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### Research Article

Preincubation of neutrophils with recombinant human tumor necrosis factor-alpha (rH TNF-alpha) enhanced the subsequent release of superoxide anion in response to various concentrations of N-formylmethionylleucylphenylalanine (FMLP). Enhanced superoxide anion production was evident by 5 min and had reached a plateau by 15 min. Not only was the total amount of superoxide anion released greater, but the rate of release was also enhanced threefold by rH TNF-alpha. In contrast, rH TNF-alpha reduced or abolished neutrophil locomotion under agarose in response to a gradient of FMLP. Binding studies of f-Met-Leu-[3H]Phe to purified human neutrophils revealed a heterogeneous binding to unstimulated cells. The high affinity component consisted of approximately 2,000 sites per cell and had an average Kd of  $2 \pm 0.7$  nM (n = 4). The low affinity component consisted of approximately 40,000 sites per cell and had an average Kd of  $180 \pm 50$  nM (n = 4). rH TNF-alpha caused conversion to a linear Scatchard plot showing no significant change in total binding sites but a single Kd of  $40 \pm 10$  nM (n = 4). These data indicate that rH TNF-alpha may influence neutrophil responses to FMLP by regulating the affinity of FMLP receptors.

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# Recombinant Human Tumor Necrosis Factor- $\alpha$

## Regulation of *N*-Formylmethionylleucylphenylalanine Receptor Affinity and Function on Human Neutrophils

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### Abstract

Preincubation of neutrophils with recombinant human tumor necrosis factor- $\alpha$  (rH TNF- $\alpha$ ) enhanced the subsequent release of superoxide anion in response to various concentrations of *N*-formylmethionylleucylphenylalanine (FMLP). Enhanced superoxide anion production was evident by 5 min and had reached a plateau by 15 min. Not only was the total amount of superoxide anion released greater, but the rate of release was also enhanced threefold by rH TNF- $\alpha$ . In contrast, rH TNF- $\alpha$  reduced or abolished neutrophil locomotion under agarose in response to a gradient of FMLP. Binding studies of f-Met-Leu- $^3$ H]Phe to purified human neutrophils revealed a heterogeneous binding to unstimulated cells. The high affinity component consisted of  $\sim 2,000$  sites per cell and had an average  $K_d$  of  $2 \pm 0.7$  nM ( $n = 4$ ). The low affinity component consisted of  $\sim 40,000$  sites per cell and had an average  $K_d$  of  $180 \pm 50$  nM ( $n = 4$ ). rH TNF- $\alpha$  caused conversion to a linear Scatchard plot showing no significant change in total binding sites but a single  $K_d$  of  $40 \pm 10$  nM ( $n = 4$ ). These data indicate that rH TNF- $\alpha$  may influence neutrophil responses to FMLP by regulating the affinity of FMLP receptors.

### Introduction

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ),<sup>1</sup> identified and cloned for its cytotoxic and cytotoxic properties, has been shown to stimulate a wide range of cells, including the human neutrophil. Small doses of TNF- $\alpha$  rapidly induce an "activated" state in neutrophils characterized by an increased capacity to kill antibody-coated tumor cells (1), adhere to endothelial cells (2), and respond with an oxidative burst to unopsonized zymosan particles (3). These properties suggested that neutrophils played an important role in the inflammatory reactions seen near TNF- $\alpha$ -stimulated tumor rejection, and also that intravenous administration of TNF- $\alpha$  will have a number of deleterious effects. These findings also imply that the local accumulation

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1. Abbreviations used in this paper: rH TNF- $\alpha$ , recombinant human tumor necrosis factor- $\alpha$ .

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of neutrophils at the site of infection, or their activation, is due to the local elaboration of TNF like molecules.

In cases of bacterial infection the inflammatory response is thought to be promoted by agents such as formylmethionylleucylphenylalanine (FMLP) (4). Not only do neutrophils migrate in response to a gradient of FMLP but, at higher doses, FMLP also activates various toxic mechanisms, such as the capacity to generate superoxide anion. Since both TNF and FMLP appear to be involved in stimulating inflammatory responses, we hypothesized that TNF- $\alpha$  might alter these responses leading to an enhanced inflammatory state.

We show that the modulation of FMLP receptors by rH TNF- $\alpha$  is associated with an enhancement of an FMLP-mediated neutrophil function such as superoxide anion production. In contrast, rH TNF- $\alpha$  inhibits FMLP-stimulated motility of neutrophils. The FMLP receptor population of neutrophils is altered from a heterogeneous population of receptors exhibiting a high and a low affinity component on unstimulated neutrophils to a homogenous population displaying an intermediate affinity on rH TNF- $\alpha$ -stimulated neutrophils. Although the affinities of the FMLP receptor population were altered by rH TNF- $\alpha$  stimulation, the total numbers of receptors remained unchanged. We hypothesize that changes in receptors and in function are related.

### Methods

**Purification of human neutrophils.** Neutrophils were obtained from the peripheral blood of healthy volunteers after dextran sedimentation (Dextran T500, Pharmacia Fine Chemicals, Uppsala, Sweden) and density gradient centrifugation at 450 *g* on Lymphoprep (Nyegaard, Oslo, Norway) followed by hypotonic lysis of erythrocytes using sodium chloride. The cell preparations, which always consisted of  $> 95\%$  neutrophils, were resuspended to  $10^7$ /ml in RPMI 1640 and 2% fetal calf serum (FCS), 20 mM Hepes buffer, and antibiotics. The assays were carried out in this medium unless otherwise stated. The lipopolysaccharide (LPS) content of this medium was determined to be  $< 1.9$  pg/ml using the *Limulus* amoebocyte lysate assay (5).

**rH TNF- $\alpha$ .** The rH TNF- $\alpha$  used was from lot numbers 3056-55 and 4260-1, containing  $\sim 5 \times 10^7$  U/mg and  $1.2 \times 10^8$  U/mg, respectively. Both were generously provided by Genentech, South San Francisco, CA. The molecule was produced in *Escherichia coli* (6) and purified to  $\sim 99.8\%$ . The LPS content of this solution was determined to be  $< 0.125$  endotoxin units/ml as measured by the manufacturers using the *Limulus* amoebocyte lysate assay. Dilutions from stock material were made weekly into HBSS with calcium and magnesium plus 1% BSA (Commonwealth Serum Laboratories, Parkville, Australia) and stored at 4°C. The final concentration of LPS present in incubation suspensions was  $6.25 \times 10^{-7}$  endotoxin units/ml.

**Superoxide production.** Purified neutrophils ( $10^7$ /ml) were incubated with medium or different concentrations of rH TNF- $\alpha$  for various times at 37°C. After this, 150  $\mu$ l of cells ( $10^6$ ) was added to a mixture of 100  $\mu$ l freshly prepared cytochrome *c* (type VI, 12.4 mg/ml, Sigma Chemical Co., St. Louis, MO) and 100  $\mu$ l FMLP (Sigma Chemi-

cal Co.) and made up to 1 ml with medium. The mixtures were then incubated at 37°C for 5 min, after which the cells were rapidly cooled and pelleted at 4°C, and the supernatants were transferred to plastic disposable cuvettes. Superoxide production was measured by the reduction of cytochrome *c* using an extinction coefficient of 21.1 nM<sup>-1</sup> (7) at 550 nm (8).

In experiments where the rate of superoxide anion production was measured, cells were preincubated with  $1.4 \times 10^{-10}$  M rH TNF- $\alpha$  for 45 min at 37°C. 150  $\mu$ l of cells was then added to disposable plastic cuvettes containing 100  $\mu$ l FMLP ( $10^{-7}$  M final) and 100  $\mu$ l freshly prepared cytochrome *c* and made up to 1 ml with medium. This suspension was incubated in a thermostatically controlled cuvette holder at 37°C for 10 min. The change in OD 550 nm was continuously monitored and recorded at 30-s intervals in a DU-50 spectrophotometer (Beckman Instruments, Inc., Berkeley, CA). The baseline readings decreased during these experiments because of the settling of cells.

In control experiments the release of superoxide anion was confirmed by allowing the reaction to FMLP to proceed in the presence of freshly prepared superoxide dismutase (280 U/ml final, Sigma Chemical Co.). The release of superoxide anion was completely abolished in the presence of superoxide dismutase. To ensure the change in OD 550 nm was not limited by cytochrome *c* concentration, maximum reduction was determined by adding a few grains of sodium dithionite (British Drug House, Ltd., Poole, England) to the final mix.

**Chemotaxis.** The chemotactic response of neutrophils to a gradient of FMLP was tested under agarose as described (9). Briefly, 5 ml of 0.5% agarose (type II, Sigma Chemical Co.) dissolved in RPMI 1640 and 2% FCS with 20 mM Hepes, and 375  $\mu$ g/ml sodium bicarbonate (Flow Laboratories, North Ryde, New South Wales) was set in a plastic petri dish (50 mm, Kayline, Adelaide) and 2.4-mm diam wells were formed 2.4 mm apart in a horizontal line from the center to the edge of the plate. Neutrophils at  $2.5 \times 10^7$ /ml in medium were preincubated 45 min at 37°C in the presence or absence of  $1.4 \times 10^{-10}$  rH TNF- $\alpha$ . This period of incubation was chosen to ensure maximal stimulation by rH TNF- $\alpha$ . 5  $\mu$ l of cells was then added to the center well, 5  $\mu$ l of medium was added to the inner well, and 5  $\mu$ l of  $10^{-7}$  M FMLP was added to the outer well. The petri dish was incubated for 2 h at 37°C in 5% CO<sub>2</sub>. The cells were fixed with methanol at 4°C overnight, followed by 40% formalin for 30 min at 25°C before the agarose was removed.

Migration in the absence of any stimuli (random migration), with FMLP in both cell-containing and adjacent medium-containing wells (chemokinetic stimulus) and with FMLP only in the non-cell-containing well (chemotactic stimulus) were measured using an ocular micrometer. Results are expressed in arbitrary units as either chemotactic migration, chemokinetic migration, or random movement.

**High-performance liquid chromatography of f-Met-Leu-[<sup>3</sup>H]Phe.** To ensure that impurities were not present in the commercially available f-Met-Leu-[<sup>3</sup>H]Phe used (50.9–55.3 Ci/mmol, New England Nuclear, Boston, MA), the preparation was analyzed by high-performance liquid chromatography. The system consisted of a model 334 gradient system (Beckman Instruments, Inc., Palo Alto, CA) with a variable wavelength detector. Spectrophotometric detection was at 220 nm. The sample was run through a reverse-phase, C18, 5- $\mu$ m particle size Ultrasphere column (Beckman Instruments, Inc., Berkeley, CA). Chromatography was run under the following conditions: flow rate 1.0 ml/min, temperature 25°C, gradient elution over 20 min from 20% to 60% CH<sub>3</sub>CN in 0.1% phosphoric acid. Under these conditions, f-Met-Leu-[<sup>3</sup>H]Phe appeared as a single peak at 16 min, corresponding to the retention time of unlabeled FMLP. 1-min fractions were collected and radioactive content was determined in a Tricarb 2000CA liquid scintillation counter. Greater than 95.5% of radioactivity corresponded to the 16-min peak in all preparations tested.

**f-Met-Leu-[<sup>3</sup>H]Phe-binding assay.** Binding of f-Met-Leu-[<sup>3</sup>H]Phe to purified neutrophils was measured at 4°C in siliconized 12  $\times$  75-mm glass test tubes in a total volume of 105  $\mu$ l. Cells from the same donor at a concentration of  $10^7$ /ml were incubated in the presence or absence of rH TNF- $\alpha$  for 60 min at 37°C in RPMI + 2% FCS, then

washed in cold incubation buffer (HBSS with 1.6 mM CaCl<sub>2</sub>, 10 mM sodium azide, 5  $\mu$ g/ml cytochalasin B (Sigma Chemical Co.), and 0.1% BSA (Commonwealth Serum Laboratories), and resuspended to  $10^7$ /ml. Sodium azide and cytochalasin B were included in the incubation buffer to prevent ligand internalization which may occur at 4°C (10). 100  $\mu$ l of cells was added to triplicate tubes containing 5  $\mu$ l of f-Met-Leu-[<sup>3</sup>H]Phe at various concentrations (100 nM to 0.5 nM) in incubation buffer, or to duplicate tubes containing, in addition, unlabeled FMLP. (100  $\mu$ M). Bound f-Met-Leu-[<sup>3</sup>H]Phe was measured after 30 min by filtration through glass fiber filters (GF/B, Whatman, Maidstone, England) using HBSS, and quantified by liquid scintillation counting.

The binding data were analysed using LIGAND, a weighted nonlinear least squares regression analysis computer program (11). After subtracting the nonspecific binding from total binding, the specific binding data were fitted to one or more of the appropriate equations from a one-saturable-site model, a two-saturable-site model, and the Hill equation (12–14). The weighted sums of squared deviations generated by LIGAND for one saturable site, two saturable sites, and the Hill equation were compared using the *F* test (11).

## Results

**rH TNF- $\alpha$  alters the production of superoxide anion by neutrophils stimulated with FMLP.** Preliminary experiments showed that rH TNF- $\alpha$  enhanced the production of superoxide anion by neutrophils in response to FMLP. To determine the optimal concentrations of cytokine required for this effect, a titration of rH TNF- $\alpha$  was done. Neutrophils were preincubated with various concentrations of rH TNF- $\alpha$  for 45 min then stimulated with  $10^{-7}$  M FMLP. This showed that rH TNF- $\alpha$  enhanced superoxide anion production by neutrophils stimulated with  $10^{-7}$  M FMLP in a dose-dependent manner (Fig. 1). Very little or no reduction of cytochrome *c* was detected with neutrophils preincubated with rH TNF- $\alpha$  without the addition

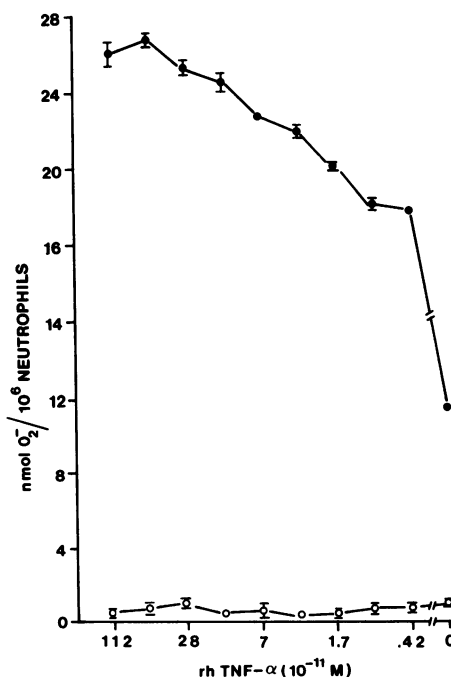


Figure 1. Stimulation by rH TNF- $\alpha$  of the production of superoxide anion by neutrophils in the presence (●) and absence (○) of  $10^{-7}$  M FMLP. Each point represents the mean of triplicate determinations from a single experiment representative of nine others. The bars represent the SEM when this is bigger than the symbols.

of FMLP. Although it was observed that the optimal concentration of rH TNF- $\alpha$  required for maximal superoxide anion production by neutrophils varied among donors,  $1.4 \times 10^{-10}$  M was the most consistent concentration of rH TNF- $\alpha$  to induce maximal levels of superoxide anion production after stimulation with FMLP and was therefore used in subsequent experiments.

In parallel experiments, a titration of FMLP in the presence or absence of rH TNF- $\alpha$  was performed. Neutrophils were preincubated in the presence or absence of  $1.4 \times 10^{-10}$  M rH TNF- $\alpha$  for 45 min then stimulated with various concentrations of FMLP. This showed that neutrophils responded with an increased production of superoxide anion at concentrations of FMLP from  $10^{-6}$  to  $10^{-8}$  M (Fig. 2). In this individual a significant response to FMLP alone was observed. The response to FMLP differed between individuals and therefore influenced the degree of enhancement observed by preincubation with rH TNF- $\alpha$ . In nine experiments, preincubation with rH TNF- $\alpha$  enhanced the response to FMLP from 0.5-fold to 18-fold. The most consistently optimal concentration of FMLP for enhanced responsiveness of neutrophils to rH TNF- $\alpha$  was  $10^{-7}$  M. No shift in the titration curve of FMLP was observed with neutrophils stimulated with rH TNF- $\alpha$ ; rather there was an increase in the total amount of superoxide anion at each concentration of FMLP tested.

To determine whether the increase in superoxide anion production from neutrophils stimulated rH TNF- $\alpha$  was associated with a change in the rate of superoxide anion production, neutrophils were incubated with cytochrome *c* and FMLP over a 10-min period. Fig. 3 shows a typical experiment in which preincubating neutrophils with  $1.4 \times 10^{-10}$  M rH TNF- $\alpha$  enhanced the rate of production of superoxide in their subsequent response to FMLP. The levels reached a plateau by

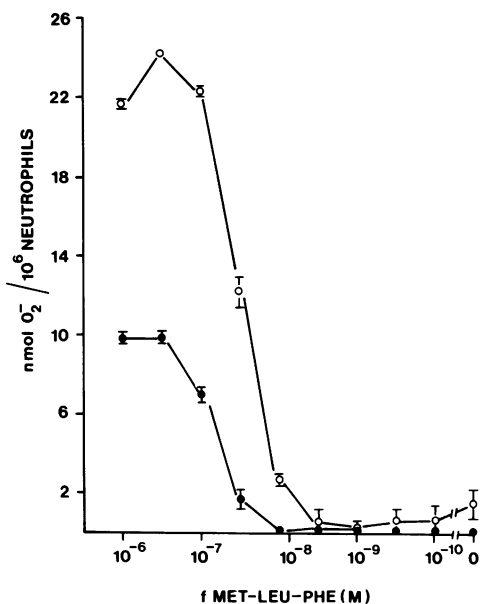


Figure 2. Stimulation by  $1.4 \times 10^{-10}$  M rH TNF- $\alpha$  of the production of superoxide anion by neutrophils in response to various concentrations of FMLP. (●) Values for unstimulated neutrophils; (○) values for rH TNF- $\alpha$ -stimulated neutrophils. Each point represents the mean of triplicate determinations from a single experiment representative of five others. The bars represent the SEM when this is larger than the symbols.

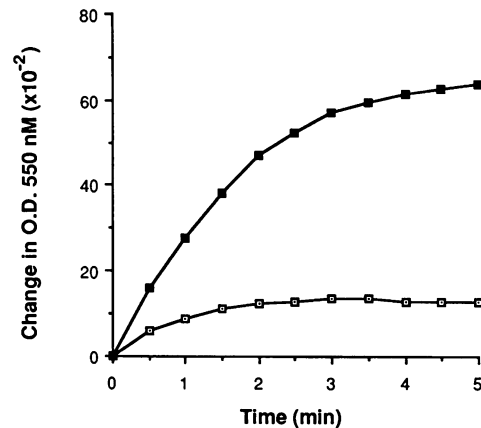


Figure 3. Stimulation by rH TNF- $\alpha$  of the rate of superoxide anion production by neutrophils in response to FMLP. After preincubation in the presence (■) and absence (□) of  $1.4 \times 10^{-10}$  M rH TNF- $\alpha$ , cells were incubated over 10 min in a thermostatically controlled cuvette holder at 37°C in the presence of cytochrome *c* and OD 550 nm continuously monitored every 30 s.

5 min. This effect was consistent over 10 experiments where the mean rate of change in OD 550 nm per minute over 5 min for rH TNF- $\alpha$ -stimulated neutrophils was  $0.094 \pm 0.005$  ( $n = 10$ ), whereas the mean rate for unstimulated neutrophils was  $0.031 \pm 0.008$  ( $n = 10$ ). Both unstimulated neutrophils and neutrophils preincubated with rH TNF- $\alpha$  exhibited an average change in OD 550 nm per minute over 5 min of  $0.001 \pm 0.001$  ( $n = 10$ ).

Time course experiments performed by preincubating neutrophils for various times with rH TNF- $\alpha$  showed that activation had occurred by 5 min and had reached optimal levels by 15 min (Fig. 4). The amount of superoxide anion released by rH TNF- $\alpha$ -stimulated neutrophils remained constant up to 60 min preincubation. Unstimulated neutrophils became progressively more responsive to FMLP with time, a phenomenon that was observed in six experiments. At every time point tested, cells incubated with rH TNF- $\alpha$  alone released very little superoxide anion.

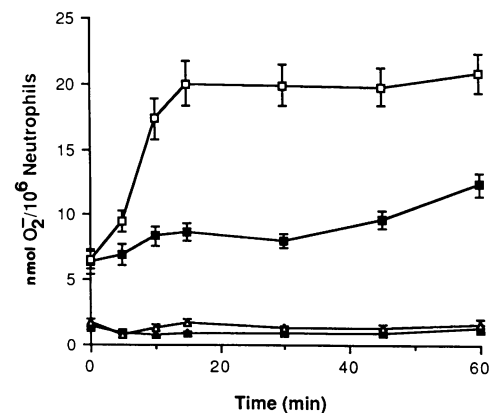


Figure 4. Time-dependent stimulation by rH TNF- $\alpha$  of superoxide anion production by neutrophils. Cells were incubated in the presence (open symbols) or absence (closed symbols) of  $1.4 \times 10^{-10}$  M rH TNF- $\alpha$  for various times then for 5 min with (squares) or without (triangles)  $10^{-7}$  M FMLP. Each point represents the mean of 18 determinations (triplicates from six different experiments). Bars represent the SEM when this is larger than the symbols.

rH TNF- $\alpha$  inhibits the chemotactic migration of neutrophils in response to a gradient of FMLP. To determine whether rH TNF- $\alpha$  influences a second FMLP-mediated response, the chemotaxis of neutrophils towards a gradient of FMLP was studied. The neutrophils were preincubated with rH TNF- $\alpha$  then washed and placed in the agarose-containing dishes in the presence or absence of a gradient of FMLP. It was shown that preincubating neutrophils with rH TNF- $\alpha$  inhibited random, chemokinetic, and chemotactic migration (Table I). As with superoxide anion release, the response varied among different individuals.

To ensure the inhibition of chemotaxis in rH TNF- $\alpha$ -stimulated cells was not due to oxidant toxicity, chemotaxis was allowed to proceed in the presence of 100  $\mu$ g/ml superoxide dismutase and 70  $\mu$ g/ml catalase. In the absence of these enzymes, chemotaxis was reduced from  $82.5 \pm 2.5$  in unstimulated cells to  $45 \pm 5$  in rH TNF- $\alpha$ -stimulated cells. In the presence of superoxide dismutase and catalase chemotaxis was reduced from  $80 \pm 5$  to  $42.5 \pm 5$  in rH TNF- $\alpha$ -stimulated cells. The degree of inhibition of random and chemokinetic migration was similarly unaffected.

**Binding of f-Met-Leu-[ $^3$ H]Phe to neutrophils.** Neutrophils incubated with  $1.4 \times 10^{-10}$  M rH TNF- $\alpha$  for 60 min exhibited saturable binding of f-Met-Leu-[ $^3$ H]Phe as did unstimulated neutrophils.

Statistical analysis of the binding data indicated that the two-saturable-site model was preferable to the one-saturable-site model for neutrophils incubated in medium for 60 min at 37°C. Fig. 5 A represents the Scatchard analysis of this same data generated by the program LIGAND. Unstimulated neutrophils exhibit a curvilinear Scatchard plot (Fig. 5 A), whereas neutrophils from the same donor that had been incubated with rH TNF- $\alpha$  for 60 min fitted better to a one-saturable-site model, and the Scatchard plot for these neutrophils was linear (Fig. 5 B).

A summary of the analysis of the binding models from four separate experiments, both for unstimulated and rH TNF- $\alpha$ -stimulated neutrophils is shown in Table II. The characteristics of the two-saturable-site model were a high affinity site with a  $K_d$  value of  $2 \pm 0.7$  nM and a low affinity site with a  $K_d$  value of  $180 \pm 50$  nM. Of the total number of sites detected 1–9% were of the higher affinity. In addition, these same data when applied to the Hill equation were characterized by a Hill coefficient of  $0.94 \pm 0.03$ , suggesting the curvilinearity of the Scatchard plot is not due to site-site interactions. Although the

Table I. rH TNF- $\alpha$  Inhibits the Migration of Neutrophils under Agarose

Conditions of assay	Migration		P value
	Nil*	rH TNF- $\alpha$	
	Arbitrary units		
Random	$22.8 \pm 2.2^\ddagger$	$12.4 \pm 0.75$	<0.001
Chemokinetic	$38.8 \pm 10.3$	$20.6 \pm 2.8$	<0.001
Chemotactic	$101.8 \pm 5.1$	$56 \pm 4.6$	<0.01

\* Neutrophils were preincubated in the presence or absence of  $1.4 \times 10^{-10}$  M rH TNF- $\alpha$  for 60 min at 37°C.

$^\ddagger$  Numbers represent the mean of eight determinations (duplicates of four different experiments)  $\pm$  SEM.

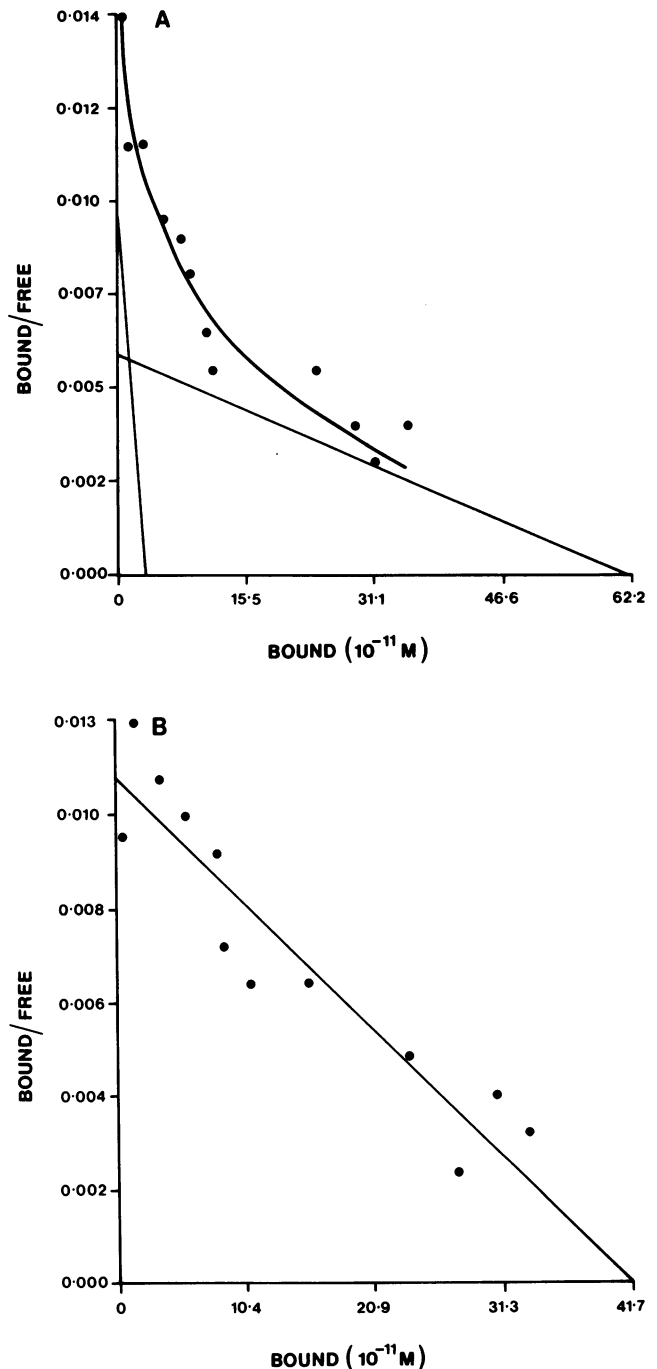


Figure 5. Scatchard analysis of f-Met-Leu-[ $^3$ H]Phe equilibrium binding to (A) unstimulated neutrophils and (B) neutrophils stimulated with  $1.4 \times 10^{-10}$  M rH TNF- $\alpha$ .

affinity was altered by rH TNF- $\alpha$  stimulation, the total number of receptors remained significantly unchanged as determined by Student's two-tailed  $\pm t$  test (data not shown).

## Discussion

In this article we show that rH TNF- $\alpha$  modulates the affinity but not the total number of FMLP receptors on human neutrophils. This may explain in part our observation that rH TNF- $\alpha$  enhances superoxide anion production by neutrophils

Table II. rH TNF- $\alpha$  ( $1.4 \times 10^{-10}$  M) Alters the Affinity of FMLP Receptors on Human Neutrophils

Binding parameters	Unstimulated neutrophils	Neutrophils + rH TNF- $\alpha$
$K_{d1}$ (nM)	$2 \pm 0.7^*$	$40 \pm 10^\ddagger$
No. sites per cell	$2,000 \pm 700^\S$	$33,000 \pm 8,000$
$K_{d2}$ (nM)	$180 \pm 50$	NA
No. sites per cell	$40,000 \pm 9,500$	NA
Significance	$P < 0.01^  $	$P > 0.05$
	(1)	(2)

\* Arithmetic means  $\pm$  standard error of computer-estimated values from four separate experiments each with a curvilinear Scatchard plot for normal neutrophils and a linear Scatchard plot for rH TNF- $\alpha$ -stimulated neutrophils.

$^\ddagger$  The  $K_d$  of the low and of the high affinity populations was found to be significantly different from the  $K_d$  of the homogeneous receptor population of rH TNF- $\alpha$ -stimulated neutrophils ( $P < 0.05$  for both).

$^\S$  The number of sites per cell was compared with those on normal neutrophils from four separate experiments and no significant change was found ( $P > 0.1$ ).

$^||$  Significance, indicated by results of  $F$ -ratio tests, pertains to the statistical difference of the data relative to those for the one-saturable-site model (1) and the two-saturable-site model (2).

in response to FMLP but inhibits their chemotaxis towards a gradient of FMLP.

Our study of the equilibrium binding of f-Met-Leu-[ $^3$ H]Phe to unstimulated neutrophils revealed a curvilinear plot when analysed as described by Scatchard (15). A nonlinear Scatchard plot is consistent with a heterologous population of FMLP receptors, and the binding of formyl peptides to the receptors of human (16–18), rabbit (19), and rat neutrophils (20) has been shown previously to result in curvilinear Scatchard plots. In general, such investigations have been interpreted as due to the presence of two binding sites consisting of a high and a low affinity component (17, 19). Several possible explanations for the curvilinearity of the Scatchard analysis of FMLP receptors on normal neutrophils include the possibility that two interconvertible states of the same receptor exist (21–23), or that the two sites may be independent (24), or the existence of negative cooperativity (20, 25).

LIGAND analysis of our binding data reveals a high affinity component consisting of  $\sim 2,000$  sites per cell with a  $K_d$  of 2 nM. The low affinity component consisted of  $\sim 40,000$  sites per cell with a  $K_d$  of 180 nM. When these data were fitted to the Hill equation (13, 14), a Hill coefficient of 0.94 was observed suggesting that a two-site binding model was more appropriate than negative site-site interactions. Despite the various interpretations placed on a curvilinear Scatchard plot, our observations thus far suggest the existence of two components of the FMLP receptor population on normal resting neutrophils in agreement with several other groups (17, 19).

The stimulation of cells with  $1.4 \times 10^{-10}$  M rH TNF- $\alpha$  caused a conversion of the characteristics of the Scatchard plot. The resulting plot was linear indicating a homogeneous population of receptors. The affinity of this receptor population was significantly different from those characteristic of unstimulated neutrophils. This suggests either that the high affinity receptors are internalized (26) and lower affinity ones are expressed from the intracellular pool (27) or that the formyl

peptide receptor exists in an interconvertible state as is the case for interleukin 2 receptors (28, 29). Such regulation of the expression of functionally important molecules is an interesting phenomenon in that bacterial products such as LPS (30) and the complement component C5a (31) exert a similar effect. Thus the regulation of functional molecules on the cell surface may be an important mechanism by which TNF- $\alpha$  activates cells in vivo.

Formyl peptide binding to neutrophils and its effect on various neutrophil functions has been previously studied (32–34). A correlation between the  $ED_{50}$ s for various neutrophil functions and the  $K_d$  for ligand binding has been observed (35). These observations are consistent with the theory that high affinity receptors play a role in chemotaxis whereas the low affinity component plays a role in secretory responses and superoxide anion production.

We found that rH TNF- $\alpha$  activates a number of parameters of superoxide anion production by neutrophils in response to FMLP. Thus it appears that cells preexposed to rH TNF- $\alpha$  are primed and their subsequent response to a bacterial product such as FMLP is both more efficient and of greater magnitude. This phenomenon caused by a natural cytokine is likely to be physiologically significant inasmuch as it could enhance the capacity of neutrophils to respond to bacterial products during the course of an inflammatory reaction. In titration experiments designed to reveal whether rH TNF- $\alpha$  influenced the FMLP concentration required to stimulate neutrophils, rH TNF- $\alpha$  activated neutrophils to release more superoxide anion. This suggests that rH TNF- $\alpha$  may be activating a previously unresponsive population of cells or that the FMLP receptors are being modulated.

A notable characteristic of the activation of neutrophils by rH TNF- $\alpha$  is that no detectable release of superoxide anion occurs in the presence of rH TNF- $\alpha$  alone. This would be advantageous in vivo because the neutrophils would not be activated to produce superoxide anion, which would cause tissue damage, until they meet the appropriate target. The inability of rH TNF- $\alpha$  to activate mature neutrophils alone is not surprising when one considers that we have observed that rH TNF- $\alpha$  does not translocate protein kinase C in mature neutrophils (unpublished observations), which is a mediator in response to FMLP (36) and phorbol myristate acetate (37).

In contrast to stimulation of superoxide anion release rH TNF- $\alpha$  inhibits both random and FMLP-stimulated motility. The inhibition of random neutrophil migration is less than the inhibition of FMLP-induced movement. This suggests some specificity in the effect of rH TNF- $\alpha$  but also illustrates that receptor modulation is not the sole cause for the changes observed. These data further suggest that rH TNF- $\alpha$  may cause a fundamental change in neutrophils resulting in a generalized decrease in motility. The possibilities include an influence on actin polymerization and the modulation of other receptors. Those possibilities will be the subject of future investigations.

The loss of FMLP-stimulated motility together with the disappearance of the high affinity formyl peptide receptors nevertheless supports previous data suggesting that high affinity receptors mediate chemotaxis whereas low affinity receptors mediate superoxide anion release (4, 35). Although receptor modulation may explain the functional observations, the change in low affinity receptors observed here cannot be directly equated with the changes in cell function for two reasons. Firstly, stimulus-response coupling is a complex process

that depends on receptor occupancy rather than total receptor numbers. Secondly, the intracellular transduction mechanisms are known to amplify the initial signal from the receptor, and it is not known what the magnitude of this effect is.

Although the mechanism of action is unknown, previous studies have associated the formyl peptide receptor with a family of proteins that regulate adenylate cyclase known as the guanine nucleotide-binding proteins or G proteins (38). Specifically, the formyl peptide receptor is coupled to a pertussis toxin-sensitive, G protein termed Ni. There is evidence to suggest that the G proteins modulate the affinity of the formyl peptide receptor on human (21, 39, 40), rabbit (41, 42), and guinea pig (43) neutrophils. Considering this and the recent finding that some cytokines interact with G proteins (44), it is feasible that this is one mechanism by which rH TNF- $\alpha$  modulates the affinity of FMLP receptors, although the TNF- $\alpha$  receptor may not necessarily be coupled to the same type of G protein. It is possible that a third G protein may be involved in the transduction signal from the TNF- $\alpha$  receptor to the FMLP receptor.

In addition, the high affinity and low affinity receptors may not be distinct molecules—rather they may represent a single interconvertible molecule—thus the change in affinity but not numbers presented in this article can be interpreted as supportive of this theory. Although both the respiratory burst and chemotaxis are sensitive to pertussis toxin, new G proteins are still being discovered and it is feasible that other pertussis toxin-sensitive, G proteins exist. Thus the loss of high affinity receptors induced by rH TNF- $\alpha$  is consistent with the loss of chemotactic responsiveness, whereas the increased capacity to generate superoxide anion may also be partially explained by the change in FMLP receptor affinity.

These findings are particularly relevant to in vivo situations where the functional activation and immobilization of neutrophils to sites of inflammation are desirable properties, and suggest that TNF- $\alpha$  may play an important role during the inflammatory reaction. It is interesting that the effects of rH TNF- $\alpha$  reported in this article are shared by another biologically important compound, granulocyte-macrophage colony-stimulating factor (manuscript submitted for publication). This compound has also been implicated in the processes of inflammation, and these findings support the relevancy of our observations to in vivo situations. In addition, these findings are important in considerations of the clinical use of TNF- $\alpha$  as an antineoplastic agent. In these situations the route of administration of rH TNF- $\alpha$  may become critical because it could be anticipated that immobilization of neutrophils proximal to the desired site of action might occur.

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