The Tumor Necrosis Factor Receptor and Human Neutrophil Function
Deactivation and Cross-deactivation of Tumor Necrosis Factor–induced
Neutrophil Responses by Receptor Down-regulation

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Abstract

Despite numerous reports, the role of tumor necrosis factor (TNF) in polymorphonuclear leukocyte (PMN) function remains controversial. We found TNF to be a potent, pertussis toxin–independent stimulator of PMN adhesion (ED50 2.6 pM). TNF-stimulated PMN under adherent conditions released up to 65% of their transcobalamin content (ED50 3.9 pM) and increased their burst activity 10-fold (ED50 3.2 pM) as measured by the hexose monophosphate shunt, whereas PMN held in suspension hardly degranulated at all and only little burst activity was demonstrable. However, preincubation of PMN with TNF in suspension led to a decrease in cellular adhesiveness, degranulation, and burst activity in response to a secondary stimulus of TNF under adherent conditions, although cells remained fully responsive toward phorbol myristate acetate. A concomitant dose-dependent decline of TNF receptor numbers that correlated well with the inhibition of PMN function (r = 0.91) suggests receptor down-regulation as the mechanism of functional PMN deactivation. Remarkably, preincubation with other PMN stimuli such as N-formyl-methionyl-leucyl-phenylalanine, platelet-activating factor, leukotriene B4, complement component fragment 5a (C5a)/C5a (desargadated), and endotoxin also led to a reduction of TNF-specific PMN responses (cross-deactivation) from 35% (LTB4) to 90% (endotoxin), corresponding with the down-regulation of TNF receptors. Deactivation and receptor down-regulation are independent of pertussis toxin–sensitive G proteins and protein kinase C but seemed to depend on changes in calcium metabolism. Granulocyte hypersensitivity towards TNF in sepsis (with elevated blood levels of endotoxin and TNF) might be a mechanism of self-protection or, to the contrary, might impair a possibly central mod of host defense. (J. Clin. Invest. 1990. 86:184-195.) Key words: deactivation • function polymorphonuclear leukocyte • receptor • tumor necrosis factor

Introduction

Tumor necrosis factor (TNF)1 or cachectin, a glycoprotein of 17,000 mol wt, has, apart from its cytotoxicity towards certain tumor cells (1) and the suppression of lipoprotein lipase activity in adipocytes (2, 3), pleiotropic effects on a large number of different cell types including fibroblasts (4), osteoclasts (5), and endothelial cells, in which TNF induces procoagulant activity (6), adhesion (7), and transmigration of granulocytes (8, 9). Its role in the stimulation of polymorphonuclear leukocytes (PMN), however, has been controversial. Some authors found TNF to be only a weak direct stimulus of the neutrophil respiratory burst and degranulation and to act essentially as a primer of PMN phagocytosis (10), antibody-dependent cellular cytotoxicity (ADCC) (11), or PMN responses to secondary stimuli such as N-formyl-methionyl-leucyl-phenylalanine (FMLP) (12). In contrast, others presented data that showed TNF to be a potent stimulus of the respiratory burst of neutrophils (13, 14). Starting out from the concept that PMN adhesion strongly promotes neutrophil functional responses (15, 16), it was suggested that differences in incubation conditions could explain these conflicting results: whereas PMN in suspension showed hardly any stimulation of degranulation or respiratory burst, adherent PMN did (with or without cytochalasin B (17)).

Although it has proven to be difficult to isolate and characterize the TNF receptor biochemically (18–20), there is good evidence that TNF effects are mediated via a ligand–receptor system. High-affinity binding sites on tumor cells are necessary, although not sufficient for tumor cell sensitivity towards TNF-induced cytotoxicity (4, 18, 20). On PMN, the expression of high-affinity binding sites for TNF could be demonstrated as well and was correlated to PMN stimulation (21). Possibly owing to the lack of unequivocal TNF-induced functional data, there has been little interest in the further characterization of the TNF receptor on neutrophils, and the amount of information on the TNF receptor on neutrophils has remained relatively scarce compared to, for example, FMLP and its receptor. Specific PMN deactivation towards FMLP, i.e., cellular desensitization by preexposure to the same stimulus, has been thoroughly investigated and has been attributed to a reduction in receptor availability (22–24). Whether deactivation processes regulate PMN responses toward stimulation by TNF as well is unknown.

In confirming previous reports, we found TNF-induced responses in PMN to be highly dependent upon the incubation conditions: whereas PMN in suspension showed only little degranulation and activity of their respiratory burst upon stimulation with TNF, TNF was a most effective stimulus in adherent granulocytes. However, this effect was largely suppressed when cells were preincubated with TNF in suspension desargadated; DAG, diacylglycerol; HIP, heat-inactivated plasma; HMPs, hexose monophosphate shunt; PAF, platelet-activating factor; TC, transcobalamin (vitamin B12 binding protein); TNF, tumor necrosis factor.

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1. Abbreviations used in this paper: C5a and C5a desarg, complement component fragment 5a and complement component fragment 5a.
before receiving a secondary dose of TNF under adherent conditions (deactivation). Similarly, preexposure of granulocytes to various PMN stimuli also led to a reduction of TNF-induced cellular functions (cross-deactivation). Both deactivation and cross-deactivation were specific and correlated with down-regulation of the number of TNF receptors.

**Methods**

**Compounds and reagents.** Recombinant TNF was the generous gift of Dr. Frickel, Knoll/BASF AG, Ludwigshafen, Federal Republic of Germany. Recombinant TNF had a specific activity of 3.9 × 10⁶ U/mg (cytotoxicity assay on murine L929 cells) and was free of endotoxin as determined by Limulus assay performed by the manufacturer. The preparation appeared as a single band at 17,000 mol wt in silver-stains of SDS-PAGE gels. Endotoxin strain R595 Salmonella minnesota, prepared according to the method of Galanos reported by Dahinden et al. (25), was purchased from SEBAG GmbH, Aidenbach, Federal Republic of Germany. FMLP was purchased from Bachem AG, Bubendorf, Switzerland. The tripeptide was dissolved in dimethyl sulfoxide (DMSO) and diluted in NaCl 0.15 M. Phorbol myristate acetate (PMA, Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO and added to the reaction sample to give a dilution > 1:1,000. 1,0-alkyl-2-acetyl-sn-glycerol-3-phosphatidylcholine (platelet-activating factor, PAF) was obtained from Bachem AG. Bioactivity of leukotrione (LT) B₄ (ICN Biomedicals, Eschwege, Federal Republic of Germany) was always checked in a chemotaxis assay. Complement component fragment 5a (C5a) was purified from human serum by the procedure of Fernandez and Hugli (26), with minor modifications. These modifications included the substitution of carboxymethyl-Sepharose for carboxymethyl-cellulose, the use of continuous gradients on all ion-exchange columns, and use of the potent serum carboxypeptidase inhibitor described by Ondetti et al. (27). Actinomycin D, cyclochalin B, cycloheximide, chlorouoquine, colchicine, monensin, and polymyxin B were from Sigma Chemical Co. Pertussis toxin was from List Biological Laboratories, Inc., Campbell, CA.

Monoclonal antibody (MAB) Mon 5005 (clone TNF2, IgG1) and polyclonal antibody PS30 (rabbit), both directed against human TNF, were purchased from Sanbio, Uden, The Netherlands. All other reagents were of analytical grade.

**Iodination of TNF.** Iodination was performed with the iodine procedure with slight modifications (28). 100 µl of NaI¹²⁵ in phosphate buffer, 0.1 M, was added to an Iodogen-coated (2 µg) reaction tube (Treff AG, Degersheim, Switzerland) on ice and left to react for 10 min at 4°C. Subsequently, the solution was transferred to a second reaction tube containing 20 µg of TNF in 10 µl of PBS and the iodination was allowed to proceed for 10 min at 4°C, when it was stopped by the addition of 1 ml of ice-cold PBS. The iodinated protein was separated from free iodine by filtration over a PD 10 (Sephadex G-25M, Pharmacia, Uppsala, Sweden) column that had been equilibrated with PBS-A. Specific activity of ¹²⁵I-TNF was first estimated by the yield of the iodination procedure and subsequently determined more accurately by self-displacement analysis (29), which, in addition, provided evidence for the similar affinity of iodinated and unlabeled TNF for its receptor (data not shown). Calculations were corrected for the portion of radiolabeled TNF (18–30%) that did not bind to its receptor as estimated by extrapolation of the binding curve of a constant concentration of ¹²⁵I-TNF (0.1 ng/ml) to an increasing concentration of cells (1–20 × 10⁶/ml [30]). Three independent iodinations yielded specific activities between 23.8 and 34.2 µCi/µg and iodinated TNF that was biologically fully active as determined by PMN adhesion assay (see below). ¹²⁵I-TNF was stable over at least 8 wk, when stored at −70°C. Autoradiography of radiiodinated TNF on SDS-PAGE gels showed a single band at 17,000 mol wt. Fractionation of both unlabeled TNF and of ¹²⁵I-TNF by gel filtration chromatography (Sephadex G 75) provided a single peak with an apparent molecular weight of ≈ 53,000, suggesting a noncovalent trimer as the form in which the glycoprotein occurs under nondenaturating conditions (19, 31). Therefore, for calculations of receptor binding data and molarity, a molecular weight of 51 kD was assumed.

**Preparation of neutrophils.** Venous blood from healthy human volunteers was collected into 60-ml syringes containing preservative-free sodium heparin (50 U/ml blood, Novo Industries, Copenhagen, Denmark) and PMN were separated as described (> 98% neutrophils [25]). Incubation medium consisted of autologous heat-inactivated plasma (40 min, 56°C; HEP) prepared from heparinized venous blood (3 U/ml). When necessary, PMN were washed in PBS, pH 7.4, supplemented with albumin (ORHA 20/21, Behringwerke AG, Marburg, Federal Republic of Germany) and glucose (138 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄·2H₂O, 1.5 mM KHzPO₄, 0.6 mM CaCl₂·2H₂O, 1.0 mM MgCl₂·6H₂O, 2.7 mM glucose, 5 mg/ml albumin; PBS-A).

**Equilibrium specific binding of ¹²⁵I-TNF to PMN.** PMN in 1 ml of HEP were incubated at 4°C in triplicates with increasing concentrations of ¹²⁵I-TNF (between 600 pg/ml and 10 ng/ml, if not indicated otherwise) in reaction tubes and vortexed gently at regular intervals. To terminate the assay and separate PMN-bound ¹²⁵I-TNF from unbound ligand, 850 µl of the cell suspension was laid over 500 µl of Dextran-T70 (Pharmacia, 10%, wt/vol in PBS) and centrifuged at 13,000 g, 4°C. This procedure allows separation of bound from unbound ligand within seconds. Reaction tubes were then frozen in an isopropanol bath on dry ice, tips containing the cell pellet were cut off and radioactivity was quantitated in a γ-counter (Cobra model 5005, Packard Instrument Co., Inc., Downers Grove, IL) with automatic half-time correction and a counting efficiency of 80%. The assay was found to be linear between 1 × 10⁵ and 1 × 10⁶ PMN/ml, when a concentration of 600 pg/ml ¹²⁵I-TNF was used (r = 0.986, P ≤ 0.0001).

In standard assays, a cellular concentration of 5 × 10⁶ PMN/ml was used to provide for sufficient accuracy at the given specific activity of radiiodinated TNF and yet keep the total receptor concentration smaller than the Kd of the receptor (Kd < Kd [32]). Nonspecific binding was determined in parallel in duplicates by adding a 100-fold excess of unlabeled TNF to a second set of matched reaction tubes. Specific binding was defined as the difference between total binding and nonspecific binding, which was consistently < 10% of total binding.

**Adherence and suspension assays.** PMN adhesiveness was tested as outlined (25, 33), with slight modifications as to the quantification of adherent neutrophils. Briefly, 1-ml aliquots of neutrophil suspensions (4.0 × 10⁶ PMN/ml) were incubated in 10 × 35-mm Petri dishes (Falcon Labware, Becton, Dickinson & Co., Oxnard, CA) for 40 min at 37°C, 100% humidity/5% CO₂. Immediately before plating, the adhesion stimulus was added; dilution by stimulus solution was 1% (vol/vol). After incubation, the dishes were washed thoroughly and nonadherent PMN were removed by passing the dishes three times through an air-fluid interface sequentially in four beakers containing NaCl (0.15 M) at room temperature. The dishes were decanted and rest fluid was dried. 1 ml of Zap-oglobin (Couler Electronics Ltd., Luton, England) was added to each dish and the nuclei, which were set free by the detergent counted in a Coulter flow cytometer (Model ZB) in an adaptation of the method of Nagakawara and Nathan (34). Quantification of adherence, induced by various stimuli including FMLP, endotoxin, PMA, and TNF and expressed as the percentage adherence of totally added cells, correlated well with results obtained with ¹¹¹In-labeled PMN (r = 0.994, P ≤ 0.0001 [9]). All adherence experiments were verified by phase-contrast microscopy.

In degranulation experiments with cells held in suspension, PMN were incubated in overhead rotation (4–6 rpm; multi-purpose rotator model 151, Scientific Industries, Inc., Bohemia, NY) in polypropylene tubes (Falcon Labware) under otherwise identical conditions as used for the adhesion experiments.

**Granule exocytosis.** Transcobalamin (vitamin B₁₂ binding protein; TC), an excellent indicator of secondary granule exocytosis, which we measured by the relative absorption of ¹³⁵Co-B₁₂ (35), was determined in supernatants of PMN stimulated to adherence on pla-
tic dishes and of PMN held in suspension after pelleting the cells by centrifugation at 400 g, 4°C.

Glucose oxidation via the hexose monophosphate shunt (HMPS). Glucose oxidation via the HMPS, which yields NADPH necessary for the formation of oxygen radicals during the respiratory burst and thus serves to quantify the activity of the respiratory burst, was determined by measuring the generation of $^{14}\text{CO}_2$ from [1-$^{14}$C]glucose as described (25). Cells were assayed either in suspension (dishes shaken on a IKA-Vibrax [VXR7, IKA, Staufen, Federal Republic of Germany] at 600 rpm) or under adherent conditions.

Superoxide release. Superoxide release was determined by measuring superoxide dismutase (10 ng/ml, Sigma Chemical Co.) inhibitable cytochrome c (80 $\mu$M, horse heart type VI, Sigma Chemical Co.) reduction in a dual-wavelength spectrophotometer (model UV 160, Shimadzu, Seisakusho Ltd., Kyoto, Japan) at 550-nm wavelength, $\epsilon$ = 21.6 mM$^{-1}$ (36, 37).

Results

TNF is a potent stimulus of PMN function. TNF increased PMN adhesiveness in a dose-dependent manner (Fig. 1). PMN responded to as little as 33 pg/ml (0.6 pM) TNF and showed maximal responses at 1 ng/ml (20 pM), with an ED$_{50}$ of 135±41 pg/ml (2.6±0.8 pM, n = 3). Under adherent conditions, cells were induced by TNF to degranulate (ED$_{50}$ = 200±28 pg/ml [3.9±0.6 pM], n = 3) and to activate their respiratory burst (ED$_{50}$ = 164±80 pg/ml [3.2±1.5 pM], n = 3) to an extent comparable with that of PMA (100 ng/ml; see Figs. 3 and 4 below). However, when PMN were stimulated in suspension under otherwise identical conditions as under adherence, only slight increases in exocytosis or HMPS activity could be elicited even at concentrations of TNF up to 100 ng/ml (Fig. 2). Similar results were obtained for O$_2$ release: PMN (adherent) 0.43±0.86 nmol/10$^6$ PMN per 30 min; PMN (in suspension), 0.10±1.72 nmol/10$^6$ PMN per 30 min; PMN plus TNF (10 ng/ml; adherent), 8.06±1.82 nmol/10$^6$ PMN per 30 min; PMN plus TNF (in suspension), 0.33±0.40 nmol/10$^6$ PMN per 30 min (mean±SD of triplicates, n = 3).

In contrast to PMN activation by FMLP (38), TNF-induced cellular responses were insensitive to pertussis toxin as preincubation (37°C, 2 h) with pertussis toxin (1 $\mu$g/ml) revealed the following inhibition profile (percent reduction compared to control cells): FMLP (adhesion), 87.1±6.0%; FMLP (TC release), 89.3±4.2%; PMA (adhesion), 8.3±6.6%; PMA (TC release), 2.0±2.3%; TNF (adhesion), 9.4±7.4%; TNF (TC release), 6.9±1.1%.

Remarkably, TNF (10 ng/ml)-induced release of transcobalamin, both in adhesion and in suspension, was unaltered by pre- and coincubation of PMN with 5 $\mu$g/ml cytochalasin B: adhesion (without cytochalasin B), 80.6%; (with cytochala-
was (with cytochalasin B), 14.0%; (with cytochalasin B), 16.8%.

Endotoxin contamination, as the source of the observed effects, was ruled out by experiments, in which PMN were stimulated by TNF (10 ng/ml), pretreated either at 100°C, 30 min (79.9% inhibition of adhesion, compared to controls) or with polymyxin B (10 μg/ml, 3.7% inhibition).

Deactivation and cross-deactivation of TNF-induced responses in PMN. Because TNF proved to be such a powerful activator of PMN function under adherent conditions, while inducing only little functional activity as long as cells were held in suspension, we proceeded to investigate whether preexposure of PMN to TNF in suspension might influence their responses under adherent conditions toward a second dose of TNF, and we found that preincubation of PMN in suspension resulted in a dose-dependent inhibition of adherence, degranulation, and HMPS activity (Fig. 3) as well as superoxide production induced under adherent conditions by a second dose of 10 ng/ml TNF: preincubation without TNF—without secondary TNF, 1.24±0.37 nmol/10⁶ PMN per 30 min; with secondary TNF (10 ng/ml), 8.56±1.99 nmol/10⁶ PMN per 30 min; preincubation with TNF (500 pg/ml)—without secondary TNF, 2.73±1.10 nmol/10⁶ PMN per 30 min; with secondary TNF (10 ng/ml); 4.32±0.58 nmol/10⁶ PMN per 30 min. It should be noticed, that the activity due to secondary TNF was regarded as the difference between total response (prestimulating TNF plus secondary TNF) and the response evoked by the prestimulating dose alone. Inhibition (optimal after 2 h of preincubation) was specific, as PMN were fully vital (98% by trypan blue) and showed undiminished responses, when PMA (100 ng/ml, Fig. 3) or FMLP (2.5 μM, not shown) were used as second stimuli. In other words, deactivation exists for TNF as well as it does for FMLP. Probably owing to secondary metabolic changes, PMN, which had been preincubated and not restimulated by TNF, showed reduced functional responses under adherent conditions compared with cells, which were incubated for adhesion immediately after addition of the stimulus (compare Figs. 1 and 3).

Surprisingly, preincubation of granulocytes with stimuli other than TNF resulted also in a specific, dose-dependent desensitization toward secondary stimulation by TNF with respect to adherence, degranulation, HMPS activity (Fig. 4), and superoxide release (preincubation without LPS—without secondary TNF, 1.28±0.26 nmol/10⁶ PMN per 30 min; with secondary TNF (10 ng/ml), 4.80±0.38 nmol/10⁶ PMN per 30 min; preincubation with LPS (40 ng/ml)—without secondary TNF, 2.46±0.31 nmol/10⁶ PMN per 30 min; with secondary TNF (10 ng/ml), 3.62±0.10 nmol/10⁶ PMN per 30 min). Again, it must be emphasized that the response due to the secondarily added TNF is equivalent to the difference between the total response (prestimulus plus secondary TNF) and the response elicited by the prestimulus alone. In the case of endotoxin, this response elicited by the prestimulus alone was generally greater at maximally deactivating doses than with TNF (Figs. 4 and 5). In the experiment depicted in Fig. 4 C, cellular activity due to secondary TNF approximated zero, whereas in other experiments (Fig. 4, A and B) a considerable rest activity remained. In contrast to these at least partly donor-specific variations in the degree of cross-deactivation, particularly in the case of endotoxin and FMLP, the degree of tachyphylaxis caused by TNF itself varied only little between experiments. Stimuli so different in origin, chemical structure, and pattern of biological functions as LTB₄, C₅a, C₅a desarg, endotoxin, and FMLP caused specific cross-deactivation to TNF (Fig. 5).

In contrast, preincubation with PMA (5 ng/ml), a direct activator of protein kinase C, was without effect, indicating that deactivation and cross-deactivation of TNF-induced responses in human granulocytes are not mediated by protein kinase C (Fig. 5). This concentration of PMA was chosen, because 5 ng/ml PMA stimulates PMN adhesion, degranulation, and respiratory burst, whereas heavy aggregation, which is common with higher concentrations of PMA, is avoided.

Characterization of high-affinity binding sites for TNF on neutrophils. Before the question could be addressed, whether alterations in receptor–ligand binding might be responsible for deactivation and cross-deactivation, high-affinity binding of TNF to PMN in our system had to be characterized (Fig. 6). At 37°C, ¹²⁵I-TNF binding to PMN peaked early (10 min) to decrease relatively fast thereafter, whether azide (0.1%) had

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Deactivation of Tumor Necrosis Factor-induced Neutrophil Stimulation

187
been added to the cell suspension or not (Fig. 6 A). At 37°C, 125I-TNF bound to its receptor appears to be rapidly internalized and then intracellularly degraded at a steady rate; PMN incubated at 4°C for 2 h showed only little 125I-TNF binding, which could not be dissociated by low pH (Fig. 6 B [31, 39]), and hence had to be regarded as internalized. When cells that had 125I-TNF bound to their cell surface receptors at 4°C were reincubated after three washes (PBS-A) in HIP at 37°C, surface-bound 125I-TNF was rapidly internalized and hence was no longer dissociable at low pH (Fig. 6 B). Furthermore, when PMN that were preincubated with 125I-TNF at 37°C for 60 min were reincubated after three washes (PBS-A) in HIP at 37°C, concomitant with the gradual decrease of PMN-associated radioactivity, an increase in radioactivity in the supernatant could be detected, most of which was non-TCA-precipitable (Fig. 6 C). Taken together, these results suggest that at 37°C PMN internalize 125I-TNF via its receptor and degrade it thereafter. They explain the rapid rise and gradual decline of cell-associated radioactivity when cells are incubated with 125I-TNF at 37°C (Fig. 6 A).

In contrast to incubations at 37°C, binding at 4°C reached equilibrium after 8 h (Figs. 6 and 7). Consequently, these conditions were used for all receptor binding studies. The $K_{\text{app}}$ at 4°C was remarkably low, so that after three washes (PBS-A) over 45 min, only little dissociation of 125I-TNF from PMN could be detected (Fig. 7 A). Moreover, the rate of dissociation was hardly influenced by the addition of a 100-fold excess of unlabeled TNF (Fig. 7 A). The possibility that 125I-TNF might become internalized even at 4°C was excluded by experiments, which showed that 125I-TNF bound to PMN at 4°C could be dissociated by exposure to low pH (98% vitality, no reduction in cell numbers; Fig. 7 B).

In Scatchard plots (40) of saturation binding experiments, carried out under the conditions described above, only a single class of TNF receptors could be detected on PMN: receptor number (N) = 516±94; n = 28 (iodination 1,557±90 [n = 9]; iodination 2,522±80 [n = 14]; iodination 3,426±91 [n = 5]), $K_d$ = 11.7±2.9 pM; n = 28. When receptor assays were performed in PBS-A similar values were obtained (N = 637±54; $K_d$ = 9.3±0.1 pM; n = 2, figures denote mean±1/2 range).

Deactivation and cross-deactivation of PMN toward TNF are correlated to a reduction in TNF surface receptors. When PMN were preincubated with TNF or FMLP in exactly the
same way as described for functional deactivation and cross-deactivation experiments, and were subsequently examined for equilibrium receptor binding, they showed a strong reduction in the number of TNF receptors together with a concomitant decrease in apparent receptor affinity (Figs. 8 and 9, Table 1). Notably, on PMN preincubated with 500 pg/ml TNF, specific binding was too low to be differentiated from background noise. Three cell washes proved to be optimal to remove cold TNF before the equilibrium studies. When stimuli (other than TNF) were added to the controls during the receptor assay, no competition with 125I-TNF for the receptor could be detected (not shown).

Preincubation of PMN at 37°C, without the addition of stimulus, did not alter TNF receptor expression, when compared to cells held at 4°C.

The role of calcium, protein kinase A, protein kinase C, and pertussis toxin-sensitive G proteins in signal transduction leading to TNF-specific deactivation/cross-deactivation of PMN. Pertussis toxin inhibited TNF-specific deactivation only with such stimuli as FMLP (Fig. 10 B), which cause PMN adhesion, degranulation, or chemotaxis via pertussis toxin-sensitive G proteins (38), whereas deactivation by TNF and lipopolysaccharide (LPS), which activate PMN independently from pertussis toxin-sensitive G proteins (see above), was left uninfluenced (Fig. 10, A and C). Neither TNF or LPS nor pertussis toxin-sensitive G protein-linked FMLP seemed to

Figure 6. 125I-TNF binding to PMN under different conditions. (A) PMN in HIP (3 × 10⁶/ml) were incubated for varying periods of time with 125I-TNF (1 ng/ml) at 4°C (0.1% azide, o), at 37°C (0.1% azide, c) in suspension, or at 37°C in suspension without the addition of azide (●). At the indicated times, triplicate samples were taken, and the receptor assay performed as detailed in Methods. Counts were normalized to 1 × 10⁶ PMN. (B) After incubation in HIP (5.0 × 10⁶/ml) with 125I-TNF (4 ng/ml) for 2 h at 4°C PMN were washed three times with PBS-A, resuspended in HIP, and reincubated at 37°C. At the indicated times, samples were taken, washed twice in PBS-A, and finally exposed to pH 3.0 (glycine buffer, 50 mM in 0.1 M NaCl) for 3 min to detach surface bound ligand. After neutralizing pH by PBS-A dilution cells were pelleted and cell-associated radioactivity was determined as described above. (C) PMN in HIP (6.3 × 10⁶/ml) were incubated with 125I-TNF (10 ng/ml) in suspension for 60 min at 37°C, washed three times in PBS-A, and finally resuspended and reincubated in HIP (2.4 × 10⁶/ml). At the indicated times, triplicate samples were taken, layered onto Dextran-T70 (10%, wt/vol) in 1.5-ml reaction tubes, and separated by centrifugation. Cell-associated radioactivity (not normalized, ●) was determined as described above. Additionally, after Dextran centrifugation supernatant was taken, and total (o) and non-TCA-precipitable radioactivity (c) was measured. Values were corrected for different sample volumes. Mean±SD of triplicates are shown.

Figure 7. At 4°C, 125I-TNF is bound to PMN in a saturable manner, and once bound, is not dissociated by excess unlabeled TNF, but is not internalized. (A) PMN in HIP (6.6 × 10⁶/ml) were incubated with 125I-TNF (1 ng/ml) at 4°C. At the indicated times, samples were taken and PMN were either directly pelleted through Dextran (total binding, dashed line) or subsequently to a 60-min exposure to a 100-fold excess of unlabeled TNF (●). A third batch was washed three times (PBS-A, ○) over 60 min and cell-bound radioactivity was determined as described for Fig. 6. Symbols denote mean of triplicates, normalized to 5 × 10⁶ PMN, SD (12%) are not shown for the sake of clarity. Two independent experiments yielded similar results. (B) PMN in HIP (5 × 10⁶/ml) were incubated with TNF (4 ng/ml) at 4°C. At the indicated times, samples were taken, washed twice in PBS-A, and either directly pelleted (●) or first exposed to pH 3.0 (glycine buffer, 50 mM in 0.1 M NaCl, ○) for 3 min. After neutralizing pH by PBS-A dilution, cells were centrifuged and cell-associated radioactivity was determined. Mean±SD of triplicates, normalized to 5 × 10⁶ PMN, are shown. Two independent experiments yielded similar results.
cause TNF-specific deactivation via the phospholipase C-dependent generation of diacylglycerol (DAG) and activation of protein kinase C, inasmuch as preincubation of PMN with PMA left both functional responses toward TNF and TNF receptors unaltered (Fig. 5, Table I). Forskolin (10^{-5} to 10^{-7} M) and isoproterenol, which activate adenylate cyclase either directly or via adrenergic receptors, led to no significant reduction of TNF receptor numbers (85.9–96% of original receptor numbers), making protein kinase A unlikely to be the secondary messenger of TNF deactivation. However, preincubation of PMN with the ionophore ionomycin resulted in a dramatic reduction of TNF receptors on the cellular surface (Table I). Testing ionomycin in functional deactivation of PMN was not possible because the ionophore caused by itself 77% adhesion at 5 μM with no response at 1 μM (in HIP). It thus appears that changes in granulocyte calcium metabolism might be involved in TNF receptor down-regulation and functional deactivation/cross-deactivation.

The degree of deactivation caused by increasing concentrations of TNF correlated well with the degree of receptor down-regulation (Fig. 11 A). Similarly, TNF cross-deactivation due to either LPS (Fig. 11 B) or FMLP (r = 0.95) was highly correlated to a reduction in TNF receptor number. The functional relevance of this statistical correlation is underscored by the fact that, all stimuli, which caused cross-deactivation of PMN toward TNF, induced a reduction of TNF surface receptors, whereas PMA, which had been without effect in cross-deactivation studies, also failed to down-regulate TNF receptor surface expression (Table I). Moreover, pertussis toxin influenced deactivation or cross-deactivation and TNF receptor numbers always in parallel (Fig. 10).

Possible mechanisms of TNF receptor down-regulation. TNF receptor down-regulation induced by TNF itself can be explained by internalization of the receptor–ligand complex (Fig. 6) and insufficient replacement of receptors either by de novo synthesis or by receptor recycling. The mechanism of TNF receptor reduction due to preincubation with different stimuli such as FMLP and endotoxin, however, was not so straightforward to evaluate.

These stimuli probably do not act through the induction of TNF synthesis in PMN, which in turn might have bound to its receptor, inasmuch as inhibitors of protein synthesis (actino-
Table I. Down-regulation of TNF Receptors in Response to Preincubation of PMN with Various Stimuli

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>N*</th>
<th>Kd (pM)</th>
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<tr>
<td>PAF (1 × 10^-4 M)</td>
<td>306±27</td>
<td>21.9±4.9</td>
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<td></td>
<td>(597±33)</td>
<td>(11.2±2.6)</td>
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<td>LTB4 (250 ng/ml)</td>
<td>384±75</td>
<td>16.3±3.2</td>
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<td></td>
<td>(516±19)</td>
<td>(12.4±2.0)</td>
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<td>C5a (250 ng/ml)</td>
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<td>17.3±5.0</td>
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<tr>
<td></td>
<td>(521±58)</td>
<td>(11.4±3.5)</td>
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<tr>
<td>C5a desarg (250 ng/ml)</td>
<td>156±21</td>
<td>33.2±12.8</td>
</tr>
<tr>
<td></td>
<td>(510±62)</td>
<td>(9.8±0.6)</td>
</tr>
<tr>
<td>FMLP (100 nM)</td>
<td>167±6</td>
<td>26.4±4.9</td>
</tr>
<tr>
<td></td>
<td>(555±114)</td>
<td>(11.7±0.6)</td>
</tr>
<tr>
<td>Endotoxin (40 ng/ml)</td>
<td>222±76</td>
<td>57.7±39.4</td>
</tr>
<tr>
<td></td>
<td>(565±117)</td>
<td>(13.0±1.1)</td>
</tr>
<tr>
<td>TNF (100 pg/ml) ≡</td>
<td>191±2</td>
<td>23.6±4.0</td>
</tr>
<tr>
<td></td>
<td>(407±81)</td>
<td>(13.7±4.9)</td>
</tr>
<tr>
<td>Ionomycin (1 × 10^-3 M)</td>
<td>73±4</td>
<td>49.4±0.1</td>
</tr>
<tr>
<td></td>
<td>(598±110)</td>
<td>(14.0±1.1)</td>
</tr>
<tr>
<td>PMA (5 ng/ml)</td>
<td>564±49</td>
<td>13.9±2.6</td>
</tr>
<tr>
<td></td>
<td>(543±65)</td>
<td>(9.5±0.1)</td>
</tr>
</tbody>
</table>

After preincubation in suspension for 2 h, PMN were either directly (stimuli other than TNF) or after three washes in PBS-A (TNF) assayed for receptor binding (8 h, in HIP at 4°C). In each experiment, unstimulated cells, treated otherwise exactly the same as stimulated cells, served as controls (in parentheses). Cell counts were always checked before incubation with 125I-TNF and subsequent calculations corrected accordingly (of importance only when cells had to be washed after preincubations with TNF itself). Number of receptors and Kd were derived from Scatchard plots.

* N*, number of receptors.

† On PMN preincubated with 500 pg/ml TNF, specific binding was too low to be differentiated from background noise. Values denote mean±SD of three experiments in each set.

mycin D, cycloheximide) did not abolish the down-regulation of receptors (Table II). Furthermore, pretreatment of TNF (100 pg/ml), but not of FMLP (10 nM), with anti-TNF-directed MAbs Mon 5005 (2:1) reversed receptor down-regulation (control, 2,739±222 cpm; TNF alone, 841±101 cpm; TNF with MAb, 2,476±112 cpm; FMLP alone, 467±27 cpm; FMLP with MAb, 31±50 cpm; mean±SD of triplicates).

The possibility that TNF receptors were enzymatically cleaved by proteases set free from activated granulocytes was tested by preincubating cells with the filtrate of sonication-lysed PMN at a concentration of lysosomal proteases equivalent to 10% of total cellular contents. This free concentration of lysosomal contents, which is higher than any concentration induced by the relevant stimuli in suspension, did not alter TNF receptor expression at all (102.9±13.1% of controls, n = 4).

Interference with the integrity of the cytoskeleton is said to interfere with endocytosis (41). However, the disruption of microtubules and actin filaments by colchicine and cytochalasin B did not affect receptor down-regulation, whether induced by TNF or by endotoxin (Table II), although at least half of TNF elicits receptor endocytosis of its own receptor. However, agents which are known to inhibit receptor recycling (monensin) or the degradation of the receptor–ligand complex (NH4Cl, chloroquine), seemed to reinforce receptor down-regulation (Table II). These findings would support the contention that receptor down-regulation by stimuli other than TNF is mediated by endocytosis as well.

Discussion

Confirming the results of others (42), we could show that recombinant human TNF is a very efficient and potent stimulus

Deactivation of Tumor Necrosis Factor-induced Neutrophil Stimulation 191
Figure 11. TNF receptor down-regulation and deactivation or cross-deactivation of PMN towards TNF are closely correlated. (A) PMN in HIP were incubated with increasing concentrations of TNF (33–500 pg/ml) in suspension (2 h, 37°C). Then, cells were either directly restimulated by TNF (10 ng/ml) and let to adhere (= deactivation assay), or washed three times in PBS-A and tested for receptor binding. Adhesion and TC release were determined as described for Fig. 1. Functional responses due to restimulation alone were calculated as the difference between the total responses of restimulated cells and the responses induced by the preincubation dose alone. Results are expressed as percentage of original adhesion/receptors (no TNF added during preincubation). Results of three independent experiments are shown. Transcobalamin release and TNF receptor expression were similarly correlated (r = 0.89; P ≤ 0.0001). (B) PMN in HIP were incubated with increasing concentrations of LPS (0.5–20.0 ng/ml) in suspension (2 h, 37°C). All other procedures were as described under A with the exception that PMN were not washed before receptor binding. TC release and TNF receptor expression were similarly correlated (r = 0.95; P ≤ 0.001).

All inhibitors, with the exception of actinomycin D and cycloheximide (45 min), were added to the cells at 37°C 10 min before the respective stimulus. After preincubation in suspension for 2 h at 37°C, PMN were washed three times in PBS-A, resuspended in HIP, and assayed for receptor binding (8 h, at 4°C). Cell counts were always checked before incubation with 125I-TNF (1 ng/ml) and results normalized to 5 × 10⁶ PMN/ml. Specific binding values are given as mean±SD of triplicates. Chloroquine (25 μM), colchicine (10 μg/ml), and cytochalasin B (5 μg/ml) were tested at lower concentrations as well, with similar results.

Table II. Failure to Inhibit TNF Receptor Down-regulation

<table>
<thead>
<tr>
<th>Preincubation stimulus</th>
<th>TNF (100 pg/ml)</th>
<th>Endotoxin (40 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm</td>
<td></td>
</tr>
<tr>
<td>Cycloheximide (100 μg/ml)</td>
<td>0±25*</td>
<td>36±71</td>
</tr>
<tr>
<td>Actinomycin D (10 μg/ml)</td>
<td>39±15</td>
<td>165±53</td>
</tr>
<tr>
<td>NHx (10 mM)</td>
<td>90±18</td>
<td>174±43</td>
</tr>
<tr>
<td>Chloroquine (100 μM)</td>
<td>8±21</td>
<td>84±11</td>
</tr>
<tr>
<td>Monensin (25 μM)</td>
<td>6±7</td>
<td>90±15</td>
</tr>
<tr>
<td>Colchicine (100 μg/ml)</td>
<td>115±29</td>
<td>322±39</td>
</tr>
<tr>
<td>Cytochalasin B (20 μg/ml)</td>
<td>195±52</td>
<td>158±41</td>
</tr>
<tr>
<td>Control I (stimulus, no inhibitor)</td>
<td>265±65</td>
<td>448±147</td>
</tr>
<tr>
<td>Control II (no stimulus, no inhibitor)</td>
<td>2164±39</td>
<td>2878±131</td>
</tr>
</tbody>
</table>

TNF mediates its effects by coupling to high-affinity receptors (Kd 11.7 nM), of which only 520 were found per cell. The higher number of TNF receptors found by others on PMN (14) and HL-60 cells (45) might well be explained, if these authors used the molecular weight of the monomer as the basis for the calculation of receptor number and Kd (since gel filtration chromatography suggested that TNF normally occurs as a noncovalent trimer, we calculated our data assuming a molecular mass of 51 kD; for details, see Methods). The same considerations hold true for the apparent Kd, which others, with the exception of Kull et al. (3 pM [18]), have generally found one order of magnitude higher than we did (4, 14, 45, 46). In addition, errors may arise from the failure to correct for nonspecific binding of radiolabeled TNF, different methods of estimating specific activity of iodinated TNF, or failure to let the system reach equilibrium (8 h at 4°C, in the present study). Finally, a possible interference of nonspecific binding might lead to overestimation of the apparent Kd. It must be assumed that the dissociation constant, as calculated by us from Scatchard plots, still overestimates the true Kd, since the ED50 observed for adhesion, degranulation, and HMS was well below the Kd.

Preexposure to TNF caused functional deactivation of PMN toward restimulation by TNF, which was highly correlated to a concomitant loss of TNF surface receptors by receptor endocytosis, similar to the observations made with FMLP [22, 23, 47]. Although Scatchard plots demonstrated a loss of apparent receptor affinity in parallel to the reduction of TNF receptor number, these results should be viewed with caution. The reduction of receptor expression causes a decrease in total receptor concentration [R9], which should yield an apparent Kd closer to its true value and therefore lower (32). However, at receptor densities of 120 per cell, as found in some of our experiments after receptor down-regulation, interference from...
nonspecific binding gains in relative importance. This, in turn, would lead to overestimation of the $K_d$ (52). In addition, it seems hard to conceive of the biological significance even of a fourfold rise in $K_d$ for functional deactivation and cross-deactivation, when PMN are restimulated with doses of TNF that correspond to 20-fold the apparent $K_d$.

Cross-talk between receptors has been recognized for some time: in priming, stimulation of PMN via one class of receptors increases their responses to a second stimulus due either to an increase in receptor number or to modulation of its affinity (12, 48-50). Down-regulation of one class of receptors in response to cellular activation by a second class of receptors has been reported, but either the functional relevance of this receptor modulation was not examined (51), or this loss of surface receptors was not accompanied by a specific deactivation of the cells towards the respective ligand but, surprisingly and rather difficult to interpret, subsequent responses were even enhanced (52, 53). We have shown that preexposure of granulocytes to a range of stimuli, endogenous such as C5a, PAF, or LTB4, or exogenous such as endotoxin or FMLP, down-regulated the number of TNF receptors on granulocytes and in all cases concomitantly and statistically highly correlated inhibited PMN adherence, degranulation, and respiratory burst in response to secondary exposure to TNF. In contrast, PMA, which did not alter receptor number, had no effect on TNF-induced functional PMN responses. Moreover, pertussis toxin influenced deactivation or cross-deactivation and TNF receptor numbers always in parallel (Fig. 10). This allows us to conclude that not only in TNF deactivation, similar to the results found for FMLP (47), but in cross-deactivation due to stimuli such as endotoxin or FMLP as well, is the loss of TNF-inducible function causally related to a reduction in the number of TNF receptors.

Others found a similar decrease of TNF receptors on macrophages, which had been preexposed to endotoxin, but failed to demonstrate inhibition of specific binding for PMN (54; results given in percent original binding). The discrepancy between these results and ours is possibly due to the different media used (Krebs-Ringer-phosphate buffer/10% FCS). FCS, which most likely contains C5a and C5a desarg and probably endotoxin, might cause TNF receptor down-regulation and cross-deactivation before the actual start of the experiment, and hence, cross-deactivation due to the addition of prestimuli would become impossible to evaluate. On the other hand, macrophages, cells which in contrast to PMN can be cultured in vitro, may be less sensible to the culture media used.

A final pathway common to the various stimuli that cause TNF-specific deactivation/cross-deactivation and TNF receptor down-regulation by various stimuli does not include a pertussis toxin-sensitive G protein inasmuch as pertussis toxin inhibited cross-deactivation only with those stimuli, such as FMLP (Fig. 10 B), that cause PMN adhesion, degranulation, or chemotaxis via pertussis toxin-sensitive G proteins (38), whereas deactivation by TNF or LPS, which activates PMN independently from pertussis toxin-sensitive G proteins, was left uninfluenced (Fig. 10, A and C). Furthermore, TNF-specific deactivation appears to be independent of phospholipase C-generated DAG and activation of protein kinase C, in that preincubation of PMN with PMA left both functional responses toward TNF and TNF receptors unaltered (Fig. 5, Table 1). Discrepancies with previous reports, which showed down-regulation of TNF receptors upon incubation with PMA (55, 56), may be explained by the different cell systems used. In contrast, the dramatic down-regulation of TNF receptors as seen in preincubation experiments employing the ionophore ionomycin suggests that changes in granulocyte calcium metabolism might be involved in TNF receptor down-regulation and functional deactivation/cross-deactivation.

Down-regulation of TNF receptors (and hence deactivation) induced by TNF itself was shown to result from receptor endocytosis, in agreement with studies carried out on various cell lines (31, 39, 57). However, the nature of the mechanism for the reduction of receptors owing to preincubation with stimuli other than TNF (cross-deactivation) can only be speculated on: unspecific damage to the cells could be excluded, inasmuch as PMN remained vital and fully responsive towards PMA. Receptor digestion by proteases or oxidative metabolites from activated granulocytes is very unlikely, because (a) all experiments were carried out in scavenger-rich autologous plasma, (b) preincubation of PMN with filtres of cell lysates caused no alterations in TNF binding, and (c) PMA, which even at 5 ng/ml causes the strongest degranulation and activation of the respiratory burst in suspension of all stimuli tested, left TNF receptor number and affinity unchanged. None of the effective prestimuli competed with TNF for its receptor. Inhibitors of protein synthesis abolished neither down-regulation induced by TNF nor by endotoxin. As cytochalasin B and colchicine left both endotoxin and TNF-induced receptor down-regulation unaltered, of which the latter was shown to occur by receptor internalization, the negative results obtained with these inhibitors, similar to the ones seen by Peetre et al. (45), cannot be interpreted. The enhancement of receptor down-modulation seen with inhibitors of receptor recycling for both TNF and endotoxin, however, may point to receptor endocytosis as the mechanism of non-TNF-induced receptor reduction. Although electron microscopy and subcellular fractionation techniques are faced with formidable difficulties in view of the low receptor density on PMN, these studies are planned to investigate further into the mechanism of non-TNF-induced modulation of TNF receptors on PMN.

Many of the deleterious consequences of endotoxin in septic shock have been shown to be mediated by TNF (58-60), and elevated serum levels of TNF, as found in gram-negative septicemia, were correlated to poor clinical prognosis (61, 62). Aided by the lack of response induced by TNF in suspension, endotoxin- or TNF-induced down-regulation of receptors to this potent stimulus of PMN degranulation and respiratory burst activation in sepsis may thus serve as a self-protective mechanism of the organism to limit the harm inflicted by raised levels of TNF. Under circumstances, however, in which TNF-mediated killing of microorganisms might be of central importance (63, 64), deactivation and cross-deactivation of granulocytes could decisively impair host defense. The question of whether deactivation and cross-deactivation of PMN toward TNF are beneficial or harmful to the organism will have to be answered by future in vivo studies using suitable animal models.

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References


16. Fehr, J., and C. Dahinden. 1979. Modulating influence of chemo-


