Shedding of Tumor Necrosis Factor Receptors by Activated Human Neutrophils

By Françoise Porteu and Carl Nathan

From the Beatrice and Samuel A. Seaver Laboratory, Division of Hematology-Oncology, Department of Medicine, Cornell University Medical College, New York, New York 10021

Summary

The capacity of human neutrophils (PMN) to bind tumor necrosis factor (TNF) was rapidly lost when the cells were incubated in suspension with agents that can stimulate their migratory and secretory responses. Both physiological (poly)peptides (FMLP, C5a, CSF-GM) and pharmacologic agonists (PMN, calcium ionophore A23187) induced the loss of TNF receptors (TNF-R) from the cell surface. Half-maximal loss in TNF-R ensued after only ~2 min with 10^{-7} M FMLP at 37°C, and required only 10^{-9} M FMLP during a 30-min exposure. However, there were no such changes even with prolonged exposure of PMN to FMLP at 4° or 16°C. Scatchard analysis revealed loss of TNF-binding sites without change in their affinity (K_d ~0.4 nM) as measured at incompletely modulating concentrations of FMLP, C5a, PMA, or A23187. The binding of anti-TNF-R mAbs to PMN decreased in parallel, providing independent evidence for the loss of TNF-R from the cell surface. At the same time, soluble TNF-R appeared in the medium of stimulated PMN. This inference was based on the PMN- and FMLP-dependent generation of a nonsedimentable activity that could inhibit the binding of TNF to fresh human PMN or to mouse macrophages, and the ability of mAbs specific for human TNF-R to abolish inhibition by PMN-conditioned medium of binding of TNF to mouse macrophages. Soluble TNF-R activity was associated with a protein of M_r ~28,000 by ligand blot analysis of cell-free supernatants of FMLP-treated PMN. Thus, some portion of the FMLP-induced loss of TNF-R from human PMN is due to shedding of TNF-R. Shedding was unaffected by inhibitors of serine and thiol proteases and could not be induced with phosphatidylinositol-specific phospholipase C. Loss of TNF-R from PMN first stimulated by other agents may decrease their responsiveness to TNF. TNF-R shed by PMN may be one source of the TNF-binding proteins found in body fluids, and may blunt the actions of the cytokine on other cells.

During infection (1), allograft rejection (2), and ischemic tissue damage (3), macrophages and lymphocytes produce TNF-α, a pro-inflammatory cytokine (4) with profound effects on neutrophils (PMN).[1] In rats injected with TNF intravenously, PMN invade the walls of blood vessels in organs undergoing hemorrhagic necrosis (5). In mice injected with TNF intradermally, the skin undergoes hemorrhagic necrosis only in animals that can generate or are repleted with the PMN chemoattractant C5a (6), and only if they can mobilize a radiosensitive population of non-T cells (7); PMN mass within and around blood vessels supplying the areas destined for infarction (6, 7). In vitro, TNF enhances the expression of CD11b/CD18 adhesion molecules on PMN (8), increases PMN adhesion to endothelium (8), triggers adherent (9) PMN that express CD11/CD18 (10) to release large amounts of reactive oxygen intermediates, and promotes PMN degranulation (11), phagocytosis (11, 12), and antibody-dependent cytotoxicity (12).

We set out to identify physiologic mechanisms that may counteract the potentially destructive stimulation of PMN by TNF (13, 14). One such mechanism could involve regulation of cell surface receptors for TNF (TNF-R). A single class of TNF-R with a K_d of 0.1–1 nM has been identified on PMN by ligand binding (15–17). On cells other than PMN, cross-linking experiments have demonstrated association of TNF with a variety of proteins ranging from 50 to 140 kD (reviewed in reference 18). mAbs that mimic biologic effects of TNF while inhibiting the ability of cells to bind TNF are thought to be directed against TNF-R (19). These mAbs react with cellular proteins of ~55 and 75 kD (20), but their binding to PMN has not been documented. On some cells, the number of TNF-R can be increased by treatment with IFN-γ (21), IL-2 (22), or agents that elevate cAMP (23), or

[1] *Abbreviations used in this paper:* CSF-GM, CSF for granulocyte/macrophage; DFP, diisopropylfluorophosphate; KRPG, Krebs-Ringer phosphate buffer with glucose; PMN, neutrophils; TGF-β1, transforming growth factor-β1.

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decreased by exposure to IL-1 (24), LPS (25), activators of protein kinase C (23, 26, 27), inhibitors of protein synthesis (28), and drugs that depolymerize (28) or stabilize (29) microtubules. However, binding of TNF did not change after treatment of PMN with LPS (25), cycloheximide (28), or microtubule-disrupting agents (28), and effects of the other regulators on TNF-R of PMN have not been described. Thus, there has been no evidence that the TNF-R of PMN are subject to regulation.

We demonstrate below that exposure of PMN to a variety of physiologic and pharmacologic stimuli results in rapid loss of TNF-R from the cell surface. Medium conditioned by stimulated PMN contained soluble molecules that bound TNF and reacted with mAbs to TNF-R. Thus, activated PMN shed TNF-R. Shedding of TNF-R by activated PMN may both blunt their responsiveness to TNF, and diminish the concentration of TNF able to act on other cells.

Materials and Methods

Cells. PMN were isolated from heparinized blood of healthy adults by centrifugation on Neutrophil Isolation Medium (Los Alamos Diagnostics, Los Alamos, NM) as described (9). Contaminating erythrocytes were lysed with 0.2% NaCl for 90 s. PMN were washed and resuspended at 5×10^6/ml in ice-cold Krebs-Ringer phosphate buffer with glucose, pH 7.2-7.4, 300 mosM (KRPG) containing 0.2% heat-inactivated FCS (HyClone Laboratories, Logan, UT). PMN were incubated in 1.5-ml polypropylene microfuge tubes (Brinkmann Instruments Co., Westbury, NY) at 37°C with chemotactic factors, cytokines, or medium alone, while rotating end-over-end for times varying from 1 to 90 min. For incubations of <10 min, the cells were prewarmed for 10 min at 37°C before addition of the stimulus. After incubation, the cells were washed with cold KRPG, resuspended in binding buffer, and tested for TNF-R as described below.

Peritoneal exudates were elicited in CD1 mice (Charles River Breeding Laboratories, Wilmington, MA) with thioglycolate broth (Difco Laboratories, Detroit, MI) and the cells harvested as described (25). Monolayers of adherent macrophages were obtained by plating 1×10^6 cells/well in 16-mm-diameter wells in plastic trays (Costar Data Packaging, Cambridge, MA) in α-modified MEM (αMEM; KC Biological, Lenexa, KS) with 10% FCS. Macrophages were maintained at 37°C in 5% CO_2/95% air for 1–4 d before experiments.

Stimuli and Other Reagents. All cytokines were pure, recombinant proteins of human origin. TNF (5.6×10^7 U/mg) produced in *Escherichia coli* was a gift of Genentech (South San Francisco, CA). IFN-γ (1×10^7 U/ml) and transforming growth factor-β1 (TGF-β1, 50 μg/ml) were gifts of Amgen (Thousand Oaks, CA). CSF for granulocyte/macrophage (CSF-GM, 1.4×10^7 U/ml; from Chinese hamster ovary cells) was from Genetics Institute (Cambridge, MA). IL1-β was a gift of Dr. C. Dinarello (Tufts New England Medical Center, Boston, MA). Pure, recombinant human C5a was provided by Dr. Henry Showell, Pfizer Inc. (Groton, CT).

FMLP, PMA, and the calcium ionophore A23187 were from Sigma Chemical Co. (St. Louis, MO). Stock solutions of FMLP (10^{-3} M) and A23187 (10^{-2} M) in DMSO were stored at −70°C. Protease inhibitors were from Sigma Chemical Co. Phosphatidylinositol-specific phospholipase C from *Bacillus thuringiensis* was from Dr. M.G. Low (Columbia University College of Physicians and Surgeons, New York, NY), and was a gift of Dr. A. LeBivic, Cornell University Medical College.

Antibodies. Purified mouse IgG1 mAbs Htr-5, Htr-9, and Utr-1 specific for human TNF-R, prepared as described (20), were the gift of Dr. Manfred Brockhaus, F. Hoffman-La Roche AG, Basel, Switzerland. Purified mouse IgG2a mAbs IB4 (anti-CD18) and W6/32 (anti-HLA-A, B, C) were gifts of Dr. S. Wright, The Rockefeller University, New York, NY. Purified mouse IgM mAb BH2C6 anti-p157 (not CD11) (30) was the gift of Dr. J. Michl, SUNY, Brooklyn, NY. Affinity purified $F(ab')_2$ fragments of goat IgG anti-mouse IgG were from Cappel Laboratories (Organon Teknika Corp., West Chester, PA).

Iodination of Cytokines and Antibodies. TNF and affinity-purified $F(ab')_2$ fragments of anti-mouse IgG were radiolabeled with ^{125}I according to the method of Aggarwal et al. (21) using Iodogen-coated tubes and 0.5–1 mCi of $Na^{125}I$ (100 mCi/ml; Radiochemical Center, Amersham). As before (25, 28, 29), ^{125}I-TNFα had a specific activity of 200–400 Ci/mmol monomer, migrated as a single band of 17 kD on 11% polyacrylamide gels, and retained fully its ability to enhance H_2O_2 releasing capacity of resident mouse peritoneal macrophages. Radiolabeling of rhuIFN-γ was performed as described (31).

Binding Assays. Replicate samples of 1×10^6 PMN in 0.3 ml of KRPG containing 5% FCS (binding buffer) were incubated with the indicated concentrations of ^{125}I-TNF (0.1–2 nM) or ^{125}I-IFN-γ (2 nM) in the presence or absence of a 100-fold molar excess of unlabeled ligand to determine nonspecific binding. After 3 h at 4°C (sufficient to reach equilibrium), the cells were washed three times with 0.9% NaCl by centrifugation (180 *g*, 10 min, 4°C) and solubilized with 30 μl of 1 N NaOH. The lysates were transferred into new tubes for gamma counting (Packard Instrument Co., Downers Grove, IL). Nonspecific binding was <15% of total binding and was subtracted. Protein concentration of the solubilized samples was determined by the assay of Lowry et al. (32). Results are expressed as specific cpm bound per 100 μg protein. Binding of ^{125}I-TNF-α to monolayers of adherent macrophages was measured as described (25, 28, 29).

For binding of mAbs, PMN (1×10^6 in 100 μl of KRPG with 10% FCS) were incubated with saturating amounts of mAbs (10–20 μg/ml) for 1 h at 4°C, washed twice in KRPG-10% FCS, and reacted for an additional 30 min at 4°C with ^{125}I-$F(ab')_2$ anti-mouse IgG (500,000 cpm). After three washes in saline (180 *g*, 4°C, 10 min), bound cpm and protein were measured as above. Background binding was determined by omitting the primary antibody and was subtracted. It varied from 2 to 5% of total binding for anti-HLA and anti-CD18 mAbs, to ~30% for mAbs anti-TNF-R.

Assay for Soluble TNF-R. TNF-R were detected in the conditioned medium of stimulated PMN by competition with ^{125}I-TNF for its binding to fresh PMN or macrophages. PMN ($1.0–1.2 \times 10^8$/ml) were incubated in KRPG-1% FCS for 10 min at 37°C with FMLP (10^{-6} M) or medium alone. The supernatants were harvested by centrifugation (180 *g*, 10 min, 4°C), centrifuged again (12,000 *g*, 5 min, 4°C), and incubated with 2 nM ^{125}I-TNF in the presence or absence of 200 nM unlabeled TNF and/or a mixture of the anti-human TNF-R mAbs Htr-5, Htr-9, and Utr-1 (5 μg/ml each). After 30 min at 4°C, the mixtures were transferred to wells containing monolayers of macrophages or 1×10^6 fresh PMN, and binding was allowed to proceed for 3 h at 4°C as described above. As a control, 10^{-6} M FMLP was added to supernatants from buffer-treated cells during the binding assay.

Gel Electrophoresis and Ligand Blot Analysis. Cell-free supernatants of PMN treated with buffer alone or stimulated with FMLP

were resolved by electrophoresis in 5–15% polyacrylamide gradient gels according to the procedure of Laemmli (33) under nonreducing conditions. The proteins were then transferred to nitrocellulose membranes (Schleicher & Schuell Inc., Keene, NH) at 100 V for 1 h at room temperature, using the buffer systems described by Burnette (34). The nitrocellulose sheets were blocked with 1% nonfat dry milk in 50 mM Tris-HCl pH 7.4, 140 mM NaCl, 5 mM EDTA and 1 mM sodium azide for 2 h at room temperature before addition of ^{125}I-TNF (0.5 nM) in the presence or absence of 100 nM unlabeled TNF. After 16 h at 4°C, the membranes were washed four times with PBS at room temperature, dried, and autoradiographed.

Measurement of LPS. The level of contaminating LPS in all media and reagents was monitored by a chromogenic limulus amebocyte lysate test (Whittaker Bioproducts, Inc., Walkerville, MD) with a sensitivity of 10 pg/ml.

Results

Effects of PMN Activators on Expression of TNF-R. Exposure of PMN for 45 min at 37°C to FMLP (10^{-8} to 10^{-7} M), PMA (100 ng/ml), or calcium ionophore A23187 (10^{-5} M) resulted in almost complete inhibition of the subsequent binding of ^{125}I-TNF at 4°C (Table 1). Exposure of PMN to C5a (10^{-8} M) or CSF-GM (100 ng/ml) also suppressed their subsequent binding of TNF. However, in contrast to the effect observed with FMLP, PMA, or A23187, the decrease in ^{125}I-TNF binding induced by C5a or CSF-GM was never complete; 30–50% specific binding remained even when the incubation time was prolonged to 2 h at 37°C (not shown).

In contrast, IFN-γ, IL-1β, and TGF-β1 (each 100 ng/ml) had little or no effect on TNF-R expression (Table 1). A 45-min exposure to high concentrations of LPS (0.1–1 μg/ml) reduced ^{125}I-TNF binding only slightly (20–30%), and a 10-min exposure to LPS had no detectable effect (Table 1). The measured LPS content of all reagents at the concentrations used was <10 pg/ml. Thus, downregulation of TNF-R induced by other agents could not be attributed to contamination by LPS.

Kinetic Studies of TNF-R Modulation. The decrease in binding of ^{125}I-TNF induced by FMLP was extremely rapid, 50% reduction being reached after ~3 min treatment with 10^{-8} M FMLP (Fig. 1). Complete loss of ^{125}I-TNF binding required 6–10 min exposure to 10^{-7} M FMLP or 12–15 min exposure to 10^{-8} M FMLP (Fig. 1). Likewise, binding of ^{125}I-TNF to PMN was decreased to 0–5% that of control cells by 10 min after addition of PMA or A23187, and 30–70% binding capacity of the cells was lost upon a 10-min incubation with C5a and CSF-GM (Table 1).

The decrease in TNF binding induced by FMLP was relatively long lasting. After PMN were exposed to FMLP for 15 min and washed, their capacity to bind TNF recovered only partially during a subsequent 2-h incubation at 37°C (Table 2). Recovery studies were not carried out to longer times because prolonged incubation was associated with a fall in TNF-R on control PMN (data not shown).

Temperature Dependence. In contrast to the results obtained at 37°C, pre-exposure of PMN for 30 min at 4°C to FMLP, C5a, CSF-GM, A23187, or PMA, or addition of these factors during the binding assay, did not affect TNF binding. Neither FMLP nor C5a induced a decrease in TNF binding when

Table 1. *Effects of Various PMN Activators on the Ability of PMN to Bind TNF*

Treatment*		^{125}I-TNF binding (percent of untreated control)‡ after treatment at 37°C for: 10 min	45 min
FMLP	10^{-7} M	13 ± 5 (4)	9 ± 4 (4)
	10^{-8} M	17 ± 4 (4)	12 ± 9 (2)
rC5a	10^{-7} M	41 ± 14 (4)	34 ± 3 (2)
	10^{-8} M	58 ± 2 (3)	55 ± 2 (2)
A23187	10^{-5} M	4 ± 1 (2)	1 ± 1 (2)
PMA	100 ng/ml	0 ± 0 (3)	0 ± 0 (3)
rCSF-GM	100 ng/ml	67 ± 1 (2)	50 ± 18 (2)
rIFN-γ	100 ng/ml	95	93 ± 1 (2)
rTGF-β1	100 ng/ml	100	90 ± 4 (2)
rIL-1β	100 ng/ml	ND	91
LPS	1 μg/ml	104 ± 20 (3)	73 ± 6 (3)
	100 ng/ml	104 ± 12 (3)	82 ± 7 (3)

* PMN were incubated for 10 or 45 min at 37°C with the indicated reagent, washed and assayed for ^{125}I-TNF binding at 4°C as described.

‡ TNFα-specific binding is given as a percent of specific binding seen with cells treated for 10 or 45 min at 37°C with buffer alone; the latter value averaged 5,525 ± 1,256 cpm/100 μg protein. Results are means ± SD from the number of independent experiments indicated in parentheses.

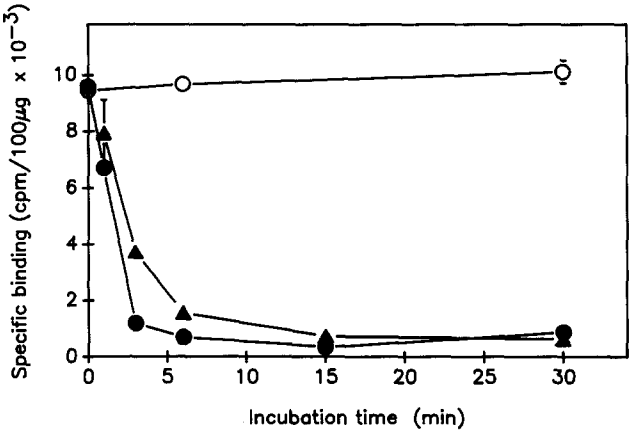

Figure 1. Time course of FMLP-induced downregulation of TNF binding capacity. PMN were incubated at 37°C for the indicated times with either 10^{-7} M FMLP (●), 10^{-8} M FMLP (▲), or buffer alone (○) before binding of ^{125}I-TNF was measured at 4°C. The results are means ± SE of triplicates. When error bars are not seen, they fall within the symbols.

Table 2. *Partial Recovery of TNF-R after Downregulation by FMLP*

Time after pulsing*	TNF binding (percent of control)‡ after exposure to:	
	Buffer	FMLP
min		
0	100 (5)	22 ± 4 (5)
45	100 ± 15 (5)	39 ± 5 (5)
120	84 ± 6 (3)	39 ± 11 (3)

* PMN were pulsed with either buffer alone or 10^{-7} M FMLP for 15 min at 37°C, washed three times, and incubated in fresh medium at 37°C for the indicated times before the TNF binding assay.
‡ Specific binding of TNF is given as a percent of specific binding obtained at time 0 for buffer-treated cells, which averaged 7,626 ± 1,407 cpm/100 μg protein. Results are means ± SE of the number of experiments in parentheses.

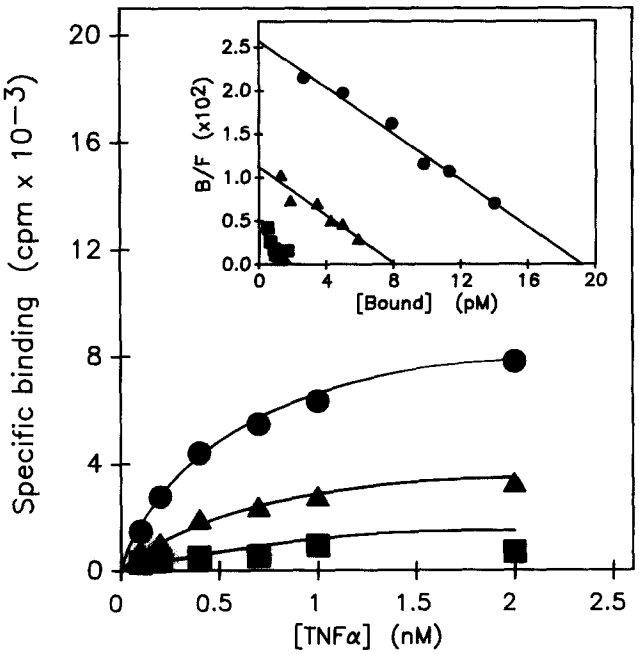

Figure 2. Equilibrium binding and Scatchard analysis of ^{125}I-TNF binding to PMN incubated at 37°C for 15 min with 10^{-8} M FMLP (■), 3 × 10^{-9} M FMLP (▲), or buffer alone (●), as described in Materials and Methods. (*Inset*) Scatchard analysis of specific binding using the LIGAND program (36). The results are means of duplicates.

added to PMN for 90 min at 16°C before the binding assay (not shown).

Selectivity of Loss of TNF-R Expression. Treatment of PMN with FMLP under conditions that led to almost complete inhibition of binding of ^{125}I-TNF did not affect the binding of another cytokine (^{125}I-IFN-γ) nor the binding of mAbs directed against HLA-A,B,C (Table 3) or a surface antigen of 157 kD (30) (not shown). Under the same conditions, cell surface expression of CD18 increased two- to threefold (Table 3). Thus, the decrease in TNF-R induced by FMLP was relatively selective.

Decreased Binding of TNF Results from Loss of TNF-R. The rapid decrease of TNF binding induced by PMN activators could be due to a conformational change of the receptor, resulting in decreased affinity for its ligand, or to a loss of receptors from the cell surface. To distinguish between these possibilities, buffer- or FMLP-treated PMN were reacted with increasing concentrations of ^{125}I-TNF and the specific binding values were analyzed according to Scatchard (35). Fig. 2 shows that FMLP reduced the total receptor number per cell from 1533 ± 132 for buffer-treated cells to 139 ± 54 and 629 ± 92 for cells incubated 15 min at 37°C with 10^{-8} M or 3 × 10^{-9} M FMLP, respectively, without any change in affinity (K_d of 0.4 ± 0.16 nM for both buffer- and FMLP-treated PMN). Results were similar after exposure of PMN to C5a, PMA, or A23187 at incompletely modulating concentrations (Table 4).

Independent evidence that TNF-R were lost from the cell surface of activated PMN was provided by reacting FMLP- and buffer-treated PMN with mAbs against TNF-R, followed by ^{125}I-F(ab')$_2$ anti-mouse IgG. As shown in Table 5, incubation with FMLP led to a decrease in binding of anti-

Table 3. *Effect of FMLP on Expression of TNF-R, IFN-γ-R HLA, and CD18 Antigens on PMN*

Treatment*	Specific binding (cpm/100 μg of protein)‡			
	TNF	IFN-γ	mAb anti-HLA-A,B,C	mAb anti-CD18
None	3,660 ± 306	5,902 ± 539	55,252 ± 4,228	35,290 ± 4,600
FMLP	896 ± 273 (24)§	6,606 ± 664 (111)	54,749 ± 10,400 (99)	78,502 ± 5,280 (222)

* PMN were incubated for 15 min at 37°C with either buffer alone or 10^{-8} M FMLP before binding assays for cytokines or mAbs as described in Methods.
‡ Mean ± SE for triplicates.
§ Percent of specific binding to cells treated with buffer alone.

Table 4. *Scatchard Analysis of TNF Binding by PMN Treated with C5a, PMA, or A23187*

Treatment	K_d (nM)*	Sites/cell*	Percent of control
	nM		
Control	0.43 ± 0.16	2,122 ± 545 (3)	-
C5a (10 nM)‡	0.36 ± 0.07	710 ± 42 (2)	33
A23187 (1 μM)§	0.35 ± 0.03	1,512 ± 461 (2)	71
PMA (1 ng/ml)§	0.56	871	41

* Parameters obtained by Scatchard analysis of specific binding by using the LIGAND program (36). Means ± SD from the number of independent experiments in parentheses.
‡,§ PMN were treated for 45 min (‡) or 10 min (§) at 37°C with the indicated reagents before the binding assay. Stimuli were used at lower concentrations than in Table 1 in order to induce incomplete modulation of TNF binding sites and permit estimation of K_d and sites/cell.

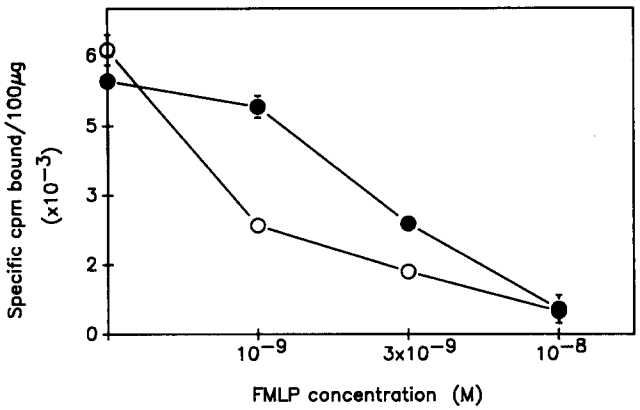

Figure 3. Effect of cytochalasin B on FMLP-induced decrease in TNF binding to PMN. PMN were incubated for 30 min at 37°C with (○) or without (●) 10 μg/ml cytochalasin B before adding the indicated concentrations of FMLP for 15 min at 37°C. Binding of ^{125}I-TNF was then assayed at 4°C. Results are means ± SE of triplicates.

TNF-R mAbs similar in extent to the decrease in binding of TNF.

Mechanism of Loss of TNF-R. TNF binding sites could disappear from the cell surface by internalization or shedding. To test for a role of endocytosis, PMN were pretreated with the microfilament-disrupting agent cytochalasin B (10 μg/ml) for 30 min at 37°C before adding FMLP. As shown in Fig. 3, cytochalasin B did not prevent the decrease in TNF binding induced by FMLP. On the contrary, cytochalasin B synergized with FMLP to augment loss of TNF-R.

To examine the possibility that activated PMN might shed TNF-R, we tested cell-free supernatants of stimulated PMN for soluble TNF-R in a radioreceptor assay. PMN were incubated for 10 min at 37°C with FMLP or buffer alone. The cell-free incubation media were collected and their ability to compete with ^{125}I-TNF for its binding to fresh cells was analyzed at 4°C, a condition in which the indicator cells could not respond to FMLP. As a control, FMLP was added to supernatants of buffer-treated PMN during the binding assay at 4°C. Fig. 4 shows that supernatants from FMLP-stimulated PMN inhibited ^{125}I-TNF binding to fresh PMN and mouse macrophages, while supernatants from buffer-treated neutrophils had no effect.

Table 5. *Effect of FMLP on Binding of TNF and Anti-TNF-R mAbs to PMN*

	Specific binding‡	
Treatment*	TNF	anti-TNF-R mAb§
Control	8,898 ± 818	2,901 ± 382
FMLP	983 ± 352 (11)‖	680 ± 263 (23)

* PMN were incubated for 10 min at 37°C with either buffer alone or 10^{-7} M FMLP and assayed for binding of TNF or mAbs as described in Materials and Methods.
‡ Specific binding is given in cpm per 100 μg protein (means ± SD for triplicates).
§ mAbs Utr-1, Htr-5, and Htr-9 at 8 μg/ml each.
‖ Percent of specific binding to buffer-treated cells.

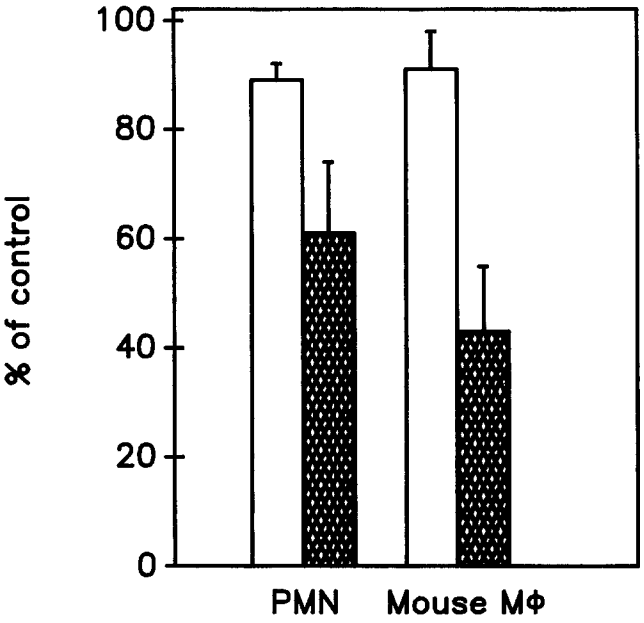

Figure 4. Competition for TNF binding to human PMN and mouse macrophages (Mϕ) by conditioned media from PMN treated with FMLP (*dashed bars*) or buffer (*open bars*). PMN were incubated with 10^{-6} M FMLP or with buffer alone for 10 min at 37°C, and cell-free supernatants were used as the medium in which to measure ^{125}I-TNF binding to untreated cells. Results are means ± SE of three independent experiments and are expressed as a percent of specific binding obtained in the absence of conditioned medium from PMN, which averaged 1,897 ± 112 on PMN and 1,083 ± 185 on macrophages.

To determine whether the moieties that blocked ^{125}I-TNF binding were related to TNF-R, we used anti-TNF-R mAbs Htr-5, Htr-9, and Utr-1, and chose murine macrophages as indicator cells in the competition assay. The specificity of these mAbs for the human as opposed to the mouse TNF-R was shown by their ability to block completely the binding of ^{125}I-TNF to human PMN while inhibiting binding to mouse macrophages by only 10–20% (Fig. 5 *A*). Preincubation of cell-free supernatants from FMLP-stimulated PMN with a mixture of these mAbs (5 μg/ml each) totally reversed their inhibitory effect on ^{125}I-TNF binding to mouse macrophages (Fig. 5 *B*).

To test if TNF-R released from stimulated PMN were soluble rather than associated with membrane fragments, supernatants of PMN treated with FMLP were ultracentrifuged (100,000 *g*, 1 h, 4°C) or passed through a 0.22-μm filter (Millipore Corp., Danvers, MA). Neither procedure diminished the ability of the conditioned media to block ^{125}I-TNF binding to cells (data not shown). Microscopic examination of both FMLP- and buffer-treated PMN revealed monodisperse cells with >95% viability by trypan blue exclusion, indicating that the generation of soluble TNF-R from PMN was not a consequence of cell death.

Characterization of Soluble TNF-R by Ligand Blotting. Supernatants from buffer- or FMLP-treated PMN were subjected to SDS-PAGE, transferred to nitrocellulose membranes and probed with ^{125}I-TNF. Two ^{125}I-TNF binding proteins were identified in the supernatants of FMLP-stimulated PMN (Fig. 6 *A*): a major species of ~95 kD and a ~28-kD molecule which appeared as a diffuse, less intense band. In the presence of a 200-fold excess of unlabeled TNF, binding of ^{125}I-TNF to the higher-M_r band was unaffected. The identity of the ~95-kD protein that binds TNF nonspecifically is unknown. In contrast, unlabeled TNF completely eliminated binding of ^{125}I-TNF to the ~28-kD protein (Fig. 6 *B*). However, no ^{125}I-TNF–binding proteins were detected in ligand blots of supernatants from PMN that were not exposed to FMLP (Fig. 6 *C*), even when autoradiography was prolonged from 3 d (Fig. 6) to 3 wk (not shown). Thus, FMLP triggered PMN to release a soluble ~28-kD protein that bound TNF specifically.

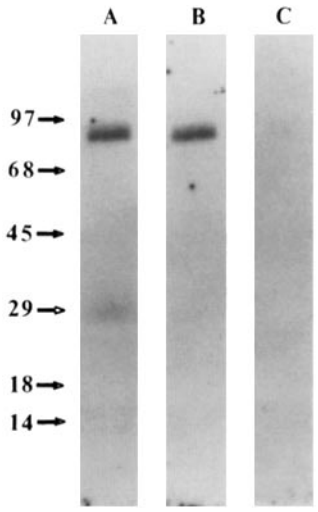

Figure 6. Ligand blot analysis of soluble TNF binding proteins released by PMN. Cell-free supernatants of PMN stimulated with 10^{-6} M FMLP (lanes *A* and *B*) or buffer alone (lane *C*) for 10 min at 37°C were fractionated by 5–15% SDS-PAGE under nonreducing conditions, electrophoretically transferred to nitrocellulose membranes and blotted with ^{125}I-TNF in the absence (lanes *A* and *C*) or presence (lane *B*) of a 200-fold excess of unlabeled TNF. Supernatants from 37 × 10^6 PMN were applied per lane. Positions of prestained M_r markers are indicated.

Effect of Protease Inhibitors. To test if proteolytic cleavage were responsible for shedding of TNF-R, PMN were stimulated with FMLP in the presence or absence of inhibitors of serine or thiol proteases. The decrease in ^{125}I-TNF binding to PMN induced by FMLP could not be prevented by addition of diisopropylfluorophosphate (DFP), trypsin inhibitor, PMSF, leupeptin, aprotinin, or pepstatin (Table 6).

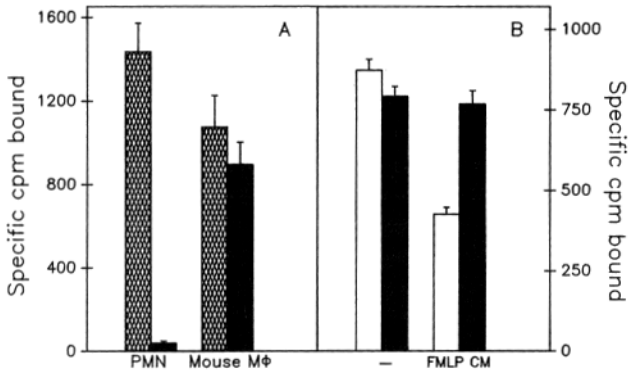

Figure 5. Presence of TNF-R in conditioned medium of FMLP-stimulated PMN. (*A*) Species specificity of inhibition of binding of TNF by anti-TNF-R mAbs. Human PMN or mouse macrophages were incubated with either buffer alone (*dashed bars*) or mAbs Htr-5, Htr-9, and Utr-1, 5 μg/ml each (*filled bars*), for 30 min at 4°C, and then assayed for ^{125}I-TNF binding. Results are means ± SE of three independent experiments. (*B*) Effect of anti-TNF-R mAbs on inhibition of TNF binding to mouse macrophages by conditioned medium from FMLP-stimulated PMN. Medium alone or cell-free supernatants of PMN treated with FMLP were incubated for 30 min at 4°C with ^{125}I-TNF in the absence (*open bars*) or presence (*filled bars*) of the same anti-TNF-R mAbs used in *A*, then added to mouse macrophages for the binding assay. Results are means ± SE of triplicates.

Discussion

Downregulation of cell surface receptors is one means by which cells control their responses to agonists. At least two mechanisms serve to decrease the number of TNF-R on various cells: internalization induced by homologous (27, 37, 38) or heterologous ligands (25, 26), and reduced rates of TNF-R synthesis and/or translocation to the plasma membrane (28). Neither of these mechanisms has been detected in PMN (25, 28). In contrast, PMN appear to use a third mechanism, shedding, to decrease rapidly the number of TNF-R on their surface. Along with FcRIII (CD16) (39) and the MEL-14 selectin (40), TNF-R is the third plasma membrane receptor that PMN are known to shed upon activation.

Activators of PMN function that induced a decrease in TNF-R included physiologic peptides (FMLP) and polypeptides (C5a, CSF-GM) of bacterial or human origin, and pharmacologic agents that activate protein kinase C (PMA, A23187). The decrease in TNF-R expression induced by all these agents was rapid, and required cell metabolism, judging by its temperature dependence. Shedding seemed to account for at least part of the decreased expression of TNF-R, in

Table 6. *Effect of Protease Inhibitors on FMLP-induced Decrease in TNF-R*

Protease inhibitor*	TNF binding (percent of control)‡
None	11 ± 5
DFP (2.5 mM)	8 ± 4
Trypsin inhibitor (1 mg/ml)	12 ± 3
PMSF (0.5 mM) + leupeptin (20 μg/ml) + aprotinin (10 μg/ml) + pepstatin (10 μg/ml)	9 ± 3

* PMN were incubated for 15 min at 37°C with 10^{-7} M FMLP alone or in the presence of the indicated protease inhibitors before the binding assay for TNF.

‡ Specific binding of TNF is given as a percent of specific binding seen with cells treated with buffer alone, which averaged 5,008 ± 1,299 cpm/100 μg protein. Results are means ± SD for triplicates.

that a loss of TNF-R binding sites (as estimated by Scatchard analysis of TNF binding, and by binding of anti-TNF-R mAbs) was coincident with the appearance in the medium of soluble molecules capable of binding both TNF and anti-TNF-R mAbs. However, we could not quantitate soluble TNF-R, and thus could not establish whether shedding accounted completely for the disappearance of TNF-R from the PMN surface. It is possible that only some plasma membrane TNF-R were shed; others may have been lost by internalization or degradation. By whatever mechanism(s), the loss of TNF binding sites often appeared to reach 100%.

Shedding of many plasma membrane receptors from cells other than PMN has been described. Examples are the antigen-binding molecules, MHC class I (41) and class II (42); the adhesion molecule CD8 (43); and receptors for IgE (44), IL-2 (45), and insulin (46). The biochemical processes involved are not well understood. In some instances, release of plasma membrane vesicles is involved (41, 42). This probably did not account for shedding of TNF-R from PMN, since the inhibitor of TNF binding in supernatants of activated PMN was not sedimented by ultracentrifugation. Perhaps selective proteolytic cleavage (45) releases TNF-R, while preserving its ability to bind both TNF and anti-TNF-R mAbs. If so, then the role of PMN activators may be to promote exocytosis of lysosomal proteases. However, we could not prevent FMLP-induced loss of TNF-R with DFP, PMSF, soybean trypsin inhibitor, leupeptin, and pepstatin. This militates against a role for serine or thiol proteases, but leaves open the possibility that a protease insensitive to these inhibitors may be involved. Proteins anchored to membranes via glycosylphosphatidylinositol can be shed after cleavage by phospholipases (47). Such a mechanism may underlie the FMLP-induced shedding of FcRIII (39). However, we were able to affect the expression of TNF-R on PMN with phosphatidylinositol-specific phospholipase C (data not shown). We are only likely to understand the mechanism of shedding of PMN TNF-R after both its membrane and soluble forms have been purified.

Soluble TNF-binding proteins of 27 to 50 kD have been isolated from the urine of normal (48) and febrile individuals (49) and the sera of patients undergoing hemodialysis (50). At least two of these proteins share immunological reactivity with TNF-R of myeloid and epithelial cell lines (48). The findings presented here raise the possibility that some TNF-binding proteins in body fluids may consist of or derive from TNF-R shed by PMN. The apparent 28,000 M_r of TNF-R shed by PMN is consistent with this possibility.

At first glance, the observations made here in vitro might appear to preclude the possibility that PMN can respond to TNF in vivo, where multiple stimuli, including C5a and formylated peptides of bacterial (51) and/or mitochondrial (52) origin, are likely to be present during infection or tissue injury. However, consideration of this issue must take into account the rapidity with which the binding of TNF to its receptor commits PMN to subsequent responses (Nathan, C., unpublished observations), the retention of a substantial number of TNF-R after exposure to C5a, and the sequence in which PMN may encounter various stimuli as they move from the circulation into tissues. Another key variable is whether the PMN are in suspension, as in the present study, or have made contact with biological surfaces before being stimulated (Porteu, F., and C. Nathan, preliminary observations).

TNF is a major mediator of host responses to endotoxin (4). Responses of PMN to TNF may contribute to the pathogenesis of septic shock and respiratory distress syndromes. Downregulation of TNF-R on PMN that first encounter another agonist in the circulation may represent an important mechanism for protecting the host against deleterious effects of TNF. At the same time, soluble TNF-R released by activated PMN might serve an immunoregulatory role by interfering with the binding of TNF to other cells. Administration of soluble, recombinant, human TNF-R might protect people against the consequences of excessive production of TNF, without the antitherapeutic immune response expected to follow the administration of mAbs against TNF or TNF-R.

It is also possible that a soluble form of TNF-R has cytokine-like actions of its own, just as soluble FcεRII mimics IL-2 (53). If so, a cell surface receptor for soluble TNF-R could be the 26-kD form of TNF present on the plasma membranes of monocytes (54) and activated T cells (55). According to this view, not only could mononuclear cells signal PMN (9), but PMN could signal mononuclear cells in the regulation of inflammatory responses.

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Address correspondence to Dr. Carl Nathan, Cornell University Medical College, 1300 York Ave., Box 57, New York, NY 10021.

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