

# Adhesion-dependent Protein Tyrosine Phosphorylation in Neutrophils Treated with Tumor Necrosis Factor

Michele Fuortes, Wen-Wen Jin, and Carl Nathan

Beatrice and Samuel A. Seaver Laboratory, Division of Hematology-Oncology, Departments of Cell Biology/Anatomy and Medicine, Cornell University Medical College, New York 10021

**Abstract.** Human neutrophils (PMN) respond to tumor necrosis factor (TNF) by releasing their granules, reorganizing their cytoskeleton, and massively secreting hydrogen peroxide. This response is dependent on adhesion to extracellular matrix proteins and expression of CD11b/CD18 integrins (Nathan, C., S. Srimal, C. Farber, E. Sanchez, L. Kabbash, A. Asch, J. Gailit, and S. D. Wright. 1989. *J. Cell Biol.* 109:1341-1349). We investigated the role of tyrosine phosphorylation in the response of PMN to TNF. PMN adherent to protein-coated surfaces but not suspended PMN showed tyrosine phosphorylation of several proteins (~150, ~115, ~75, and ~65 kD) in response to TNF. Tyrosine phosphorylation was evident 5 min after addition of TNF and lasted at least 2 h. The tyrosine kinase inhibi-

tors K252a, genistein and ST638 suppressed tyrosine phosphorylation and blocked hydrogen peroxide production in a reversible manner at low concentrations. Tyrosine kinase inhibitors also blocked the spreading of PMN in response to TNF. Dihydrocytochalasin B did not inhibit tyrosine phosphorylation, but in its presence phosphorylation was rapidly reversed. By immunocytochemistry, the majority of tyrosine phosphoproteins were localized to focal adhesions. Thus TNF-induced tyrosine phosphorylation depends on adhesion of PMN to extracellular matrix proteins, and participates in the transduction of the signals that direct the cells to spread on a biological surface and undergo a respiratory burst.

A prominent element of the mammalian response to infection, antigen, or injury is production of tumor necrosis factor (TNF),<sup>1</sup> a cytokine with both immediate (transcription independent) and differentiative (transcription dependent) effects on polymorphonuclear leukocytes (PMN), macrophages, lymphocytes, endothelial cells, hepatocytes, adipocytes, fibroblasts, and myocytes (59). TNF's signal transduction pathways remain incompletely understood (52), despite the molecular cloning of two TNF receptors, p55 (34, 50) and p75 (11, 56). The extracellular portions of these receptors are homologous to the p75 nerve growth factor (NGF) receptor, the antigens Fas (24), CD40, CD27 (9), and OX 40, and proteins of vaccinia (20) and Shope fibroma viruses (57). However, these proteins are not homologous in their intracellular portions. The cytoplasmic amino acid sequences of the TNF receptors reveal no clues to their mechanisms of action. In particular, the intrinsic tyrosine kinase activity common to many growth factor receptors (61) is not evident in this family. Nonetheless, mAb to CD40 induces tyrosine phosphorylation (60). Likewise, NGF elicits protein tyrosine phosphorylation in its target cells through the co-receptor *trk* (4), and many of the actions of nerve

growth factor are blocked by a tyrosine kinase inhibitor, K252a (5).

Human PMN respond dramatically to TNF (32, 35, 39, 41, 43, 48, 53), independently of transcription (Farber, C., and C. Nathan, unpublished observation) and translation (39). TNF-treated PMN lower their cAMP (41), reorganize their actin-based cytoskeleton to form focal adhesions (41), spread out (35, 41), discharge proteases from their granules (48) and mount a massive, prolonged respiratory burst (32, 39, 41, 43, 53). Each of these responses depends on expression by the PMN of adhesion receptors of the CD11/CD18 or  $\beta_2$  integrin family, and requires that the cells make contact with a surface coated with extracellular matrix proteins, plasma proteins, serum proteins, or other cells (39, 43, 48, 53). Responses similar in nature and magnitude ensue when PMN are stimulated with GM-CSF (40), G-CSF (40), MIP-1 (62), IL-8, C5a (C. Nathan, unpublished observations), or N-formylated peptides (39), but only if the cells are adherent. For suspended cells, the same agents are incomplete agonists that require cytochalasins as nonphysiologic cofactors, and even so, induce much smaller responses (reviewed in 39). While suspensions of PMN in vitro simulate PMN in the circulation, a state of transit, PMN actually performing their inflammatory or antimicrobial functions are usually adherent to other cells or to proteins of the extracellular matrix. We are interested in the mechanisms by which receptor-

1. *Abbreviations used in this paper:* KRPG, Krebs-Ringer phosphate buffer with glucose; NGF, nerve growth factor; PMN, polymorphonuclear leukocytes; RT, room temperature; TNF, tumor necrosis factor.

mediated signals from physiologic agonists like TNF are integrated with signals from adhesion receptors to elicit extensive morphologic and secretory responses in PMN in the absence of cytochalasins.

PMN contain tyrosine kinase activities (3, 7, 29) and transcripts for the nonreceptor tyrosine kinase *hck* (47), and *fgf* (18). Discrete sets of proteins undergo tyrosine phosphorylation in PMN in response to GM-CSF (15, 25, 33), IL3 (25, 33), formylated peptide (6, 7, 13, 15, 21, 22, 30, 37), leukotriene B<sub>4</sub> (6, 13), platelet activating factor (6), calcium ionophore (6, 21), PMA (6), Fc receptor cross-linking (10, 30), or electropermeabilization in the presence of guanosine 5'-[γ-thio]triphosphate (38) or vanadate (16). Compounds that can inhibit tyrosine kinases block the ability of some of these stimuli to prime suspended PMN for an enhanced respiratory burst to other stimuli (7, 15, 37). However, it has not been possible to detect protein tyrosine phosphorylation in PMN in response to TNF (25).

In this study, we re-examined the role of protein tyrosine phosphorylation in the signal transduction pathway of TNF. We found that TNF does induce protein tyrosine phosphorylation in PMN. The process depends on adherence of the cells to protein-coated surfaces, and is a prerequisite for their adhesion-dependent respiratory burst.

## Materials and Methods

### PMN Preparation

PMN were isolated from heparinized blood of normal human donors with a one-step, modified Ficoll-Hypaque gradient separation (Neutrophil Isolation Medium, Cardinal Associates, Santa Fe, NM) as described in detail (43). Erythrocytes were lysed by hypotonic shock and PMN resuspended in ice-cold Krebs-Ringer phosphate buffer with glucose (KRPBG).

### Tyrosine Phosphorylation

For adherent cells, PMNs were plated in 100-mm Primaria™ dishes (Falcon Labware, Becton-Dickinson & Co., Oxnard, CA) precoated with 3 ml FBS (Hyclone Systems, Logan, UT) or 3 ml RPMI 1640 containing 20 μg/ml plasminogen-free fibrinogen (Sigma Chemical Co., St. Louis, MO) at a concentration of  $5 \times 10^6$  cells/ml in 5 ml per dish of the same reaction mixture used for the H<sub>2</sub>O<sub>2</sub> assay (see below), prewarmed to 37°C. Reaction mixture consisted of KRPBG containing 40 μM scopoletin, 4.4 U/ml HRP and 1 mM NaN<sub>3</sub> to inhibit endogenous myeloperoxidase. Reaction mixture was used in order to scavenge H<sub>2</sub>O<sub>2</sub> released by the cells and to ensure comparability of conditions between the biochemical and functional assays. After 15 min, stimuli and/or inhibitors were added. At the indicated times, the dishes were placed on ice, the incubation medium was aspirated, and the residual cells were lysed in 150 μl of solubilization buffer (10 mM TrisHCl, pH 7.4, 1% SDS, 1 mM sodium vanadate, 0.1 mM sodium molybdate, 1 mM sodium pyrophosphate, 10 mM NaF, 1 mM PMSF, 5 mM diisopropyl fluorophosphate, and 5 μg/ml each of pepstatin A, leupeptin, aprotinin and chymostatin). The lysate was immediately boiled for 5 min and then stored at -80°C until further analysis. For suspended cells, 1 ml of the same concentration of PMN was incubated in FBS-coated polypropylene tubes and agitated to-and-fro at 100 cycles per min to prevent adhesion of the cells to the walls of the tube or to each other. At the indicated times, the tubes were centrifuged at 16,000 g for 10 s at room temperature (RT) and the cell pellets lysed in solubilization buffer as above. Protein concentrations of lysates were determined by the Bradford or modified Lowry methods (Bio-Rad Laboratories, Richmond, CA). Lysates were separated by SDS-PAGE (31) and transferred electrophoretically to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH). The membranes were incubated overnight at 4°C in blocking buffer (20 mM TrisHCl, pH 7.4, 137 mM NaCl, 0.1% Triton X-100, 10% FBS) and then for 3 h at RT with anti-phosphotyrosine mouse mAb 5E2 (IgG2b, κ) (12) at 1.5 μg/ml in blocking buffer. Membranes were washed in same buffer without serum, blocked

again in 20 mM TrisHCl, pH 7.4, 137 mM NaCl, 0.1% Tween 20, 10% FBS for 30 min at RT, and incubated at RT for 45 min with HRP-conjugated IgG sheep anti-mouse IgG (Amersham Corp., Arlington Heights, IL) in the same buffer. After further washing in same buffer without serum, Western blots were developed using an enhanced chemiluminescence kit (Amersham Corp.).

### Immunohistochemistry

PMN ( $10^6$  cells/ml) were plated on FBS-coated 13-mm diam glass coverslips in 12-well plates containing prewarmed reaction mixture. After 15 min to allow the cells to contact the coverslips, TNF (250 ng/ml) was added or cells were left untreated. After incubation for the indicated times at 37°C in air, the medium was gently aspirated and cells fixed either with 2.5% glutaraldehyde in KRPBG (see Fig. 6) or with 1% paraformaldehyde in cacodylate buffer, pH 7.4 (75 mM sodium cacodylate, 0.72% sucrose), (see Fig. 8) for 10 min at RT while spinning at 30 g. In the latter case, the supernatant was replaced with 3.7% formaldehyde in PBS for 10 min at RT. Cells were then washed with PBS and permeabilized with 0.05% Triton X-100 in PBS for 4 min at RT. Fixed cells were incubated with blocking buffer (PBS + 10% calf serum) for 1 h at RT and then overnight with a 1/100 dilution of rabbit anti-phosphotyrosine antibody (19) in blocking buffer, washed 3 times in PBS, and incubated for 45 min at 37°C with rhodamine-conjugated goat IgG F(ab)<sub>2</sub> anti-rabbit IgG (γ and light chain) antibody (Tago, Inc., Burlingame, CA) in blocking buffer. Coverslips were washed twice in PBS and once in distilled water, inverted, and mounted in 90% glycerol in water on a glass slide.

### Hydrogen Peroxide Release

$1.5 \times 10^4$  PMN in 20 μl KRPBG were added per well to 100 μl of prewarmed (37°C) reaction mixture in FBS-precoated 96-well Primaria™ plates. The plates were incubated for up to 2 h at 37°C in air with the appropriate stimuli and/or drugs. Production of H<sub>2</sub>O<sub>2</sub> was monitored every 15 min in a plate-reading fluorimeter by recording the stoichiometric oxidation of scopoletin to a nonfluorescent product (39).

### Other Reagents

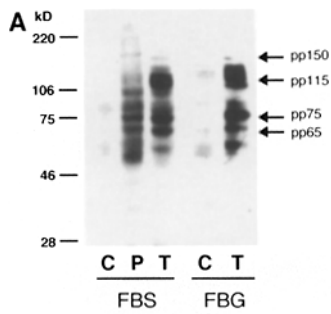
Recombinant human TNFα was a gift of Genentech Inc. (South San Francisco, CA). mAb 5E2 was a generous gift of B. M. Fendly (Genentech Inc.). ST638 was a generous gift of Dr. T. Shiraishi (Kanegafuchi Chemical Industry Co. Ltd., Takasago, Japan). Genistein was from GIBCO BRL (Gaithersburg, MD), K252a from Kamiya Biomedical Company (Thousand Oaks, CA), and all other reagents from Sigma Chemical Co.

## Results

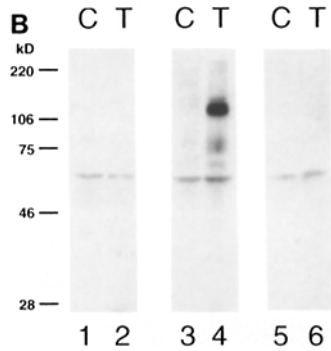
### TNF Induces Protein Tyrosine Phosphorylation in Adherent PMN

PMN were incubated in FBS-coated polystyrene dishes for 15 min at 37°C to allow them to make contact with the substratum. They were then exposed to TNF (250 ng/ml) or PMA (100 ng/ml) or left untreated. 30 min later, the incubation medium was gently aspirated. The cells were lysed, and equal amounts of cell protein subjected to SDS-PAGE and Western blotting with an anti-phosphotyrosine mAb (Fig. 1 A). Without TNF, contact with the plate for 45 min led to little or no detectable tyrosine phosphorylation. In contrast, exposure to TNF for the last 30 min of the incubation resulted in intense tyrosine phosphorylation of several species. The major species migrated with an apparent molecular mass of ~150 (pp150), ~115 (pp115), ~75 (pp75), and ~65 kD (pp65). Results were the same when the dishes were coated with fibrinogen rather than FBS (Fig. 1 A). The pattern of phosphorylation in response to PMA was almost completely distinct from that observed with TNF; only pp75 was found in common.

The specificity of the mAb was confirmed by the ability



**Figure 1.** TNF induces tyrosine phosphorylation. (A) Immunoblot with anti-phosphotyrosine mAb 5E2 of lysates of PMN adherent to FBS- or fibrinogen (FBG)-coated polystyrene dishes. After a 15-min preincubation, cells were treated with TNF (T, 250 ng/ml), PMA (P, 100 ng/ml), or left untreated (control, C) for 30 min. Cells were lysed and proteins separated by SDS-PAGE followed by Western blot with 5E2. Each lane was loaded with 65  $\mu$ g (from FBS-coated dishes) or 75  $\mu$ g (from FBG-coated dishes) of cell protein. Molecular mass markers are indicated. (B) Same procedure was used but blots were incubated with 5E2 in the presence of 1 mM phosphotyrosine (lanes 1 and 2) or a combination of tyrosine, phosphoserine, and phosphothreonine (1 mM each) (lanes 3 and 4) or with purified normal mouse IgG (1  $\mu$ g/ml) instead of mAb 5E2 (lanes 5 and 6).

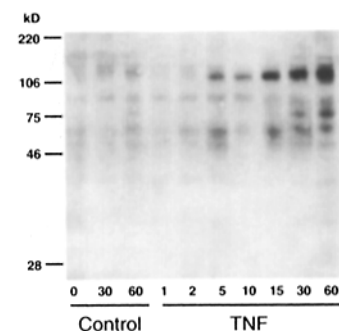


of 1 mM phosphotyrosine to abolish its reactivity, while a mixture of 1 mM each of tyrosine, phosphoserine, and phosphothreonine had no effect. Normal mouse IgG was nonreactive (Fig. 1 B).

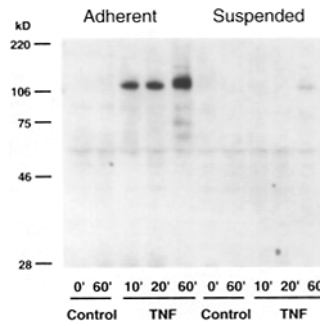
TNF-induced tyrosine phosphorylation was first evident in pp115 within 5 min of addition of TNF (Fig. 2). Tyrosine phosphorylation of pp75 was delayed until 30 min, and pp150 was prominently phosphorylated only by 60 min after addition of TNF. Over the 60- (Fig. 2) or 120-min period (not shown), no species showed a decrease in the extent of tyrosine phosphorylation. Thus, TNF-induced protein tyrosine phosphorylation in adherent PMN, like the TNF-induced respiratory burst, was relatively delayed in onset and prolonged in duration, compared with responses of suspended PMN to other agonists.

### Adherent but Not Suspended PMN Undergo Tyrosine Phosphorylation in Response to TNF

No tyrosine phosphorylation was detectable in TNF-treated, suspended PMN, beyond a minor degree of phosphorylation



**Figure 2.** Time course of TNF-induced tyrosine phosphorylation. Immunoblot with anti-phosphotyrosine mAb 5E2 of lysate of PMN adherent to FBS-coated polystyrene dishes. After a 15-min preincubation, cells were treated with TNF (250 ng/ml) for 1, 2, 5, 10, 30, and 60 min or left untreated (Control) for 0, 30, and 60 min. Each lane was loaded with 35  $\mu$ g protein. Molecular mass markers are indicated.



**Figure 3.** TNF-induced tyrosine phosphorylation in adherent and suspended cells. Immunoblot with anti-phosphotyrosine mAb 5E2 of lysate of PMN; cells were incubated with TNF (250 ng/ml) or left untreated (control) for the time indicated. Adherent cells were added to stationary FBS-coated dishes; suspended cells were added to FBS-coated polypropylene tubes and agitated at 100 to-and-fro cycles/min. Cells were lysed and 75  $\mu$ g of total cellular proteins were loaded per lane. Molecular mass markers are indicated.

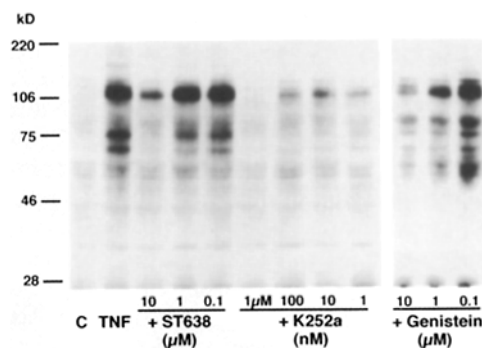
of pp115 at 60 min (Fig. 3). PMN from the same preparation underwent marked tyrosine phosphorylation when they were both exposed to TNF and allowed to spread on an FBS-coated surface (Fig. 3).

### Effect of Tyrosine Kinase Inhibitors

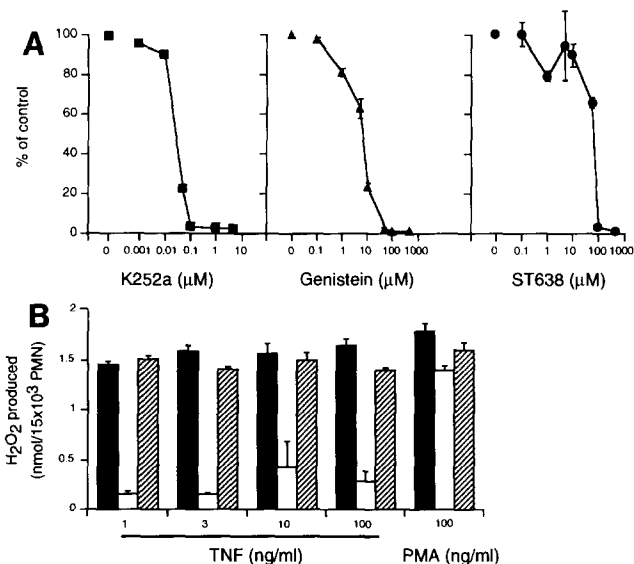
To test the physiologic consequences of tyrosine phosphorylation in TNF-treated PMN, we used three kinase inhibitors: genistein, an isoflavone (1); ST638, a cinnamide (54); and K252a, an alkaloid (26). Genistein and ST638 are specific for tyrosine kinases (1, 54), while K252a also inhibits protein kinase C (26), but is particularly potent at inhibiting tyrosine phosphorylation induced by NGF (5). ST638 (7, 15) and K252a (55), but not genistein, have been used previously to inhibit PMN activation.

First, we confirmed the ability of these agents to inhibit tyrosine phosphorylation (Fig. 4). The most potent was K252a, which was effective at 1 nM. Genistein was effective at 1  $\mu$ M and ST638 at 10  $\mu$ M (Fig. 4). Tyrosine phosphorylation of all detectable phosphoproteins was affected by each of the inhibitors.

All three inhibitors also blocked the TNF-induced respira-



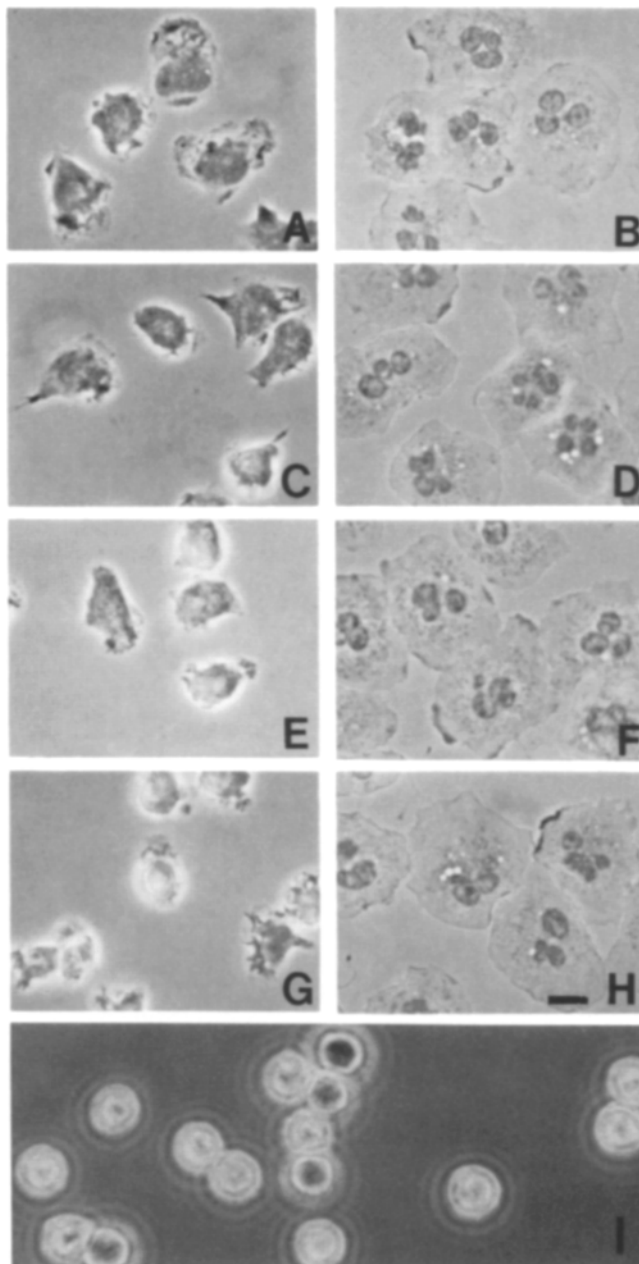
**Figure 4.** Effect of tyrosine kinase inhibitors on TNF-induced protein phosphorylation. Immunoblot with anti-phosphotyrosine mAb 5E2. PMN were plated on FBS-coated dishes and incubated for 15 min with ST638, K252a, or genistein at the concentrations indicated, or without drugs (lanes C and TNF). The cells were then stimulated with TNF (250 ng/ml) except in lane C (control). After 30 min, the cells were lysed and 75  $\mu$ g protein per lane (from genistein-treated cells) or 40  $\mu$ g protein per lane (from all other cells) separated by SDS-PAGE. Molecular mass markers are indicated.



**Figure 5.** Effect of tyrosine kinase inhibitors on H<sub>2</sub>O<sub>2</sub> release. (A) Concentration-dependent inhibition by three agents. PMN were plated on FBS-coated 96-well plates, incubated for 15 min with inhibitors, and then stimulated with TNF (250 ng/ml). Values are for H<sub>2</sub>O<sub>2</sub> release after 120 min of TNF stimulation and are expressed as percent of H<sub>2</sub>O<sub>2</sub> release by PMN stimulated with TNF and not treated with kinase inhibitors in the same experiment. The latter values were 3 nmol/1.5 × 10<sup>4</sup> PMN in the K252a experiment and 2 nmol/1.5 × 10<sup>4</sup> PMN in the ST638 and genistein experiments. Data are means of triplicates ± SEM. When not seen, error bars are smaller than the symbols denoting the means. (B) Reversibility of inhibition by genistein. PMN were preincubated for 1 h in the presence or absence of 30 μM genistein in FBS-coated polypropylene tubes with end-over-end rotation. Cells were then washed three times and plated in FBS-coated 96-well plates in the presence or absence of 30 μM genistein. 15 min later, cells were stimulated with TNF at 1, 3, 10, or 100 ng/ml or with PMA at 100 ng/ml. (■) Control PMN not exposed to genistein at any time; (□) PMN treated with genistein both during the preincubation and during stimulation with TNF or PMA; and (⊗) cells preincubated with genistein but not reexposed to genistein during stimulation with TNF or PMA. Data are means of triplicates ± SEM.

tory burst in PMN (Fig. 5 A). The concentrations causing 50% inhibition were 25 nM for K252a, 5 μM for genistein and 50 μM for ST638. The lack of toxicity of genistein was demonstrated by the reversibility of its effect on the TNF-induced respiratory burst and by its relative selectivity in inhibiting the response to TNF but not PMA (Fig. 5 B).

The ability of PMN to reorganize their actin-based cytoskeleton into focal adhesions and to spread on a protein-coated surface appears to be a prerequisite for a full-scale respiratory burst in response to soluble physiologic agonists like TNF (41). Although TNF-induced tyrosine phosphorylation was relatively slow in onset, it preceded cell spreading (see ref. 41). Thus, it was of interest to investigate whether tyrosine phosphorylation was a prerequisite for TNF-induced cell spreading. All three tyrosine kinase inhibitors blocked the extension of membranes characteristic of TNF-induced cell spreading (Fig. 6). The adherent, TNF-treated cells exposed to tyrosine kinase inhibitors had the same appearance as adherent, TNF-treated PMN exposed to the microfilament-disrupting agent, dihydrocytochalasin B (41, 58). The

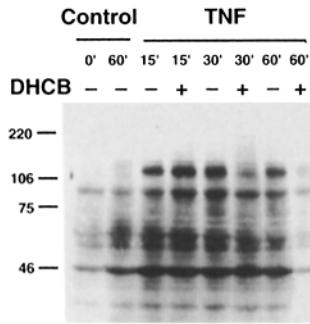


**Figure 6.** Effect of tyrosine kinase inhibitors on PMN spreading. PMN were plated on FBS-coated glass coverslips and stimulated with TNF (A, C, E, and G) or PMA (B, D, F, and H), each at 100 ng/ml, or left unstimulated (I), without tyrosine kinase inhibitors (A, B, and I) or in the presence of ST638 at 100 μM (C and D), genistein at 30 μM (E and F), or K252a at 0.1 μM (G and H). Cells were fixed 30 min later. Bar, 10 μm.

relative selectivity and lack of toxicity of the tyrosine kinase inhibitors was demonstrated by their inability to inhibit PMA-induced cell spreading in the same experiments (Fig. 6).

#### **Effect of Dihydrocytochalasin B on Tyrosine Phosphorylation**

The foregoing experiments suggested that tyrosine phosphorylation preceded and was necessary for TNF-induced cell spreading. We next investigated whether cell spreading



**Figure 7.** Effect of dihydrocytochalasin B on TNF-stimulated tyrosine phosphorylation. Immunoblot with anti-phosphotyrosine mAb 5E2 of lysate of PMN. Cells on FBS-coated dishes were stimulated with TNF (250 ng/ml) or left untreated for the number of min indicated and then lysed. Some cells were also treated with dihydrocytochalasin B (DHCB) (5  $\mu$ g/ml)

added at the same time of TNF. After cell lysis, 120  $\mu$ g protein was loaded in each lane. Molecular mass markers are indicated (kD).

in turn had any effect on TNF-induced tyrosine phosphorylation.

Microfilament-disrupting agents prevent TNF from inducing spreading (C. Nathan, unpublished observation) and a respiratory burst (39) in adherent PMN, without diminishing adherence (41). In the present experiments, treatment of PMN with dihydrocytochalasin B for 15 min simultaneously with TNF stimulation had no effect on tyrosine phosphorylation (Fig. 7), even though spreading was completely prevented (not shown). Thus, while adherence was a prerequisite for TNF to induce protein tyrosine phosphorylation (Fig. 3), spreading was not.

On the other hand, exposure to dihydrocytochalasin B for longer times (30 and 60 min) led to reversal of the pre-existent tyrosine phosphorylation (Fig. 7). Thus, cell spreading appeared to protect the predominant tyrosine phosphoproteins from being dephosphorylated.

### Immunofluorescent Localization of Tyrosine Phosphoproteins

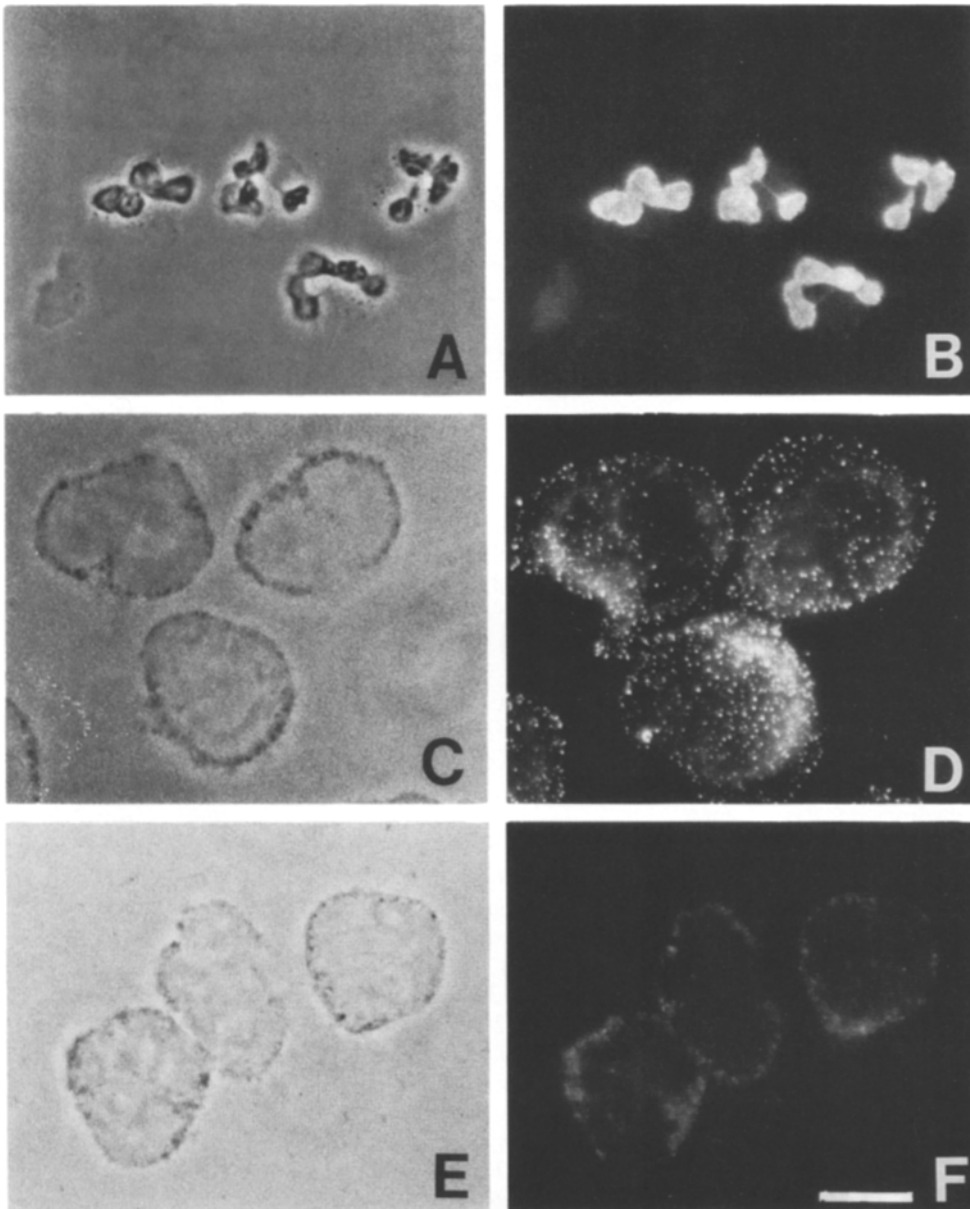
The foregoing results raised the possibility that the predominant tyrosine phosphoproteins in TNF-treated PMN might be associated with organelles involved in cell spreading, such as focal adhesions. This prompted us to examine the morphologic distribution of tyrosine phosphorylation. When PMN were in contact with a protein-coated surface but were not exposed to TNF, fluorescence was observed only at the periphery of the nuclear lobes (Fig. 8 B); this was attributable largely to the secondary antibody alone (not shown). In contrast, treatment of adherent cells with TNF led to extensive staining of punctate structures throughout well-spread cells, in some cases accentuated at the periphery (Fig. 8 D). The focal plane of this reactivity indicated that it was confined to the surface of the PMN making contact with the substratum. Some PMN lost their nuclei and cell bodies during fixation and handling; in these cases, a disk of phase-dark membrane remained behind with numerous intensely staining punctate structures (not shown). Finally, the structures staining with anti-phosphotyrosine antibody extensively colocalized with structures of similar appearance that stained with anti-vinculin antibody (not shown). TNF-treated cells exposed to an irrelevant antiserum showed minimal staining which appeared to be confined to the plasma membrane (Fig. 8 F), perhaps reflecting Fc receptor-dependent binding, a pattern dissimilar from that with anti-phosphotyrosine antibody.

### Discussion

In TNF-treated PMN, cell adherence and cell spreading were both linked to tyrosine phosphorylation. Adherence was a prerequisite for TNF to induce tyrosine phosphorylation. Tyrosine phosphorylation was a prerequisite for TNF to induce cell spreading and the respiratory burst. These findings establish protein tyrosine phosphorylation as a signal transduction step in a set of actions of TNF on human PMN that do not require transcription or translation.

TNF-induced tyrosine phosphorylation has apparently been reported in only one instance, in fibroblasts, where proteins of 41- and 43-kD were affected (27). Perhaps TNF-induced protein tyrosine phosphorylation was not detected in earlier studies of PMN, because the process depends on adherence of PMN to a surface, whereas PMN usually are studied in suspension. Dependence of tyrosine phosphorylation on adherence may reflect the critical participation of signals from CD11/CD18 adhesion receptors ( $\beta$ 2 integrins), as is the case with the other known TNF-induced responses of PMN adherent to protein-coated surfaces, including the fall in cAMP (41), spreading (35, 41), the formation of focal adhesions (41), degranulation (48), and the respiratory burst (43, 53).

It is increasingly apparent that responses of cells to cytokines or growth factors are often modulated by, and in some cases dependent on, interactions of the cells with the extracellular matrix (23, 42). An understanding of the biochemical mechanisms that underlie the joint control of cellular responses by adhesion receptors and cytokine receptors is beginning to emerge. Recently, involvement of  $\beta$ 1 and  $\beta$ 3 integrins in signaling protein tyrosine phosphorylation has been demonstrated (14, 17, 28, 46). Cross-linking of  $\beta$ 1 integrins with antibodies enhanced by  $\sim$ 3.5-fold the tyrosine phosphorylation of 115–130 kD proteins in an epidermal carcinoma line (28). Guan et al. (17) identified spreading on a fibronectin-coated surface as a stimulus for tyrosine phosphorylation of a 120-kD protein in 3T3 cells. Participation of the  $\beta$ 3 integrin gp IIb-IIIa was required for protein tyrosine phosphorylation in platelets undergoing activation by agonists (14). Finally, adherence to fibronectin fragments, or cross-linking of the  $\beta$ 1 integrin VLA-4 with antibodies, induced tyrosine phosphorylation of a pp105 in T lymphocytes (46). To our knowledge, the present study is the first to demonstrate that adhesion to protein-coated surfaces in a manner dependent on  $\beta$ 2 integrins can provide an essential costimulus for protein tyrosine phosphorylation; the first to demonstrate adhesion-dependent protein tyrosine phosphorylation in PMN, a cell type many of whose functions depend on adhesion; and the first to present evidence that adhesion-dependent protein tyrosine phosphorylation is essential to a cell's functional responses. In the context of earlier work (43), the role of  $\beta$ 2 integrins was strongly supported by the dependence of TNF-induced tyrosine phosphorylation on adherence of cells to a protein-coated surface, together with the ability of fibrinogen to provide a suitable coating;  $\beta$ 2 integrins are the only demonstrable fibrinogen receptors on PMN (2, 35). Direct evidence that an intracellular signal can arise from cross-linking  $\beta$ 2 integrins was recently provided by the demonstration that intracellular  $\text{Ca}^{2+}$  is elevated in PMN treated with mouse mAb to CD18 together with rabbit anti-mouse IgG (45).



**Figure 8.** Intracellular localization of phosphotyrosine-containing proteins. PMN were plated on FBS-coated glass coverslips. After 15 min, cells were stimulated with TNF (*C-F*) or left untreated (*A and B*); 45 min later, the medium was gently aspirated and cells fixed, permeabilized and stained with rabbit anti-phosphotyrosine antibody (*A-D*) or an irrelevant antibody (*E and F*). Photomicrographs in *C-F* were taken with the plane of focus at the surface of the PMN in contact with the coverslip. Perinuclear fluorescence in *B* was contributed mostly by the secondary antibody. *A, C, and E* are bright field photomicrographs; *B, D, and F* are corresponding fluorescence photomicrographs. Bar, 10  $\mu\text{m}$ .

A critical role for focal adhesions in mediating signal transduction via integrins has been suggested (17, 28, 36). The predominant, serologically detectable tyrosine phosphoproteins in TNF-treated PMN colocalized with focal adhesions. In other cell types, both tyrosine kinases (49) and tyrosine phosphoproteins (17, 36) have been localized to focal adhesions. Maher et al. (36) documented a punctate distribution of tyrosine phosphoproteins in adherent 3T3 and NRK fibroblasts, and stated that the tyrosine phosphoproteins colocalized with vinculin. The punctate distribution of tyrosine phosphoproteins in adherent 3T3 cells was confirmed by Guan et al. (17), who demonstrated their codistribution with  $\beta 1$  integrins.

The proteins whose tyrosine phosphorylation was enhanced via integrin ligation in fibroblasts (115–130 kD) (17, 28) resemble in apparent *M<sub>r</sub>* the p115 which was tyrosine phosphorylated in adherent, TNF-treated PMN. However, unlike Guan et al. (17), we have not been able to obtain evidence suggesting a possible association of PMN tyrosine

phosphoproteins with the focal adhesion-associated kinase of Schaller et al. (51) (unpublished observations). It will be of interest to determine whether focal adhesions, which constituted the predominant locale for tyrosine phosphoproteins in TNF-treated PMN, may also represent sites for the accumulation of  $\beta 2$  integrins, TNF receptors, and/or the cytoskeletal-associated components of the respiratory burst oxidase (44, 63).

In adherent PMN, inhibiting spreading by blocking actin polymerization with dihydrocytochalasin B led to rapid dephosphorylation of already tyrosine-phosphorylated proteins. This suggested that tyrosine phosphoproteins may have been protected from dephosphorylation when associated with the cytoskeleton. This interpretation is consistent with the speculation by Guan et al. (17) that aggregation of  $\beta 1$  integrins with cytoskeletal elements might form zones in which the 120-kD tyrosine phosphoprotein of spreading 3T3 cells may have been protected from tyrosine phosphatases. At early time points in our experiments (<5 min after addition

of TNF), before focal adhesions formed, dephosphorylation may have been rapid, perhaps accounting for the relatively slow onset of accumulation of detectable tyrosine phosphoproteins. Conversely, after formation of focal adhesions, protection of tyrosine phosphoproteins from dephosphorylation may have accounted for their persistence (>120 min).

The earliest known event in the sequence leading to a massive respiratory burst in adherent PMN is a profound drop in cAMP ( $\leq 2$  min) (41). The accumulation of tyrosine phosphoproteins (>2,  $\leq 5$  min) is the next known event in the chain, although, as noted above, the onset of protein tyrosine phosphorylation may be earlier. The relation between these two processes is unknown. In epithelial cells, a membrane-associated protein tyrosine phosphatase was activated by cAMP-dependent protein kinase (8). This raises the possibility that the drop in cAMP in adherent PMN (41) could provide an intracellular milieu conducive to the accumulation of tyrosine phosphoproteins by diminishing the activity of tyrosine phosphatase. Both the fall in cAMP and the tyrosine phosphorylation of a discrete set of proteins in response to TNF seem to be essential for the subsequent onset of cell spreading ( $\geq 10$  min), which in turn is necessary for the onset of the respiratory burst ( $\geq 15$  min) (41).

Since TNF receptors lack kinase domains, it is not clear how the binding of TNF induces tyrosine phosphorylation. Like the homologous low-affinity NGF receptor (4), TNF receptors may transmit signals by means of an associated tyrosine kinase. The findings reported here have prompted us to search in PMN for novel tyrosine kinases (Fuortes, M., manuscript in preparation). Their possible association with TNF receptors, integrins, and/or the cytoskeleton might help explain how the functions of PMN can be controlled jointly by cytokines and extracellular matrix.

We thank M. A. Palladino, Jr. (Genentech, Inc.) for his help in obtaining invaluable reagents, T. Shiraiishi for ST638, B. M. Fendly, D. Sternberg, H. Hanafusa, and T. Parsons for antibodies, and M. Berg and A. Ding for advice.

Received for publication 21 August 1992 and in revised form 28 October 1992.

## References

- Akiyama, T., J. Ishida, S. Nakagawa, H. Ogawara, S. Watanabe, N. Itoh, M. Shibuya, and Y. Fukami. 1987. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* 262:5592-5595.
- Altieri, D. C., R. Bader, P. M. Mannucci, and T. S. Edgington. 1988. Oligospecificity of the cellular adhesion receptor MAC-1 encompasses an inducible recognition specificity for fibrinogen. *J. Cell Biol.* 107:1893-1900.
- Badwey, J. A., R. W. Erickson, and J. T. Curnutte. 1991. Staurosporine inhibits the soluble and membrane-bound protein tyrosine kinases of human neutrophils. *Biochem. Biophys. Res. Commun.* 178:423-429.
- Berg, M. M., D. W. Sternberg, B. L. Hempstead, and M. V. Chao. 1991. The low-affinity p75 nerve growth factor (NGF) receptor mediates NGF-induced tyrosine phosphorylation. *Proc. Natl. Acad. Sci. USA.* 88:7106-7110.
- Berg, M. M., D. W. Sternberg, L. F. Parada, and M. V. Chao. 1992. K-252a inhibits nerve growth factor-induced *trk* proto-oncogene tyrosine phosphorylation and kinase activity. *J. Biol. Chem.* 267:13-16.
- Berkow, R. L., and R. W. Dodson. 1990. Tyrosine-specific protein phosphorylation during activation of human neutrophils. *Blood.* 75:2445-2452.
- Berkow, R. L., R. W. Dodson, and A. S. Kraft. 1989. Human neutrophils contain distinct cytosolic and particulate tyrosine kinase activities: possible role in neutrophil activation. *Biochim. Biophys. Acta.* 997:292-301.
- Brautigan, D. L., and F. M. Pinault. 1991. Activation of membrane protein-tyrosine phosphatase involving cAMP and  $Ca^{2+}$ /phospholipid-dependent protein kinases. *Proc. Natl. Acad. Sci. USA.* 88:6696-6700.
- Camerini, D., G. Walz, W. A. M. Loenen, J. Borst, and B. Seed. 1991. The T cell activation antigen CD27 is a member of the nerve growth factor/tumor necrosis factor receptor gene family. *J. Immunol.* 147:3165-3169.
- Connelly, P. A., C. A. Farrell, J. M. Merenda, M. J. Conklyn, and H. J. Showell. 1991. Tyrosine phosphorylation is an early signaling event common to Fc receptor crosslinking in human neutrophils and rat basophilic leukemia cells (RBL-2H3). *Biochem. Biophys. Res. Commun.* 177:192-201.
- Dembic, Z., H. Loetscher, U. Gubler, Y. C. Pan, H. W. Lahm, R. Gentz, M. Brockhaus, and W. Lesslauer. 1990. Two human TNF receptors have similar extracellular, but distinct intracellular, domain sequences. *Cytokine.* 2:231-237.
- Fendly, B. M., M. Winget, R. H. Hudziak, M. T. Lipari, M. A. Napier, and A. Ullrich. 1990. Characterization of murine monoclonal antibodies reactive to either the human Epidermal Growth Factor receptor or HER2/*neu* gene product. *Cancer Res.* 50:1550-1558.
- Gaudry, M., A. C. Caon, C. Gilbert, S. Lille, and P. H. Naccache. 1992. Evidence for the involvement of tyrosine kinases in the locomotory response of human neutrophils. *J. Leukocyte Biol.* 51:103-108.
- Golden, A., J. S. Brugge, and S. J. Shattil. 1990. Role of platelet membrane glycoprotein IIb-IIIa in agonist-induced tyrosine phosphorylation of platelet proteins. *J. Cell Biol.* 111:3117-3127.
- Gomez-Cambronero, J., C. K. Huang, V. A. Bonak, E. Wang, J. E. Casnellie, T. Shiraiishi, and R. I. Sha'afi. 1989. Tyrosine phosphorylation in human neutrophil. *Biochem. Biophys. Res. Commun.* 162:1478-1485.
- Grinstein, S., W. Furuya, D. J. Lu, and G. B. Mills. 1990. Vanadate stimulates oxygen consumption and tyrosine phosphorylation in electroporated human neutrophils. *J. Biol. Chem.* 265:318-327.
- Guan, J. L., J. E. Trevithick, and R. O. Hynes. 1991. Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein. *Cell Regul.* 2:951-964.
- Gutkind, J. S., and K. C. Robbins. 1989. Translocation of the FGR protein-tyrosine kinase as a consequence of neutrophil activation. *Proc. Natl. Acad. Sci. USA.* 86:8783-8787.
- Hamaguchi, M., C. Grandori, and H. Hanafusa. 1988. Phosphorylation of cellular proteins in Rous sarcoma virus-infected cells: Analysis by use of anti-phosphotyrosine antibodies. *Mol. Cell. Biol.* 8:3035-3042.
- Howard, S. T., Y. S. Chan, and G. L. Smith. 1991. Vaccinia virus homologues of the Shope fibroma virus inverted terminal repeat proteins and a discontinuous ORF related to the tumor necrosis factor receptor family. *Virology.* 180:633-647.
- Huang, C. K., V. Bonak, G. R. Laramee, and J. E. Casnellie. 1990. Protein tyrosine phosphorylation in rabbit peritoneal neutrophils. *Biochem. J.* 269:431-436.
- Huang, C. K., G. R. Laramee, and J. E. Casnellie. 1988. Chemotactic factor induced tyrosine phosphorylation of membrane associated proteins in rabbit peritoneal neutrophils. *Biochem. Biophys. Res. Commun.* 151:794-801.
- Hynes, R. O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell.* 69:11-25.
- Itoh, N., S. Yonehara, A. Ishii, M. Yonehara, S. Mizushima, M. Sameshima, A. Hase, Y. Seto, and S. Nagata. 1991. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell.* 66:233-243.
- Kanakura, Y., B. Druker, S. A. Cannistra, Y. Furukawa, Y. Torimoto, and J. D. Griffin. 1990. Signal transduction of the human granulocyte-macrophage colony-stimulating factor and interleukin-3 receptors involves tyrosine phosphorylation of a common set of cytoplasmic proteins. *Blood.* 76:706-715.
- Kase, H., K. Iwahashi, S. Nakanishi, Y. Matsuda, K. Yamada, M. Takahashi, C. Murakata, A. Sato, and M. Kaneko. 1987. K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochem. Biophys. Res. Commun.* 142:436-440.
- Kohno, M., N. Nishizawa, M. Tsujimoto, and H. Nomoto. 1990. Mitogenic signaling pathway of tumor necrosis factor involves the rapid tyrosine phosphorylation of 41,000-Mr and 43,000-Mr cytosol proteins. *Biochem. J.* 1990:91-98.
- Kornberg, L. J., H. S. Earp, C. E. Turner, C. Prockop, and R. L. Juliano. 1991. Signal transduction by integrins: increased protein tyrosine phosphorylation caused by clustering of beta 1 integrins. *Proc. Natl. Acad. Sci. USA.* 88:8392-8396.
- Kraft, A. S., and R. L. Berkow. 1987. Tyrosine kinase and phosphotyrosine phosphatase activity in human promyelocytic leukemia cells and human polymorphonuclear leukocytes. *Blood.* 70:356-362.
- Kusunoki, T., H. Higashi, S. Hosoi, D. Hata, K. Sugie, M. Mayumi, and H. Mikawa. 1992. Tyrosine phosphorylation and its possible role in superoxide production by human neutrophils stimulated with FMLP and IgG. *Biochem. Biophys. Res. Commun.* 183:789-796.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680-685.
- Laudanna, C., S. Miron, G. Berton, and F. Rossi. 1990. Tumor necrosis factor-alpha/cachectin activates the  $O_2^-$ -generating system of human neutrophils independently of the hydrolysis of phosphoinositides and the release of arachidonic acid. *Biochem. Biophys. Res. Commun.* 166:

- 308-315.
33. Linnekin, D., and W. L. Farrar. 1990. Signal transduction of human interleukin 3 and granulocyte-macrophage colony-stimulating factor through serine and tyrosine phosphorylation. *Biochem. J.* 271:317-324.
  34. Loetscher, H., Y. C. Pan, H. W. Lahm, R. Gentz, M. Brockhaus, H. Tabuchi, and W. Lesslauer. 1990. Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor. *Cell.* 61:351-359.
  35. Loike, J. D., B. Sodeik, L. Cao, S. Leucona, J. I. Weitz, P. A. Detmers, S. D. Wright, and S. C. Silverstein. 1991. CD11c/CD18 on neutrophils recognizes a domain at the N terminus of the A alpha chain of fibrinogen. *Proc. Natl. Acad. Sci. USA.* 88:1044-1048.
  36. Maher, P. A., E. B. Pasquale, J. Y. J. Wang, and S. J. Singer. 1985. Phosphotyrosine-containing proteins are concentrated in focal adhesions and intercellular junctions in normal cells. *Proc. Natl. Acad. Sci. USA.* 82:6576-6580.
  37. Naccache, P. H., C. Gilbert, A. C. Caon, M. Gaudry, C. K. Huang, V. A. Bonak, K. Umezawa, and S. R. McColl. 1990. Selective inhibition of human neutrophil functional responsiveness by erbstatin, an inhibitor of tyrosine protein kinase. *Blood.* 76:2098-2104.
  38. Nasmith, P. E., G. B. Mills, and S. Grinstein. 1989. Guanine nucleotides induce tyrosine phosphorylation and activation of the respiratory burst in neutrophils. *Biochem. J.* 257:893-897.
  39. Nathan, C. 1987. Neutrophil activation on biological surfaces. Massive secretion of hydrogen peroxide in response to product of macrophages and lymphocytes. *J. Clin. Invest.* 80:1550-60.
  40. Nathan, C. 1989. Respiratory burst in adherent human neutrophils: triggering by colony-stimulating factors CSF-GM and CSF-G. *Blood.* 73:301-306.
  41. Nathan, C., and E. Sanchez. 1990. Tumor necrosis factor and CD11/CD18 ( $\beta$ 2) integrins act synergistically to lower cAMP in human neutrophils. *J. Cell Biol.* 111:2171-2181.
  42. Nathan, C., and M. Sporn. 1991. Cytokines in context. *J. Cell Biol.* 113:981-986.
  43. Nathan, C., S. Srimal, C. Farber, E. Sanchez, L. Kabbash, A. Asch, J. Gailit, and S. D. Wright. 1989. Cytokine-induced respiratory burst of human neutrophils: dependence on extracellular matrix proteins and CD11/CD18 integrins. *J. Cell Biol.* 109:1341-1349.
  44. Nauseef, W. M., B. D. Volpp, S. McCormick, K. G. Leidal, and R. A. Clark. 1991. Assembly of the neutrophil respiratory burst oxidase. Protein kinase C promotes cytoskeletal and membrane association of cytosolic oxidase components. *J. Biol. Chem.* 266:5911-5917.
  45. Ng-Sikorski, J., R. Andersson, M. Patarroyo, and T. Andersson. 1991. Calcium signaling capacity of the CD11b/CD18 integrin on human neutrophils. *Exp. Cell Res.* 195:504-508.
  46. Nojima, Y., D. M. Rothstein, K. Sugita, S. F. Schlossman, and C. Morimoto. 1992. Ligand of VLA-4 on T cells stimulates tyrosine phosphorylation of a 105-kD protein. *J. Exp. Med.* 175:1045-1053.
  47. Quintrell, N., R. Lebo, H. Varmus, J. M. Bishop, M. J. Pettenati, B. M. M. Le, M. O. Diaz, and J. D. Rowley. 1987. Identification of a human gene (HCK) that encodes a protein-tyrosine kinase and is expressed in hemopoietic cells. *Mol. Cell. Biol.* 7:2267-2275.
  48. Richter, J., S. J. Ng, I. Olsson, and T. Andersson. 1990. Tumor necrosis factor-induced degranulation in adherent human neutrophils is dependent on CD11b/CD18-integrin-triggered oscillations of cytosolic free  $Ca^{2+}$ . *Proc. Natl. Acad. Sci. USA.* 87:9472-9476.
  49. Rohrschneider, L. R. 1980. Adhesion plaques of Rous sarcoma virus-transformed cells contain the *src* gene product. *Proc. Natl. Acad. Sci. USA.* 77:3514-3518.
  50. Schall, T. J., M. Lewis, K. J. Koller, A. Lee, G. C. Rice, G. H. Wong, T. Gatanaga, G. A. Granger, R. Lentz, H. Raab, W. J. Kohr, and D. Goeddel. 1990. Molecular cloning and expression of a receptor for human tumor necrosis factor. *Cell.* 61:361-370.
  51. Schaller, M. D., C. A. Borgman, B. S. Cobb, R. R. Vines, A. B. Reynolds, and J. T. Parsons. 1992. pp125FAK, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci. USA.* 89:5192-5196.
  52. Schutze, S., T. Machleidt, and M. Kronke. 1992. Mechanisms of tumor necrosis factor action. *Semin. Oncol.* 2:16-24.
  53. Shappell, S. B., C. Toman, D. C. Anderson, A. A. Taylor, M. L. Entman, and C. W. Smith. 1990. Mac-1 (CD11b/CD18) mediates adherence-dependent hydrogen peroxide production by human and canine neutrophils. *J. Immunol.* 144:2702-2711.
  54. Shiraishi, T., T. Domoto, N. Imai, Y. Shimada, and K. Watanabe. 1987. Specific inhibitors of tyrosine-specific protein kinase, synthetic 4-hydroxycinnamamide derivatives. *Biochem. Biophys. Res. Commun.* 147:322-328.
  55. Smith, R. J., J. M. Justen, and L. M. Sam. 1988. Effects of a protein kinase C inhibitor, K-252a, on human polymorphonuclear neutrophil responsiveness. *Biochem. Biophys. Res. Commun.* 152:1497-1503.
  56. Smith, C. A., T. Davis, D. Anderson, L. Solam, M. P. Beckmann, R. Jerzy, S. K. Dower, D. Cosman, and R. G. Goodwin. 1990. A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. *Science (Wash. DC).* 248:1019-1023.
  57. Smith, C. A., T. Davis, J. M. Wignall, W. S. Din, T. Farrah, C. Upton, G. McFadden, and R. G. Goodwin. 1991. T2 open reading frame from the Shope fibroma virus encodes a soluble form of the TNF receptor. *Biochem. Biophys. Res. Commun.* 176:335-342.
  58. Southwick, F. S., G. A. Dabiri, M. Paschetto, and S. H. Zigmond. 1989. Polymorphonuclear leukocyte adherence induces actin polymerization by a transduction pathway which differs from that used by chemoattractants. *J. Cell Biol.* 109:1561-1569.
  59. Tracey, K. J., H. Vlassara, and A. Cerami. 1989. Cachectin/tumour necrosis factor. *Lancet.* 1:1122-1126.
  60. Uckun, F. M., G. L. Schieven, I. Dibirdik, L. M. Chandan, A. L. Tuel, and J. A. Ledbetter. 1991. Stimulation of protein tyrosine phosphorylation, phosphoinositide turnover, and multiple previously unidentified serine/threonine-specific protein kinases by the pan-B-cell receptor CD40/Bp50 at discrete developmental stages of human B-cell ontogeny. *J. Biol. Chem.* 266:17478-17485.
  61. Ullrich, A., and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell.* 61:203-212.
  62. Wolpe, S. D., G. Davatelis, B. Sherry, B. Beutler, D. G. Hesse, H. T. Nguyen, L. L. Moldawer, C. F. Nathan, S. F. Lowry, and A. Cerami. 1988. Macrophages secrete a novel heparin-binding protein with inflammatory and neutrophil chemokinetic properties. *J. Exp. Med.* 167:570-581.
  63. Woodman, R. C., J. M. Ruedi, A. J. Jesaitis, N. Okamura, M. T. Quinn, R. M. Smith, J. T. Curnutte, and B. M. Babior. 1991. Respiratory burst oxidase and three of four oxidase-related polypeptides are associated with the cytoskeleton of human neutrophils. *J. Clin. Invest.* 87:1345-1351.