Hydrolysis of [γ -³²P]GTP (0.02–0.05 μ Ci per tube) was determined in a reaction mixture (100 μ l) containing 2–10 μ g of membrane protein, 50 mM-triethanolamine/HCl, pH 7.4, 1 mM-dithiothreitol, 0.1 mM-ATP, 5 mM-MgCl₂, 1 mM-EDTA, 100 mM-NaCl, 0.1 μ M-GTP, 5 mM-phosphocreatine, 0.4 mg of creatine kinase/ml and 0.2 % BSA. Reactions were performed for 10 min at 30 °C in the absence or presence of FMLP. Reactions were terminated and high-affinity GTPase activity was determined as previously described [25]. Results were expressed as pmol of free P_i generated/min per mg of protein.

O₂⁻ assay

O₂⁻ generation was measured by cytochrome *c* (type VI, Sigma) reduction. All experiments were performed in duplicate in a volume of 1 ml in micro-centrifuge tubes containing 4×10^6 cells and 80 mM-cytochrome *c*. At 10 min after addition of the stimulus, tubes were centrifuged at 2000 *g* for 30 s. The amount of reduced cytochrome *c* was calculated from the A_{550} of the supernatant by using the absorption coefficient of $2.1 \text{ M}^{-1} \cdot \text{cm}^{-1}$. O₂⁻ generation was expressed as nmol of reduced cytochrome *c*/4 $\times 10^6$ cells.

Pertussis-toxin-stimulated ADP-ribosylation

Pertussis toxin (100 μ g/ml) was activated by dilution with an equal volume of 100 mM-dithiothreitol and incubated for 10 min at 30 °C. Membranes (40–100 μ g of protein) were incubated for 60 min at 37 °C in a volume of 50 μ l containing 200 mM-Tris/HCl, pH 7.5, 6.3 mM-MgCl₂, 2 mM-ATP, 2 mM-thymidine, 2 mM-arginine, 100 μ g of activated toxin/ml and 1 μ M-[³²P]NAD⁺ (20 μ Ci/nmol). After this incubation, 50 μ l of detergent buffer (125 mM-Tris/HCl, pH 6.8, 4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol) was added, the samples were boiled for 5 min at 95 °C, and then subjected to SDS/PAGE (10 % gels). Autoradiography of the dried gels was performed.

Immunoblotting

Membrane protein was subjected to SDS/PAGE, and the separated proteins were transferred electrophoretically to nitrocellulose paper. An antibody to G-protein subunits α_1 and α_2 (AS/7) produced in rabbits was used at a dilution of 1:500. Peroxidase-labelled anti-rabbit IgG was used to detect protein bands.

Prostaglandin E₂ (PGE₂) assay

The concentrations of PGE₂ were determined by radioimmunoassay. Antibodies to PGE₂ were obtained from Sigma, and cross-reacted by < 1 % with all prostaglandins, except for 3.2 % with PGE₁. The radioimmunoassay was performed in a reaction mixture (final volume 100 μ l) containing 0.1 M-phosphate buffer (pH 7.4), 0.15 M-NaCl, 0.1 % NaN₃, antibody and [³H]PGE₂. After incubation for 10 min at room temperature and then at 16 h at 4 °C, a charcoal-dextran mixture was added and the suspension centrifuged at 1000 *g* for 10 min. The radioactivity in the supernatant was determined by scintillation spectrometry. All assays were performed in duplicate.

RESULTS

Superoxide and PGE₂ response

We have reported that cultivation of HL-60 granulocytes with 100 units of TNF/ml for 24 h is optimal for TNF-enhanced superoxide release from HL-60 cells upon stimulation by FMLP [26]. PGE₂ production stimulated by FMLP in control cells and HL-60 cells cultivated with 100 units of TNF/ml for 24 h is shown in Table 1. Cultivation with TNF did not alter the PGE₂ production by unstimulated HL-60 cells. PGE₂ released upon stimulation with an optimal (1 μ M) and a sub-optimal (30 nM) concentration of FMLP was increased about 4-fold in cells pretreated with TNF.

To determine if TNF enhanced cell responses by altering transmembrane signalling or intracellular effector enzymes, superoxide production after stimulation with FMLP or the diester phorbol myristate acetate (PMA) was compared. Table 2 demonstrates that FMLP-stimulated superoxide production is enhanced 3-fold by cultivation with TNF. The HL-60 cell superoxide response was greater after PMA stimulation than

Table 1. PGE₂ production following FMLP stimulation

Results are presented as PGE₂ concentrations (pg/ml) in the cell supernatant 15 min after addition of stimuli as means $\pm$ S.E.M. for four separate experiments: * significantly different from control cells ($P \leq 0.05$). Differentiated HL-60 cells pretreated with TNF were cultivated with 100 units of TNF/ml for 24 h, whereas control cells were treated identically except for omission of TNF.

[FMLP]	Pretreatment ...	PGE ₂ (pg/ml)	
		Control	TNF
0		41.2 $\pm$ 16.2	39.0 $\pm$ 7.0
30 nM		126.1 $\pm$ 30.3	480.2 $\pm$ 96.4*
1 μ M		168.4 $\pm$ 35.2	665.5 $\pm$ 132.2*

Table 2. Effect of TNF on superoxide production in response to FMLP or PMA

Results are presented as nmol of reduced cytochrome (Cyt.) *c*/4 $\times$ 10⁶ cells, as means $\pm$ S.D. for the numbers of experiments in parentheses: * significantly different from control cells ($P < 0.05$). Differentiated HL-60 cells pretreated with TNF were cultivated with 100 U/ml TNF for 24 hr, while control cells were treated identically except for the omission of TNF.

Stimulus	Pretreatment ...	Cyt. <i>c</i> reduced (nmol/4 $\times$ 10 ⁶ cells)	
		Control	TNF
Basal	(8)	2.8 $\pm$ 1.0	3.4 $\pm$ 2.0
FMLP (0.1 μ M)	(8)	10.4 $\pm$ 2.1	31.1 $\pm$ 2.2
PMA			
100 nM	(5)	35.6 $\pm$ 10.2	41.2 $\pm$ 7.0
10 nM	(4)	42.4 $\pm$ 9.6	47.0 $\pm$ 6.5
1 nM	(3)	2.8 $\pm$ 1.0	4.6 $\pm$ 2.8

after FMLP stimulation. Cultivation with TNF did not result in a significant increase in superoxide release stimulated by optimal or sub-optimal concentrations of PMA. The threshold concentration at which PMA induced superoxide release was not decreased by TNF pretreatment. Exposure of cells to TNF alone failed to stimulate superoxide production. These results suggest that TNF enhances HL-60 cell responses to FMLP by altering receptor-mediated transmembrane signalling. Therefore formyl-peptide-receptor number and G-protein response to FMLP were determined in isolated membranes.

Regulation of formyl-peptide receptors

Membranes were prepared from differentiated HL-60 cells cultivated with 100 units of TNF/ml for 24 h (TNF-M) and from normal HL-60 cells from the same cell culture (NM). Formyl-peptide-receptor levels in TNF-M and NM were examined by radioligand-binding assays. Scatchard transformation of the binding data gave curvilinear plots (Fig. 1). A computerized non-linear least-squares curve-fitting procedure indicated that a two-site model was a significantly better fit for both membranes than a one site model ($P < 0.001$). Table 3 compares the dissociation constants and $B_{\max}$ for high- and low-affinity binding sites

Fig. 1. Scatchard transformation of FMLP binding to TNF-M (■) and NM (▲)

A two-site model provided a better fit than a one-site model. The dissociation constants for high-affinity binding sites were not different (0.334 versus 0.379 nM, TNF-M versus NM), whereas the dissociation constants of low-affinity binding sites were different in this experiment (2.88 versus 27.6 nM, TNF-M versus NM). The number of high-affinity binding sites was 837 fmol/mg for TNF-M and 506 fmol/mg for NM. The number of low-affinity binding sites was 1066 fmol/mg for TNF-M and 715 fmol/mg for NM.

Table 3. Comparison of formyl-peptide receptor number and affinities between TNF-M and NM

Results are presented as means $\pm$ S.E.M. for four separate experiments: * significantly different from NM ($P < 0.05$). K_d is expressed as nM, and $B_{\max}$ as fmol/mg.

	TNF-M	NM
K_{d1}	0.323 $\pm$ 0.032	0.264 $\pm$ 0.079
$B_{\max 1}$	797 $\pm$ 22*	408 $\pm$ 100
K_{d2}	9.6 $\pm$ 5.2	11.5 $\pm$ 5.5
$B_{\max 2}$	827 $\pm$ 82	696 $\pm$ 116
Total receptor no.	1624 $\pm$ 99*	1104 $\pm$ 125

between TNF-M and NM. The total number of formyl-peptide receptors was increased by about 50 % in TNF-M. The dissociation constants for both the high- and low-affinity binding sites were similar in NM and TNF-M. To determine if TNF affected formyl-peptide receptors directly, FMLP binding to NM was performed in the absence or presence of 1000 units of TNF/ml. Addition of TNF to the binding assay did not alter either the dissociation constants (0.209 and 3.11 nM without TNF, versus 0.149 and 2.74 nM with TNF) or $B_{\max}$ (448 and 948 fmol/mg without TNF, versus 320 and 978 fmol/mg with TNF, for high-affinity and low-affinity binding sites respectively).

To determine if the increased receptor number found in membranes represented receptors accessible to FMLP on intact cells, binding of FMLP[³H] to formyl-peptide receptors on HL-60 granulocytes was compared on cells cultivated with or without 100 units of TNF/ml for 24 h. Fig. 2 shows the binding data for the two cell groups. Cells cultivated with TNF demonstrated an increase in specific binding at all concentrations of FMLP[³H].

Under the conditions of our membrane assay, all of the increased formyl-peptide receptors demonstrated high-affinity binding. The proportion of FMLP-binding sites expressing high and low affinities has been shown to be regulated by guanine nucleotides and by Mg²⁺ [27,28]. To determine if the high-affinity

Fig. 2. FMLP specific binding to differentiated HL-60 cells cultivated in the presence (●) or absence (▲) of 100 units of TNF/ml for 24 h

Results are means of triplicate determinations at each concentration of free FMLP [^3H]P, expressed as fmol of FMLP bound per 1×10^6 cells.

Fig. 3. Dose-response of the inhibition of FMLP binding by increasing concentrations of GTP[S] in TNF-M (■) and NM (▲)

Results represent means of triplicate samples from one experiment representative of three experiments. Results are expressed as fmol of FMLP [^3H]P bound per mg of membrane protein.

binding sites expressed on TNF-M were convertible into low-affinity sites, the ability of GTP[S] and MgCl_2 to alter FMLP binding to TNF-M was examined. Fig. 3 shows the dose-response of FMLP binding to increasing concentrations of GTP[S]. As expected from the equilibrium binding data, TNF-M demonstrated increased FMLP binding in the absence of GTP[S]. Increasing concentrations of GTP[S] inhibited FMLP binding in both TNF-M and NM. The optimal concentrations and half-maximal concentrations of GTP[S] were not different between TNF-M and NM. Optimal concentrations of GTP[S] decreased FMLP binding to similar levels in the two membrane preparations. To determine if the decreased FMLP binding to TNF-M in the presence of GTP[S] was due to alterations in the dissociation constants or in the numbers of high- and low-affinity binding sites, equilibrium binding was performed in the absence or presence of $10 \mu\text{M}$ -GTP[S]. Scatchard transformation of the binding data showed that GTP[S] decreased the number of

Fig. 4. (a) Autoradiogram of pertussis-toxin-mediated [^{32}P]ADP-ribosylation of TNF-M and NM, (b) immunoblot of α_2 content of TNF-M (right lane) and NM (left lane) using AS/7 antibody

(a) Equal amounts of membrane protein ($100 \mu\text{g}$) were incubated with activated pertussis toxin, subjected to SDS/PAGE (10% gel), and autoradiography of the dried gel was performed. The autoradiograms of NM are in the upper lane, and of TNF-M in the lower panel. Densitometry of the two lanes is also shown: that of the lane containing NM is shown in the upper panel and that of TNF-M in the lower panel. (b) Equal amounts of membrane protein ($50 \mu\text{g}$) were subjected to SDS/PAGE, transferred to nitrocellulose paper, and then probed with a 1:500 dilution of AS/7 antisera. Peroxidase-labelled anti-rabbit IgG was used to detect bound antisera.

binding sites expressing high affinity (70 versus 746 fmol/mg for $10 \mu\text{M}$ -GTP[S] versus 0 GTP[S]), whereas the number of sites expressing low affinity was similar (505 versus 749 fmol/mg for $10 \mu\text{M}$ -GTP[S] versus 0 GTP[S]). Dissociation constants for the high-affinity binding sites were similar (0.166 nM versus 0.370 nM for $10 \mu\text{M}$ -GTP[S] versus 0 GTP[S]), whereas GTP[S] increased the dissociation constant of low-affinity binding sites (31.1 nM versus 3.57 nM for $10 \mu\text{M}$ -GTP[S] versus 0 GTP[S]).

Scatchard transformation of the binding data from TNF-M in the presence of 0 and 4 mM- Mg^{2+} showed that the proportion of binding sites expressing a high affinity decreased from about 50% to 8% in the absence of Mg^{2+} (774 and 695 fmol/mg at 4 mM- Mg^{2+} , 85 and 915 fmol/mg at 0 Mg^{2+} , for high- and low-affinity binding sites respectively). The dissociation constants were not different (0.170 and 10.3 nM versus 0.233 and 8.26 nM for 0 versus 4 mM free Mg^{2+}). Thus the high-affinity formyl-peptide receptors in TNF-M were convertible into low-affinity sites by altering the concentrations of Mg^{2+} and guanine nucleotides.

Regulation of G-proteins

Formyl-peptide receptors have been shown to couple to G_{i2} and G_{i3} in HL-60 cells [29]. Pertussis toxin abolishes FMLP activation of G-proteins in HL-60 cell membrane [19] and catalyses the ADP-ribosylation of $\text{G}_{i\alpha}$ subunits, which displayed an M_r of 40 000–41 000 on SDS/polyacrylamide gels. The quantity of pertussis-toxin substrate in NM and TNF-M was compared by autoradiography and densitometry after [^{32}P]ADP-ribosylation of $\text{G}_{i\alpha}$ catalysed by pertussis toxin (Fig. 4a). The amount of pertussis-toxin-catalysed labelling was not different between the two membranes.

Membrane content of $\text{G}_{i\alpha 2}$ was also quantified by immunoblot analysis using the anti-peptide antiserum AS/7 [30]. This antibody recognizes both $\text{G}_{i\alpha 1}$ and $\text{G}_{i\alpha 2}$; however, $\text{G}_{i\alpha 1}$ is not

Fig. 5. Dose-response of GTP[S] binding (a) and GTP hydrolysis (b) to FMLP in TNF-M (■) and NM (▲)

Results are averages of triplicate determinations in a single experiment representative of four to nine experiments. GTP[S] binding was determined after a 20 min incubation of 2–10 μ g of membrane protein with FMLP and GTP[35 S] at 30 °C. Results are expressed as fmol of GTP[35 S] bound per mg of protein. Hydrolysis of [γ - 32 P]GTP was determined after a 10 min incubation at 30 °C of 2–10 μ g of membrane protein with the indicated concentrations of FMLP, by the release of free [32 P]P $_i$. Results are expressed as pmol of P $_i$ generated/min per mg of protein.

present in HL60 cells. In agreement with pertussis-toxin-catalysed ADP-ribosylation, levels of G α 2 were not different between NM and TNF-M (Fig. 4b). Thus the membrane expression of relevant G-proteins was not altered by cultivation with TNF or by the increase in receptor expression.

Functional receptor–G-protein coupling

The functional consequences of the increased number of formyl-peptide receptors induced by TNF was examined by measuring G-protein activation by FMLP. The ability of FMLP to stimulate GTP–GDP exchange, as measured by GTP[S] binding, and to stimulate GTP hydrolysis were compared in TNF-M and NM. Fig. 5 demonstrates that both GTP[S] binding and GTPase activity were enhanced in TNF-M. GTP[S] binding in response to 10 μ M-FMLP was enhanced by $56.8 \pm 13.1\%$ ($n = 4$) in TNF-M, and GTPase activity was enhanced by $46.9 \pm 11.9\%$ ($n = 9$). One explanation for the increased G-protein activation by FMLP is the increased formyl-peptide receptor number, as enhanced G-protein activation by FMLP was similar to the percentage increase in receptors (47%). A second possible explanation is more efficient G-protein activation

by individual formyl-peptide receptors. To determine if TNF altered receptor–G-protein interaction, the efficiency was calculated by dividing GTP hydrolysis (pmol/min per mg of membrane protein) by receptor number (pmol/mg of membrane protein). GTP molecules hydrolysed per receptor per min were 9.4 ± 1.4 (mean $\pm$ S.E.M.) in TNF-M and 9.3 ± 1.7 in NM ($n = 9$). Thus there was no difference in formyl-peptide-receptor efficiency of G-protein activation between TNF-M and NM.

DISCUSSION

TNF is known to enhance neutrophil response to a variety of stimuli [1–11]. Our results indicate that cultivation with TNF for 24 h enhances HL-60 granulocyte responses to FMLP. Both superoxide release and PGE $_2$ production in response to FMLP stimulation were significantly enhanced by pretreatment with TNF. Neither superoxide release nor PGE $_2$ production was stimulated by TNF alone in these non-adherent cells. Previous studies have shown that the optimal TNF concentration and time of exposure for enhanced HL-60 cell responses was 100 units/ml for 24 hr [26]. In order to localize the site of action of TNF, the superoxide responses to FMLP and PMA in control and TNF-treated cells were compared. TNF induced a 3-fold increase in superoxide release, as well as a 4-fold increase in PGE $_2$ production, in response to stimulation by FMLP. However, no significant increase in superoxide production after stimulation with optimal or sub-optimal concentrations of PMA was found. These results suggested that TNF may alter formyl-peptide-receptor transmembrane signalling. Therefore the levels of components of this signalling pathway and their functional interaction were studied.

Atkinson *et al.* [4] showed that TNF modulated the affinity, but not the number, of FMLP receptors on human neutrophils. They showed that incubation with TNF for 60 min converted receptors expressing high (2 nM) and low (180 nM) affinity into a single population with an intermediate affinity (40 nM). They suggested that this alteration in affinity may be responsible, in part, for the priming effect of TNF. Using isolated membranes, we show that formyl-peptide receptors exist in high- and low-affinity states on both NM and TNF-M. In contrast with the results of Atkinson *et al.* [4], we find that TNF increased membrane expression of formyl-peptide receptors by about 50% without altering the affinity of these receptors (Table 2). The increased formyl-peptide receptor expression found on TNF-M presents surface receptors accessible to FMLP in intact cells. FMLP binding to intact HL-60 granulocytes was increased after pretreatment with TNF. Additionally, HL-60 cells are reported to lack specific granules, which serve as an intracellular pool of receptors in neutrophils [31].

Under the conditions of our assay, the new formyl-peptide receptors detected on TNF-M expressed a high affinity. To determine if the new receptors induced by TNF were fundamentally different from those on normal HL-60 granulocytes, the ability of these receptors to be converted into a low-affinity state by guanine nucleotides was studied [28]. GTP[S] decreased FMLP binding to TNF-M and NM with similar dose-response curves by decreasing the percentage of receptors expressing a high affinity. It has recently been shown that low [Mg^{2+}] decreases the percentage of high-affinity formyl-peptide receptors [27]. Low concentrations of $MgCl_2$ ($[Mg^{2+}] = 0$ mM) resulted in a marked decrease in the percentage of high-affinity binding sites. The total number of FMLP receptors was decreased in the absence of Mg^{2+} and in the presence of GTP[S], as previously reported [27]. The new receptors induced by TNF can therefore express a high or low affinity, depending on the concentrations of guanine nucleotides and Mg^{2+} . These results suggest that the new

receptors induced by TNF are coupled to G-proteins and are not fundamentally different from those on normal HL-60 granulocytes.

Since formyl-peptide receptors have been shown to be coupled to G₂ and G₁₃ in HL-60 cells [29], the membrane expression of these G-proteins was examined. As opposed to the effects on formyl-peptide receptors, TNF did not alter the level of G_i proteins. No differences in G_i levels were detected by pertussis-toxin-catalysed ADP-ribosylation. The relative quantities of G₂ in NM and TNF-M were determined by immunoblotting. Although the antiserum used detects both subunits α_1 and α_2 , α_1 is not expressed by HL-60 cells [29]. There was no difference in the levels of α_2 between NM and TNF-M. These results suggest that G-protein levels are not regulated by the number of G-protein-coupled receptors.

The functional interaction between formyl-peptide receptors and their G-proteins was examined by measuring FMLP-stimulated G-protein activation. Receptor-specific GTP[S] binding was enhanced by 57% in TNF-M, whereas receptor-specific GTP hydrolysis was enhanced by 47%. In the absence of FMLP, GTPase activity did not differ between TNF-M and NM, whereas GTP[S] binding was greater in TNF-M. The increased basal GTP[S] binding in TNF-M may result from the increased receptor number, also. We have previously shown that pretreatment of HL-60 cell membranes with either pertussis toxin or cholera toxin decreases both stimulated and basal GTP[S] binding and GTPase activity. Gierschik & Jakobs [32] have explained this finding by proposing that unoccupied receptors are responsible for part of the basal activity observed. Therefore the increase in formyl-peptide receptors induced by TNF may be sufficient to increase basal G-protein turnover in TNF-M.

The mechanism by which TNF enhances transmembrane signalling to FMLP is likely to be an increase in formyl-peptide receptors. The percentage increase in receptors was similar to the increases in FMLP-stimulated G-protein activation in TNF-M. Calculation of receptor efficiency of G-protein activation indicates that formyl-peptide receptors on TNF-M are no more efficient at G-protein activation than those on NM. However, alteration in transmembrane signalling may not be the only mechanism by which TNF enhances HL-60 cell responses. The 50% increase in G-protein activation stimulated by FMLP is less than the 3–4-fold increase in superoxide production and PGE₂ release. It is possible that G-protein activation of effector enzymes is also enhanced by TNF. The failure of TNF to enhance PMA-stimulated superoxide release suggests that TNF has no effect on protein kinase C or the NADPH oxidase. Examination of other possible mechanisms of action of TNF will require measurement of effector enzyme activity in TNF-treated intact cells following stimulation by FMLP.

The basis for TNF regulation of formyl-peptide-receptor expression was not examined. Hormonal regulation of β -adrenergic receptor expression has been reported for glucocorticoids, thyroid hormones and sex steroids [33]. Our results suggest that hormonal regulation of other G-protein-coupled receptors can occur. Immunologically active cytokines, of which TNF is a prominent example, should be added to the list of hormones which regulate receptor expression.

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REFERENCES

- Gamble, J. R., Harlan, J. M., Klebanoff, S. J. & Vadas, M. A. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8667–8671.
- Seow, W. K., Thong, Y. H. & Ferrante, A. (1987) *Immunology* **62**, 357–361.
- Shalaby, M. R., Aggarwal, B. B., Rinderknecht, E., Svedersky, L. P., Finkle, B. S. & Palladino, M. A. (1985) *J. Immunol.* **135**, 2069–2073.
- Atkinson, Y. H., Marasco, W. A., Lopez, A. F. & Vadas, M. A. (1988) *J. Clin. Invest.* **81**, 759–765.
- Berkow, R. L., Wang, D., Larrick, J. W., Dodson, R. W. & Howard, T. H. (1987) *J. Immunol.* **139**, 3783–3791.
- Ferrante, A., Nandoskar, M., Walz, A., Goh, D. H. B. & Kowanko, I. C. (1988) *Int. Arch. Allergy Appl. Immunol.* **86**, 82–91.
- Klebanoff, S. J., Vadas, M. A., Harlan, J. M., Sparks, L. H., Gamble, J. R., Agosti, J. M. & Waltersdorff, A. M. (1986) *J. Immunol.* **136**, 4220–4225.
- She, Z., Wewers, M. D., Herzyk, D. J., Sagone, A. L. & Davis, W. B. (1989) *Am. J. Physiol.* **257**, L338–L343.
- Yuo, A., Kitagawa, S., Suzuki, I., Urabe, A., Okabe, T., Saito, M. & Takaku, F. (1989) *J. Immunol.* **142**, 1678–1684.
- Berger, M., Wetzler, E. M. & Wallis, R. S. (1988) *Blood* **71**, 151–158.
- Pichyangkul, S., Schick, D., Schober, W., Dixon, G. & Khan, A. (1988) *Exp. Hematol.* **16**, 588–593.
- Girardin, E., Grau, G. E., Dayer, J.-M., Roux-Lombard, P. & Lambert, P.-H. (1988) *N. Engl. J. Med.* **319**, 397–400.
- Michie, H. R., Manogue, K. R., Spriggs, D. R., Revhaug, A., O'Dwyer, S., Dinarello, C. A., Cerami, A., Wolff, S. M. & Wilmore, D. W. (1988) *N. Engl. J. Med.* **318**, 1481–1486.
- Mathison, J. C., Wolfson, E. & Ulevitch, R. J. (1988) *J. Clin. Invest.* **81**, 1925–1937.
- Tracy, K. J., Fong, Y., Hesse, D. G., Manogue, K. R., Lee, A. T., Kuo, G. C., Lowry, S. F. & Cerami, A. (1987) *Nature (London)* **330**, 662–664.
- Blanchard, D. K., Djeu, J. Y., Klein, T. K., Friedman, H. & Stewart, W. E. (1988) *J. Leukocyte Biol.* **43**, 429–435.
- Becker, E. L. (1979) *J. Reticuloendothel. Soc.* **26**, (suppl.), 701–709.
- Krause, K. H., Schlegel, W., Wollheim, C. B., Andersson, T., Waldvogel, F. A. & Lew, P. D. (1985) *J. Clin. Invest.* **76**, 1348–1354.
- McLeish, K. R., Gierschik, P., Schepers, T., Sidiropoulos, D. & Jakobs, K. H. (1989) *Biochem. J.* **260**, 427–434.
- Thompson, B. Y., Sivam, G., Britigan, B. E., Rosen, G. M. & Cohen, M. S. (1988) *J. Leukocyte Biol.* **43**, 140–147.
- Gierschik, P., Sidiropoulos, D., Spiegel, A. & Jakobs, K. H. (1987) *Eur. J. Biochem.* **165**, 185–194.
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
- Rodbard, D. H., Lenox, H., Way, H. L. & Ramseth, D. (1976) *Clin. Chem.* **22**, 350–358.
- Munson, P. J. & Rodbard, D. (1980) *Anal. Biochem.* **107**, 220–239.
- Aktories, K., Schultz, G. & Jakobs, K. H. (1982) *Mol. Pharmacol.* **21**, 336–342.
- Klein, J. B., Sonnenfeld, G. & McLeish, K. R. (1991) *Lymphokine Res.* **10**, 173–176.
- Gierschik, P., Steisslinger, M., Sidiropoulos, D., Herrmann, E. & Jakobs, K. H. (1989) *Eur. J. Biochem.* **183**, 97–105.
- Koo, C., Lefkowitz, R. J. & Snyderman, R. (1983) *J. Clin. Invest.* **72**, 748–753.
- Gierschik, P., Sidiropoulos, D., Jakobs, K. H. (1989) *J. Biol. Chem.* **264**, 21470–21473.
- Cerione, R. A., Kroll, S., Rajaram, R., Anson, C., Goldsmith, P. & Spiegel, A. (1988) *J. Biol. Chem.* **263**, 9345–9352.
- Fletcher, M. P. & Gallin, J. I. (1983) *Blood* **62**, 792–799.
- Gierschik, P. & Jakobs, K. H. (1990) In *G-proteins as Mediators of Cellular Signalling Processes* (Houslay, M. D. & Milligan, G., eds.), pp. 67–82, John Wiley and Sons, Chichester.
- Haddock, J. R. & Malbon, C. C. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5021–5025.