Neutrophil Activation on Biological Surfaces

Massive Secretion of Hydrogen Peroxide in Response to Products of Macrophages and Lymphocytes

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Abstract

Recombinant tumor necrosis factor alpha (rTNFα) and beta (rTNFβ) did not trigger H_2O_2 release from PMN in suspension. However, when PMN were plated on polystyrene surfaces coated with serum, fibronectin, vitronectin, laminin, or human umbilical vein endothelial cells (HUVEC), rTNFs induced a massive, prolonged secretory response, similar to that elicited by phorbol myristate acetate (PMA) or bacteria. On serum-coated plates, the maximum sustained rate of H_2O_2 release in response to rTNFα was 2.6±0.2 nmol/min per 10^6 PMN, the same as that with PMA; release continued for 73±4 min. On laminin-coated surfaces or HUVEC, release of H_2O_2 in response to rTNFs was slower, but lasted ~ 3.5 h, reaching the same total (> 100 nmol/10^6 PMN). Not only was this response far longer and larger than for other soluble stimuli of the respiratory burst studied with PMN in suspension, but the concentration necessary to elicit a half-maximal response (EC_{50}) for rTNFα was orders of magnitude lower (55 pM). Responses were similar with FMLP, but ranged from zero to small with recombinant IFNα, recombinant IFNβ, recombinant IFNγ, platelet-derived growth factor, recombinant IL-1β, or bacterial lipopolysaccharide. Adherent monocytes did not secrete H_2O_2 in response to rTNFs. H_2O_2 secretion by adherent PMN was first detectable 15–90 min after addition of rTNFs or FMLP. This lag period was unaffected by prior exposure of PMN to rTNFα in suspension, by allowing PMN to adhere before adding rTNFα, or by incubating adherent PMN in medium conditioned by rTNFα-treated PMN. Cytochalasins abolished H_2O_2 secretion in response to rTNFs, but not FMLP, if added during, but not after, the lag period. Thus, H_2O_2 secretion from rTNFα-treated PMN appears to be a direct but delayed response that requires assembly of microfilaments during exposure to the cytokine. These results suggest that PMN adherent to intra- or extravascular surfaces may undergo a massive, prolonged respiratory burst at the command of macrophages and lymphocytes reacting to microbial products and antigens.

Introduction

The secretory products of PMN, including reactive oxygen intermediates (ROI),[1] can be a major source of damage to inflamed tissues that contain few or no microorganisms (1–4). How this occurs is not well understood. Except for phagocytizable microbes, few agents are known that are likely to trigger a full-scale respiratory burst from PMN in vivo, despite the fact that a multitude of agents can trigger some level of response in vitro. Thus, many soluble secretagogues, including the most potent, phorbol myristate acetate (PMA), are nonphysiologic. The physiologic soluble secretagogues, such as immune complexes (5, 6), C5a (5, 7), *N*-formylated peptides (8, 9), leukotrienes (10), platelet-activating factor (11), arachidonic acid (12), and retinal (13), elicit transient responses that lead to the cumulative release of only a small percentage of the ROI elicited by microbes or PMA; some are active at concentrations unlikely to be generated in vivo or sustained in the presence of plasma proteins that bind or degrade the stimuli. Recently, cytokines have emerged as a new class of potentially physiologic triggers of the respiratory burst (14–20). However, the release of ROI from PMN in response to IL-1 (14), recombinant IFNγ (rIFNγ) (15, 16), tumor necrosis factor alpha (rTNFα) (16–20), or tumor necrosis factor beta (rTNFβ, lymphotoxin)) (16) is reportedly quite small, averaging 0.13±0.05 nmol H_2O_2 equivalents/10^6 PMN per min for rTNFα (16–20).[2] This is only 5% of the response observed in the present work with PMA.

Most studies of the PMN respiratory burst record short term observations (1–15 min) on dense cell suspensions (≥ 10^6/ml). These conditions bear little resemblance to the stages of inflammation in which PMN stick to endothelium, penetrate basement membranes and migrate through tissues, adherent the whole while to other types of cells and/or proteins of extracellular matrix. Attempts to analyze the respiratory burst of adherent PMN have been thwarted by the propensity of nonbiologic surfaces to trigger the burst (6), and of biologic surfaces to suppress it (22).

In the experiments described below, adherence to surfaces coated with serum, laminin, vitronectin, fibronectin, or endothelial cells prepared human PMN for a massive respiratory burst lasting 1–3 hours in response to rTNFα, rTNFβ, or FMLP, but not several other cytokines. The concentration of rTNFα necessary to elicit a half-maximal response (EC_{50}) was three to five orders of magnitude lower than for other potentially physiologic, soluble secretagogues studied with PMN in

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1. *Abbreviations used in this paper:* CGD, chronic granulomatous disease; DHCB, dihydrocytochalasin B; EC_{50}, concentration necessary to elicit a half-maximal response; FBS, fetal bovine serum (heat inactivated); HPO, horseradish peroxidase; HUVEC, human umbilical vein endothelial cells; KRPG, Krebs–Ringer phosphate buffer with glucose; MNL, mononuclear leukocytes; nIFNα, natural IFNα; NS, 0.9% NaCl; PDGF, platelet-derived growth factor; r, recombinant; ROI, reactive oxygen intermediates (O_2^-, H_2O_2, $OH^\cdot$, HOCl, etc.); TNFα, tumor necrosis factor alpha or cachectin; TNFβ, tumor necrosis factor beta or lymphotoxin.

2. For purposes of comparison, results reported elsewhere have been corrected for the ROI released without added stimuli, and values for O_2^- have been divided by two for expression as H_2O_2 equivalents (21).

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suspension. In further contrast to previous observations, secretion of H_2O_2 from adherent PMN in response to rTNFα or rTNFβ began after an extraordinarily long lag period (15–90 min), and required assembly of microfilaments. These findings suggest that when PMN leave the circulation, their cytotoxic potential can come under the control of macrophages and lymphocytes, as the latter release TNFα or TNFβ in response to bacterial lipopolysaccharide (LPS), microbes, alloantigens, or tumor antigens.

Methods

Cells. 8–10 ml venous blood was collected from healthy adults into a Vacutainer tube containing 147 U. S. Pharmacopeia units of heparin (Becton Dickinson & Co., Rutherford, NJ). Following the procedure of Ferrante and Thong (23), the blood was layered on top of 4 ml Hypaque–Ficoll (density 1.114 g/ml; 8.2% Ficoll) (Neutrophil Isolation Medium; Packard Instrument Co., Inc., Downers Grove, IL) and centrifuged at 22°C for 30 min at 400 *g* in a 15-ml conical polystyrene tube (Costar, Data Packaging Corp., Cambridge, MA). Platelet-rich plasma was discarded and the mononuclear leukocytes (MNL) and PMN were collected from separate layers above the erythrocyte pellet. The MNL and PMN fractions were placed into separate 15-ml tubes, the volume in each tube was brought to 12 ml with ice-cold Krebs–Ringer phosphate buffer with glucose (KRPG; pH 7.3–7.4; 300 mosM) formulated as described (24), and the tubes were centrifuged for 10 min at 350 *g*, 8°C. The pellets were carefully resuspended, first in 1 ml and then in 12 ml cold KRPG, and the tubes were centrifuged for 10 min at 180 *g*, 8°C. The pellets were again carefully resuspended in cold KRPG, and aliquots removed for counting in a hemocytometer and for differential counting of cytocentrifuged preparations. The cells were diluted to 7.5×10^5 PMN/ml or 10^7 MNL/ml in KRPG and kept on ice until use. The procedure required ~ 80 min and resulted in monodisperse suspensions. Per milliliter of blood, yields averaged 2.5 ($\pm$0.2) $\times$ 10^6 cells in the PMN fraction, of which 97.8$\pm$0.7% were PMN, 1.5$\pm$0.4% lymphocytes; and 0.3$\pm$0.2% monocytes (mean$\pm$SE in 18–26 experiments); and 1.2 ($\pm$0.2) $\times$ 10^6 cells in the MNL fraction, of which 25.7$\pm$2.2% were monocytes, 73.2$\pm$2.3% lymphocytes, and 1.1$\pm$0.4% PMN (6–8 experiments). In preliminary experiments, functional results were the same if the blood was anticoagulated with citrate instead of heparin, and whether or not erythrocytes in the PMN pellet were lysed by hypotonic NaCl. Because hypotonic shock may partially activate PMN, in all experiments reported below, the contaminating erythrocytes (mean 0.9$\pm$0.2 per PMN) were not lysed. Human umbilical vein endothelial cells (HUVEC) were the gift of Dr. E. Jaffe, Cornell University Medical College, New York, NY, and were prepared as described (25). Confluent monolayers derived from the second passage were used 24 h after addition to the plates described below.

Reagents. rTNFα and rTNFβ produced in *Escherichia coli* from the human gene were gifts of Genentech, Inc. (South San Francisco, CA). They were electrophoretically > 99% and > 95% pure, respectively, and had specific activities of 7.6×10^7 and 1.2×10^8 U/mg protein as titered by dye staining of actinomycin D–treated L929 cells (26, 27). Molar concentrations are expressed in terms of monomers. rIL-1β (human) was a gift of Dr. C. Dinarello, Tufts New England Medical Center, Boston, MA. It was electrophoretically 99.9% pure, with a specific activity of 5×10^7 U/mg protein in the thymocyte proliferation assay. The sources and characteristics of pure rIFNγ, rIFNβ, rIFNαA, and natural IFNα (nIFNα) were as previously detailed (28). Human platelet derived growth factor (PDGF) was from PDGF, Inc. (Boston, MA) (75% pure). Porcine PDGF was from Bethesda Research Laboratories, Gaithersburg, MD (90% pure). LPS was purified from *E. coli* 011:B4 (List Biologicals, Campbell, CA). Fetal bovine serum (FBS; heat inactivated; 13 pg LPS/ml) was from HyClone Laboratories (Logan, UT). Mouse laminin was purified 95% from Engelbreth–Holm–Swarm tumor basement membranes (Collaborative Research, Inc., Waltham, MA). Fibronectin purified from human plasma at the New York Blood Center and vitronectin purified from human serum by Dr. E. Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA) were gifts of Dr. S. Wright (The Rockefeller University, New York, NY). Dr. Wright also contributed purified IgG of monoclonal antibodies OKM1, OKM10, and IB4. Neutralizing IgG1 mouse monoclonal antibody against rTNFα (human) was the gift of Drs. A. Cerami and K. Manogue (The Rockefeller University). Bacteria from clinical isolates were grown to mid-log phase on trypticase soy broth, washed three times by centrifugation in KRPG, and stored at −80°C. Aliquots were thawed for each experiment and for colony counts on trypticase soy agar plates. All other reagents, including additional preparations of fibronectin, were from Sigma Chemical Co. (St. Louis, MO).

LPS contamination. A chromogenic limulus amebocyte lysate assay (M. A. Bioproducts, Walkersville, MD) with a sensitivity of 10 pg/ml was performed periodically. LPS was undetectable in the water used to prepare all reagents, as well as in KRPG, 0.9% NaCl (NS), and the working dilutions of rTNFα and rTNFβ. LPS was detected in Neutrophil Isolation Medium at 20 pg/ml. Priming of the PMN respiratory burst by LPS has been reported to require > 100 pg/ml LPS (29). Nonetheless, a possible effect of LPS at levels near or below the limit of detection cannot be excluded.

H_2O_2 assay. For adherent cells, assays were conducted in 6-mm-diam wells of 96-well, flat-bottomed, polystyrene tissue culture plates (Costar, Data Packaging Corp.). The wells were pretreated with 25 μl of serum or of RPMI 1640 medium containing the indicated amounts of extracellular matrix proteins (in some experiments, 100 μl of RPMI was used containing various amounts of serum). The plates were incubated in 5% CO_2 at 37°C for 50–100 min and then flicked empty, flooded with NS, and flicked empty again three times. Wells containing confluent monolayers of HUVEC were washed three times immediately before use in warm KRPG. 100 μl of reaction mixture containing 2.4 nmol scopoletin, 0.5 μg horseradish peroxidase (HPO), and 1 mM NaN_3 were then added to each well and the temperature brought to 37°C in a tissue culture incubator without CO_2. 10 μl of test agents were added, followed by 20 μl of cells (1.5×10^4 PMN or 2×10^5 MNL, corresponding to ~ 5×10^4 monocytes). This number of PMN per well was on the linear portion of the PMN dose-response curve for detectable responses in this system. A time-zero reading was taken over ~ 30 s in a plate-reading fluorometer (Microfluor; Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, VA). The oxidation of fluorescent scopoletin to a nonfluorescent product was then monitored at intervals, usually every 15 min, for 2–5 h. Control experiments indicated that 92% of the loss in fluorescence observed in response to rTNFα was abolished by addition of 3,000 Sigma units/ml of catalase, and 100% of the loss in fluorescence was abolished by omission of HPO. Subsequent readings were compared well-for-well to the time-zero readings, corrected for changes in cell-free wells, and converted to nanomoles H_2O_2 by microcomputer, as described (24). Nanomoles H_2O_2 was graphed as a function of time, and the lag period estimated to the nearest 15 min as the last time point after which the rate of H_2O_2 release differed significantly from that of the contemporaneous control (no added stimulus), or alternatively, to the nearest minute as the time at which back-extrapolation of the maximum sustained rate (lasting $\geq$ 15 min) intersected the line for the control. Estimations by these two methods averaged over all experiments were statistically indistinguishable. All conditions were tested in triplicate. When displayed graphically, the standard error was often smaller than the symbols denoting the means, so that standard errors have been omitted from the figures.

For PMN in suspension, 4-ml quartz cuvettes were coated with FBS, washed three times in NS, and filled with a total of 3 ml of reactants in the same proportions as used in the plates. The suspension was stirred magnetically with a Teflon-coated bar in the thermostatted (37°C) sample compartment of a spectrofluorometer (Fluorolog; Spex Industries, Inc., Edison, NJ). A cooled photomultiplier tube continuously detected fluorescence at 365 nm excitation and 473 nm emission. The count of photons per second was averaged by computer over 3-s intervals. Alternatively, 1.5-ml polypropylene microfuge tubes

(Sarstedt, Numbrecht, FRG) were coated with FBS, washed with NS, and filled with a total of 1.3 ml of reactants in the same proportions. The tubes were then placed on their sides on a to-and-fro horizontal shaker at 3.7 cycles/s at 37°C. At 30 min, the suspensions, or the supernatants from a 30-s microcentrifugation (Eppendorf Microfuge; Brinkmann Instruments, Inc., Westbury, NY), were transferred to plates or cuvettes to record fluorescence compared with cell-free controls.

Cytochrome b_{559}. Heparinized blood from a male patient with chronic granulomatous disease (CGD), his mother, and a normal donor was collected in California by Dr. M. Palladino (Genentech, Inc.) and flown overnight at ambient temperature to New York, where blood from an additional normal donor was collected. The content of spectrally normal cytochrome b_{559} in the PMN from all four donors was then estimated from their reduced-oxidized difference spectra, using a method that minimizes interference from hemoglobin (Ding, A., manuscript in preparation).

Results

Inability of rTNFα to trigger H_2O_2 release from PMN in suspension. When dilute suspensions of human PMN (10^5/ml) were vigorously stirred in cuvettes (3 experiments) or agitated in tubes (3 experiments), rTNFα or rTNFβ at 10–100 ng/ml elicited no detectable H_2O_2 release over periods of observation of 30–60 min. Yet, the cells were capable of responding when PMA was subsequently added, as illustrated by the fluorometer tracing in Fig. 1. Since others have reported release of ROI from rTNFα-treated PMN in denser suspensions or stationary cultures (16–20), the possibility was considered that adherence of PMN to each other or to the vessel containing them might be necessary to capacitate their response to rTNFα. At first, this proved difficult to test, because PMN incubated in polystyrene tissue culture plates in the absence of any exogenous protein, except HPO, released large amounts of H_2O_2 (~ 1.5 nmol/1.5 × 10^4 PMN per 60 min), presumably in response to the polystyrene itself, similar to what has been observed by others (6). rTNFα increased this response only ~ 20%. Thus, it was necessary to find a way to pretreat the polystyrene surface that would prevent triggering by the plastic, but still permit study of the actions of physiologic triggering agents on adherent PMN.

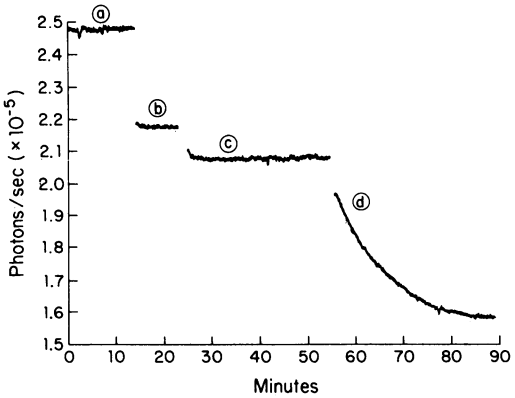

Figure 1. Failure of rTNFα to trigger H_2O_2 release from PMN in a rapidly stirred suspension. H_2O_2 release was detected as a decrease in the fluorescence of scopoletin at 37°C before (*a*) and after (*b*) adding 4.5 × 10^5 PMN to the cuvette, initially without (*b*) and then with (*c*) 100 ng/ml rTNFα. After 30 min, the ability of the PMN to release H_2O_2 was confirmed by adding 30 ng/ml PMA (*d*). The step changes at the moment of each addition are dilution artefacts.

Preparation of biological surfaces. Precoating the wells with FBS and then washing out unbound FBS suppressed polystyrene-triggered H_2O_2 release from PMN almost completely for the 1st 60 min of incubation (Table I). Human serum or plasma, and the plasma proteins fibronectin, vitronectin, and fibrinogen, were as effective as FBS. However, ~ 10-fold less fibronectin or vitronectin was required than fibrinogen. Quantitatively, fibronectin and vitronectin could probably account for the effect of serum or plasma. Not all proteins had this effect. Thus, there was little or no suppression with two other plasma proteins, albumin and IgG, and one constituent of extracellular matrix, elastin. The two most effective agents were the basement membrane protein laminin (EC_{50}, 1–5 μg/well before washing) and confluent monolayers of HUVEC. With the latter two surfaces, little or no H_2O_2 release was observed when PMN were incubated without added stimuli for up to 4 h. This could not be attributed to interference with the scopoletin assay by residual protein (30) because H_2O_2 release from PMN in response to PMA was undiminished as compared with that in uncoated wells. All subsequent experiments used polystyrene surfaces coated with FBS, fibronectin, vitronectin, laminin, or HUVEC.

H_2O_2 release in response to rTNFα from PMN incubated on FBS-coated plates. The agents considered to trigger the release of the largest cumulative amounts of ROI from PMN are the nonphysiologic soluble secretagogue PMA and physiologic particulates such as bacteria and yeasts. Optimal doses of PMA and of three different unopsonized bacteria were determined in preliminary experiments (the maximal response to bacteria was not increased by opsonization). Then all four stimuli, each at an optimal dose, were compared with 1 ng/ml of rTNFα. As seen in Fig. 2, all the stimuli induced comparable maximal sustained rates and total cumulative release of H_2O_2. However, there was a 39-min lag before the start of the

Table I. Effect of Various Coatings on the Ability of Polystyrene to Trigger the PMN Respiratory Burst

Agent	Suppression*	Duration‡	Dose/well§	IC_{50}‖
	%	*min*		
FBS	~ 100	~ 60	25 μl	0.3–1 μl
HuS	~ 100	~ 60	100 μl	~ 60 μl
HuP	~ 100	~ 60	100 μl	ND
Laminin	~ 100	>240	13–23 μg	1–5 μg
Vitronectin	~ 100	~ 60	10 μg	0.5–2.5 μg
Fibronectin	~ 100	~ 60	1 μg	<1 μg
Fibrinogen	~ 100	~ 60	100 μg	~ 10 μg
HUVEC	~ 100	>240	confluent	—
Elastin	0	—	10 μg	—
IgG	0	—	1000 μg	—
BSA	0–50	—	1000 μg	⩾1000 μg

HuS, human serum; HuP, human plasma.
* Decrease in H_2O_2 release from 1.5 × 10^4 PMN over the 1st 60 min in the absence of PMA, compared with that in uncoated wells.
‡ Duration of suppression.
§ Optimal or highest dose used per well (surface area, 32 mm^2).
‖ Concentration inhibiting the response by 50%.

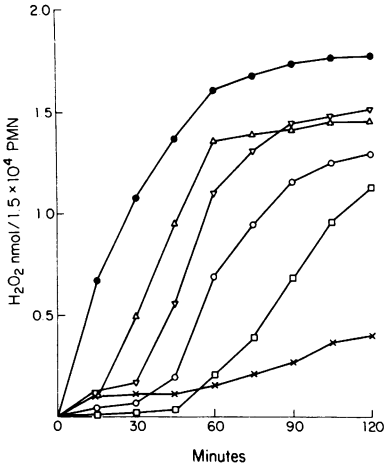

Figure 2. Comparison of H_2O_2 release from PMN on FBS-coated plates in response to rTNFα and the maximal stimuli, PMA (30 ng/ml) and unopsonized bacteria. (●) PMA; (▽ *E. coli* (10^6); (△) *Listeria monocytogenes* (10^7); (○) rTNFα (1 ng/ml); (□) *Staphylococcus aureus* (10^6); (×) no stimulus.

full response to rTNFα. The lag of ~ 15–45 min observed in response to the bacteria might have been due to their settling onto the PMN, but no such delay was anticipated for a soluble agent, nor was any seen with PMA. Because of this discrepancy, it could be questioned whether the H_2O_2 released in response to rTNFα arose from the same oxidase complex that generates ROI when triggered by other stimuli.

The same oxidase complex is triggered by rTNFα and PMA. In X-linked CGD, the leukocyte defect in ROI production is attributable to alterations in a gene at band p21 (31), and is closely linked to loss of the characteristic spectral signal from cytochrome b_{-245} (cytochrome b_{559}) in the plasma membrane (32). As shown in Table II, PMN from a boy with CGD lacked spectrally normal cytochrome b_{559} and were unable to release H_2O_2 in response to rTNFα, as well as to PMA. PMN from his mother, a presumed carrier, were intermediate between the CGD cells and cells from normal donors in all three tests. Thus, the oxidase producing ROI in response to rTNFα is regulated by the same gene and associated with the same cytochrome as the enzyme producing ROI in response to other triggers of the respiratory burst.

Table II. H_2O_2 Release in Response to rTNFα is Absent in PMN from a Subject with CGD

Cell donor*	Triggering agent (H_2O_2 nanomoles/1.5 × 10^4 PMN per 45 min) None	rTNFα‡	PMA§	Cytochrome b_{559} (picomoles/10^6 PMN)
Normal (NY)	0.23	1.44	1.75	5.4
Normal (SF)	0.93‖	1.66	1.76	7.9
CGD (SF)	0.03	0.01	0.01	0.0
Mother (SF)	0.10	0.19	0.87	2.3

* Normal (NY) donated PMN in New York immediately before testing. The other three subjects donated blood in San Francisco the day before. The bloods were flown to New York and all samples processed in parallel for testing on FBS-coated plates. The CGD patient is male; his mother is a carrier.
‡ 100 ng/ml.
§ 30 ng/ml.
‖ An unusually high value possibly related to the ~ 24-h delay in separating PMN from the blood.

Characteristics of responses on FBS-coated plates. Both the rate of secretion of H_2O_2 and the lag period before the onset of H_2O_2 release were dependent on the dose of rTNFα (Fig. 3). Table III summarizes the results of 18 such experiments with rTNFα and 8 with rTNFβ. The lag time with both agents at optimal doses averaged 27 min, 10-fold longer than that with PMA. The EC_{50} for rTNFα averaged 55 pM, 100-fold lower than that for PMA. With rTNFβ, the EC_{50} was ~ 12-fold higher than that with rTNFα. Secretion of H_2O_2 in response to rTNFα or rTNFβ continued for 1.2–1.3 h. The maximal sustained rate (lasting at least 15 min) was the same for all three agents (2.5–2.6 nmol/10^6 PMN per min), and the cumulative amounts of H_2O_2 secreted were also similar (105–124 nmol/10^6 PMN). Results were comparable if the wells were precoated with fibronectin or vitronectin instead of FBS (data not shown).

Using wells precoated with serum, fibronectin, or vitronectin, the maximal sustained rates of H_2O_2 release in response to rTNFα averaged 15-fold higher within the first 60 min of the assay than when no stimulus was added. However, the eventual onset of a substantial response without an added stimulus complicated the interpretation of apparent triggering by rTNFα. Accordingly, the next experiments were conducted on wells precoated with laminin.

Responses on laminin-coated plates. When PMN were incubated in laminin-coated wells in the absence of another stimulus, there was very little H_2O_2 release throughout the 4-h period of observation (Fig. 4). Nonetheless, rTNFα or rTNFβ elicited substantial secretion of H_2O_2, beginning 54–90 min after their addition. Although the maximal sustained rate was not as rapid as with PMA, PMN incubated on laminin-coated plates continued to secrete H_2O_2 in response to rTNFα for 3.5 h after its addition (not shown), so that in some experiments

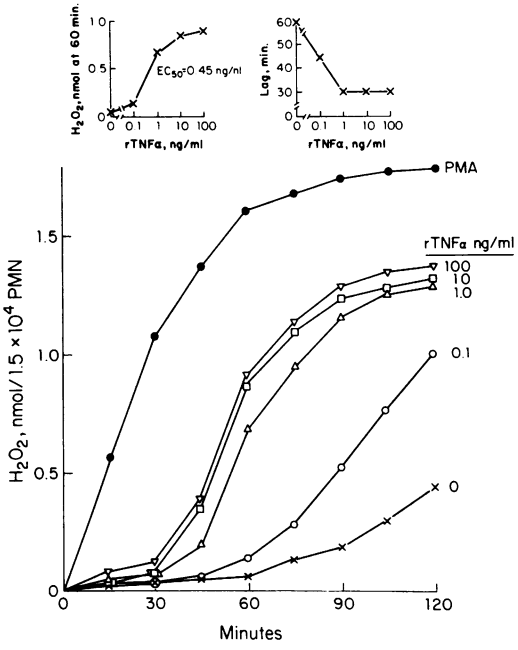

Figure 3. Effect of concentration of rTNFα on the time course of H_2O_2 release on FBS-coated plates. The left inset shows the dose-response curve for rTNFα at the 60-min time point. The right inset shows the lag period, estimated to the nearest 15 min (see Methods), as a function of dose. PMA was 30 ng/ml.

Table III. Characteristics of PMN H_2O_2 Release on FBS-Coated Plates

Feature	rTNFα	rTNFβ	PMA	No stimulus
EC_{50} (*pM*)	55±15 (16)*	648±274 (8)	5460±2200 (6)	—
Lag (*min*)‡	27±2 (15)	27±2 (8)	2.7±0.5 (19)	55±5 (20)
Duration (*min*)§	73±4 (18)	79±5 (8)	100±5 (20)	—
Rate (*nmol/min/10⁶*)‖	2.58±0.17 (18)	2.53±0.14 (8)	2.63±0.17 (20)	0.17±0.04 (20)¶
Total (*nmol/10⁶*)§	105±7 (18)	116±7 (8)	124 ± 7 (20)	—

* Means±SE (No. experiments); cells were from seven different donors. ‡ Estimated by back extrapolation from period of maximal rate to intercept with baseline. § Includes observed values for responses that were incomplete at the end of the experiment or went off scale, hence, an underestimate. ‖ Maximum rate sustained for ≥ 15 min. ¶ Measured over the 1st 60 min.

105 nmol $H_2O_2/10^6$ PMN were eventually released, the same final cumulative value seen 2.3 h sooner on FBS-coated plates. In 3 experiments, the EC_{50} for stimulation of H_2O_2 release from PMN on laminin-coated plates was $1.9\pm0.8 \times 10^{-11}$ M for rTNFα, and $3.7\pm2.5 \times 10^{-10}$ M for rTNFβ. These values did not differ from the results for cells tested on FBS-coated plates in the same experiments.

FMLP is a model for physiologic, phlogistic peptides released from bacteria (33) and damaged mitochondria (34). In terms of its EC_{50} with PMN in suspension ($3–8 \times 10^{-8}$ M), FMLP is comparable with C5a (7) as the most potent known physiologic, soluble stimuli of the respiratory burst. With PMN in suspension, there is a lag period of several seconds after addition of FMLP. Thereafter, release of ROI lasts 3–5 min and totals ~ 4–6 nmol H_2O_2 equivalents/10^6 PMN (8, 9).[2] It was thus of interest to test the response to FMLP using PMN on laminin-coated surfaces. Surprisingly, as shown in Fig. 4, there was a 60-min lag period after addition of optimal doses of FMLP ($\geq 6 \times 10^{-7}$ M), culminating in massive H_2O_2 release at the same rate as with PMA. ROI secretion lasted 2.5 h and totaled 108 nmol $H_2O_2/10^6$ PMN. In 2 experiments, the EC_{50} for stimulation of H_2O_2 release from PMN on laminin-coated plates averaged 5×10^{-8} M FMLP.

Responses on HUVEC. The first visible change in PMN during inflammation is their adherence to endothelium (35). Therefore, it was of interest to determine whether rTNFα and rTNFβ could trigger a respiratory burst from PMN incubated on endothelial monolayers. This was of further interest because PMN on protein-coated polystyrene might digest off the protein and make contact with the stimulatory plastic; HUVEC might prevent such contact. As shown in Fig. 5, the H_2O_2 secretory responses of PMN on HUVEC closely resembled their responses on laminin-coated wells. The HUVEC monolayers were carefully inspected by phase contrast microscopy and photomicroscopy before and after these experiments. No discontinuities in the monolayers could be detected.

Analysis of the long lag period. The lag period of 15–90 min before the onset of H_2O_2 secretion by adherent PMN in response to rTNFα was unprecedented for a soluble stimulus, and thus invited closer study.

A paradigm for two stimuli that cooperatively affect the PMN respiratory burst is priming and triggering. Priming

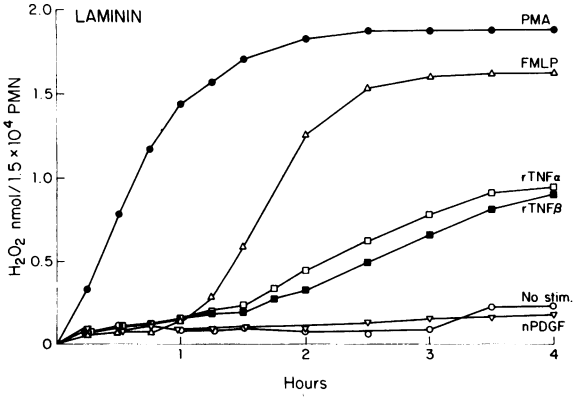

Figure 4. Time course of H_2O_2 release from PMN on laminin-coated plates in response to PMA (30 ng/ml), FMLP (5.6×10^{-6} M), rTNFα (100 ng/ml), rTNFβ (100 ng/ml), and natural human PDGF (100 ng/ml).

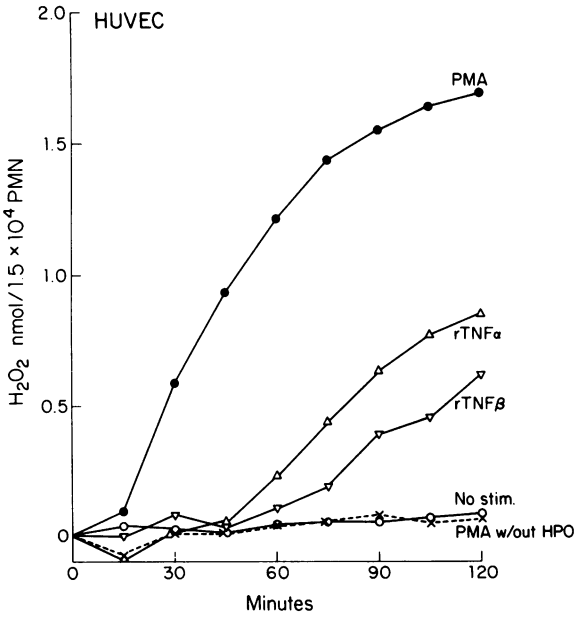

Figure 5. Ability of HUVEC monolayers to support the H_2O_2 secretory response of PMN in response to PMA (30 ng/ml), rTNFα (100 ng/ml), or rTNFβ (100 ng/ml). Omission of HPO from the reaction mixture (or addition of catalase, not shown) abolished the fluorescence change in response to PMA, demonstrating that decreased fluorescence reflected oxidation of scopoletin by H_2O_2, and not some other effect caused by HUVEC. Controls without PMN (HUVEC alone with and without each stimulus) were negative. The HUVEC monolayers were morphologically intact both at the beginning and end of each such experiment.

agents, such as LPS (29, 36), muramyl dipeptide (36), or colony stimulating factors (37, 38), do not trigger the respiratory burst themselves, but enhance the ability of subsequently added triggering agents to do so. Priming is time dependent (30–60 min [29]) and thus might account for the lag period. One way to determine if either adherence or rTNFα might be acting as a priming agent, and the other as a triggering agent, was to ask whether one of these two stimuli was required first and the other second. In the experiment shown in Fig. 6, the lag period remained 15–24 min whether PMN were plated 0, 15, 30, 45, or 60 min in advance of adding rTNFα. Conversely, as shown in Fig. 7, prior exposure of PMN to rTNFα for 30 min in suspension did not diminish the lag period when the same cells were subsequently allowed to adhere to FBS-coated wells. Thus, neither adherence nor rTNFα appeared to be an effective stimulus if provided before the other.

It remained to determine whether either stimulus could be removed before the other. Efforts to remove rTNFα already bound to PMN were technically unsatisfactory (not shown). However, it was possible to terminate adherence at various times during the lag period by addition of the microfilament-disrupting agent, dihydrocytochalasin B (DHCB). DHCB ablated the response to rTNFα if added at any time during the lag period (Fig. 8), even the last minute (not shown). Added later, DHCB had little or no effect. Results were similar with rTNFβ (Fig. 8), and using cytochalasin B instead of DHCB (not shown). In contrast, with FMLP, the effect of adding DHCB any time during the lag period was to permit immediate onset of H_2O_2 release at the maximal rate (Fig. 8). Finally, with PMA as a stimulus, DHCB had little or no effect, whenever it was added. The contrasting effects of cytochalasins on H_2O_2 release in response to rTNFα, FMLP, and PMA ruled out the possibility that the cytochalasins interfered with the oxidase or the assay.

Potential explanations for the lag other than priming were then considered. If the lag simply reflected the time taken for PMN to settle and adhere, then plating the PMN before adding rTNFα should shorten the lag. As already shown in Fig. 6, this was not the case. The lag might be required for rTNFα to adhere, thereby acquiring stimulatory properties through

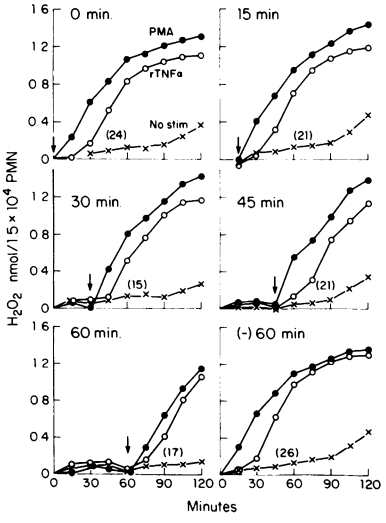

Figure 6. Insensitivity of the lag period to the order or time of addition of PMN or rTNFα. PMN were added to FBS-coated plates at zero time. rTNFα (100 ng/ml) or PMA (30 ng/ml) were added at the times indicated at the top of each panel and marked by the vertical arrows. In the last panel, rTNFα or PMA were added 60 min before PMN. The numbers in parentheses are the lag periods in min for the response to rTNFα, estimated by back extrapolation (see Methods).

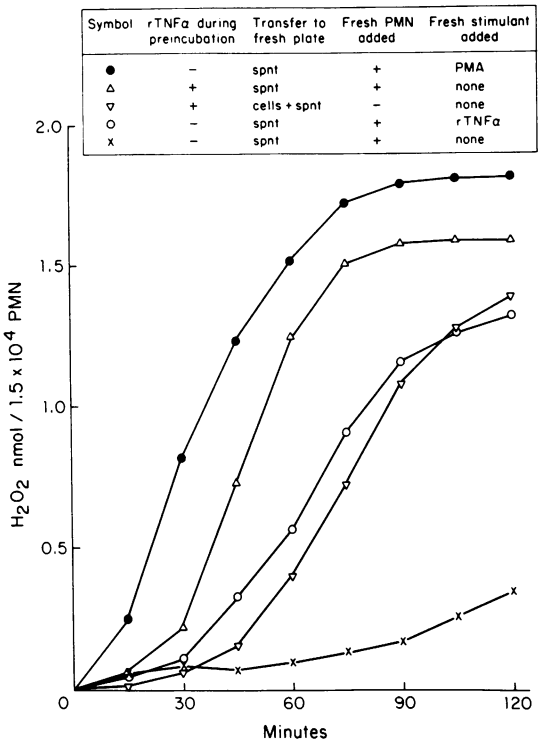

Figure 7. Inability to shorten the lag period by preincubating PMN with rTNFα in suspension, or by adding medium conditioned by rTNFα-treated PMN. PMN were agitated in tubes for 30 min at 37°C with or without 100 ng/ml rTNFα, then transferred directly to FBS-coated plates, or centrifuged. In the latter case, the supernatants were transferred to FBS-coated plates and fresh PMN added, with or without fresh rTNFα (100 ng/ml) or PMA (30 ng/ml). Supernatants from static cultures of PMN gave similar results. All combinations of these maneuvers were tested but only the key results are illustrated: preincubation of PMN with rTNFα did not abolish the lag period when the PMN were subsequently plated; preincubation of rTNFα with PMN did not make the rTNFα-containing reaction mixture rapidly stimulatory for fresh PMN.

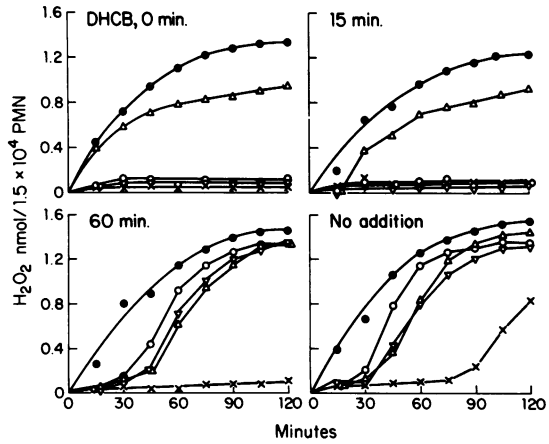

Figure 8. Contrasting effect of DHCB on the H_2O_2 secretory response of PMN to rTNFα (○) or β (▽) (100 ng/ml), FMLP (△) (5.6 × 10^{-6} M), or PMA (●) (30 ng/ml). PMN were added to FBS-coated plates at time zero. DHCB (5 μg/ml) was added at the times indicated at the top of each panel. There was no effect of adding 0.17% dimethylsulfoxide (solvent for DHCB) at time zero (not shown). (×) No stimulus.

Table IV. Effect of Cycloheximide on the Response to rTNFα

Feature	Cycloheximide*	PMA (30 ng/ml)	rTNFα (100 ng/ml)	No stimulus
	μg/ml			
Lag (*min*)	0	0	30	60
	10	0	30	60
H_2O_2‡	0	1.46±0.03	0.92±0.06	0.31±0.08
	10	1.41±0.11	0.93±0.06	0.28±0.05

* Added to the PMN in suspension for 15 min before they were plated and exposed to PMA or rTNFα.
‡ Nanomoles/1.5 × 10^4 PMN per 60 min; means±SE for triplicates.

inter- or intramolecular conformational changes. However, adding rTNFα up to 60 min in advance of PMN had no effect on the lag period (Fig. 6). PMN might need time to modify rTNFα to an active form, or to respond to rTNFα by elaborating another molecule that could elicit a respiratory burst rapidly. However, as shown in Fig. 7, rTNFα-containing medium conditioned for 30 min by PMN triggered a respiratory burst in fresh PMN with approximately the same lag as rTNFα not previously exposed to PMN. It appeared unlikely that the lag was required for synthesis of new protein (such as additional TNF receptors) because exposure of PMN to 10 μg/ml cycloheximide for 15 min before and throughout their incubation with rTNFα had no effect on the lag period or the magnitude of the response (Table IV). rTNFα might induce a transient suppressive state that requires 15–90 min to wane, for example by activating an inhibitor of protein kinase C (39). However, PMA added to PMN during the lag phase of the rTNFα response elicited H_2O_2 release with no detectable lag, at the same rate as for PMA-triggered cells not exposed to rTNFα (not shown). Finally, rTNFα is known to mobilize intracellular pools of CD11 leukocyte adhesion molecules (40), and these might trigger a response through interaction with the extracellular matrix proteins coating the wells. Accordingly, PMN were exposed for 15 min in suspension and during the plate assay to a mixture of 2 μg/ml each of three monoclonal antibodies directed to different epitopes on the complement receptor type 3 α-chain (OKM1 and OKM10) or the β-chain shared by the CD11 family (IB4), or to each antibody alone. This affected neither the lag, rate, nor duration of the response to rTNFα (not shown).

Thus, it appeared that H_2O_2 secretion was a direct yet delayed response to rTNFα, which required that PMN be adherent and exposed to rTNFα simultaneously.

Other potential triggering agents and responding cells. To gauge whether the response of PMN to rTNFα and rTNFβ was in some way selective, a panel of additional cytokines was tested with PMN (Table V; see also Fig. 4), and rTNFα and rTNFβ were tested on monocytes (Table VI). rIFNγ, rIFNβ, nIFNα and rIFNα, rIL1β, and natural PDGF stimulated little or no H_2O_2 release from adherent PMN, in contrast to reports using different experimental conditions (e.g., reference 41). LPS was stimulatory at 10–1000 ng/ml. However, LPS could not account for stimulation by rTNFα because rTNFα contained, at most, 1,000 times less than the minimal stimulatory dose of LPS, was far more stimulatory than optimal doses of LPS, was completely prevented from eliciting H_2O_2 release in the presence of monoclonal antibody against rTNFα, and did not synergize with LPS when both were tested together (not shown). Monocytes released no H_2O_2 in response to rTNFα or rTNFβ, although they were tested simultaneously with rTNFα-responsive PMN from the same donors, and responded briskly to PMA.

Discussion

Extended observations of PMN adherent to biological surfaces give a different view of the respiratory burst than short-term studies of PMN in suspension. Some major differences are in the identity of substances that act as triggering agents, the duration and magnitude of the secretory response, the kinetics of signal transduction, the role of the cytoskeleton, the mechanisms by which stimuli interact, the extent to which MNL can control the behavior of PMN, and implications for pathogenesis and therapy.

It has apparently not been appreciated that adherence of PMN can convert them from a nonresponsive to a responsive state with regard to their ability to secrete ROI when exposed to certain soluble ligands, in this case rTNFα and rTNFβ. However, three other effects of adherence have been described. First, adherence of PMN to uncoated polystyrene itself triggers

Table V. Other Potential Triggering Agents

	H_2O_2 nanomoles/1.5 × 10^4 PMN*					
Concentration	rIL-1β	PDGF‡	LPS	rIFNγ	n/rIFNα§	rIFNβ
ng/ml						
0	0.32±0.03 (4)	0.23±0.03 (3)	0.34±0.03 (3)	0.34±0.03 (3)	0.31±0.03 (3)	0.32±0.04 (2)
1	0.23±0.05 (3)	0.20±0.01 (2)	0.35±0.09 (3)	0.08 (1)	0.18±0.03 (3)	0.28±0.07 (2)
10	0.28±0.05 (4)	0.15±0.04 (3)	0.71±0.21 (3)	0.53±0.15 (3)	0.18±0.06 (5)	0.32±0.04 (2)
100	0.41±0.05 (4)	0.15±0.01 (2)	0.73±0.09 (3)	0.38±0.13 (3)	0.12±0.04 (5)	0.47±0.03 (2)
1,000	0.63±0.06 (3)	—	0.80±0.05 (3)	0.10±0.00 (2)	—	—

* Mean±SE (No. experiments) on FBS-coated plates, with responses scored at 60 min, except as indicated for PDGF. In the same experiments, results with rTNFα were 1.52±0.14 (6), and with PMA, 1.87±0.09 (6). ‡ Combined results for one experiment with porcine PDGF and two experiments with human PDGF. Plates were coated with laminin (1 experiment) or FBS (2 experiments). Responses were scored at 60, 90, or 240 min. § Results combined for 3 experiments with affinity-purified nIFNα (mixture of subtypes) and 2 experiments with rIFNαA.

Table VI. Inability of rTNFα or rTNFβ to Trigger H_2O_2 Release from Monocytes

Stimulus	Dose	H_2O_2 nanomoles/120 min*
	ng/ml	
None	—	0.10±0.04 (8)
rTNFα	100	0.07±0.05 (6)
rTNFβ	100	0.15±0.01 (2)
PMA	30	1.74±0.09 (8)

* Means±SE (No. experiments) for experiments with four cell donors, using 2×10^5 MNL (~ 5×10^4 monocytes) per well. Plates were untreated (1 experiment) or coated with FBS (6 experiments) or laminin (1 experiment). Responses remained negative for at least 4 h and at doses up to 1,000 ng/ml rTNFα.

Table VII. Comparison of Triggering of the PMN Respiratory Burst by Some Potentially Physiologic, Soluble Triggering Agents

Agent	EC_{50}	Lag	Duration	Total	Reference
	M	*min*	*min*	*nmol**	
rTNFα‡	6×10^{-11}	27	73	105	Present work
rTNFβ‡	7×10^{-10}	27	79	116	Present work
FMLP§	3×10^{-8}	0	3–5	6	8
C5a	3×10^{-8}	0	2	9.4	7
$C5a_{desarg}$	2×10^{-6}	0	2	2.2	7
Leukotriene B_4	10^{-6}	0	1	0.7	10
PAF§	10^{-6}	0	3	1.3	11
Retinal	3×10^{-6}	4	**	**	13
20:4‖	2×10^{-5}	0	**	**	12

* Nanomoles H_2O_2 equivalents/10^6 PMN at optimal doses.[2]
‡ Tested with adherent PMN on FBS-coated plates.
§ Platelet activating factor; tested in the presence of cytochalasins.
‖ Arachidonic acid.
** Data not presented.

the respiratory burst (6). Second, adherence of PMN to plastic in the presence of plasma, which quenches stimulation by polystyrene, markedly augments the rate and duration of O_2^- release in response to FMLP and C5a (42), an effect confirmed here for FMLP on plates coated with FBS or laminin. Third, monolayers of endothelium, fibroblasts, fibronectin, collagen, or endothelial cell-derived extracellular matrix were reported to abolish the respiratory burst of PMN in response to FMLP (22). The present results stand in direct contrast to this last point. Perhaps the 40-min period of observation in the earlier study was shorter than the lag period.

The reason why a large respiratory burst in the absence of triggering agents is rarely reported with PMN in polystyrene vessels probably relates to the practice of adding gelatin, albumin, or cytochrome *c* to the reaction mixture and/or the use of stirred or agitated cell suspensions. Collagen (from which gelatin is derived) was a strong suppressant of the polystyrene-induced respiratory burst in the present work (not shown). Albumin was a weak suppressant, but unbound albumin was washed away before the reaction mixture was added. The small burst triggered with rTNFα in earlier studies, 0.7–12.3% of that observed here, may reflect minimal adherence of PMN and/or the relative inability of a surface coated with albumin or cytochrome *c* to capacitate the PMN respiratory burst in response to rTNFα. It is likely that capacitation is mediated by specific PMN receptors that interact with matrix proteins (43–45). Such receptors are probably lacking for albumin and cytochrome *c*.

The potency of rTNFα was remarkable. Compared with most of the potentially physiologic, soluble stimuli of ROI secretion from PMN (Table VII), the EC_{50} for rTNFα was ~ 1,000 to 330,000 times lower, and the cumulative release of H_2O_2 ~ 10–100 times higher. Even compared with PMA, the most potent of the nonphysiologic triggering agents, the EC_{50} for rTNFα was 100 times lower; the cumulative response to rTNFα was nearly the same as that to PMA. As a bioassay for rTNFα, the secretion of H_2O_2 by adherent PMN was 50 times more sensitive than a radioreceptor assay (46) and 10 times more sensitive than an ELISA (46), but ~ 70-fold less sensitive than an 18-h cytotoxicity assay using actinomycin D–treated mouse L929 cells (26). However, in contrast to the latter assay, the effect studied here was a rapid response by human cells that were neither clonally selected for sensitivity, subjected to prolonged culture, nor treated with a metabolic poison.[3] The EC_{50} for rTNFα was 20 times less than the peak level of TNF in the serum of mice injected with LPS (47), and 5,600 times less than that in the serum of rabbits primed by infection before injection with LPS (48).

Soluble stimuli generally induce ROI secretion from PMN in suspension with lag periods < 1 min. In contrast, initiation of H_2O_2 secretion from adherent PMN in response to rTNFα required 15–90 min. The following explanations were tested but received no experimental support: that the lag was required for PMN to adhere, for rTNFα to adhere, for PMN to modify rTNFα or elaborate another molecule in response to rTNFα that could elicit a respiratory burst more rapidly, for synthesis of a protein, for the waning of a suppressive state induced by rTNFα, or for mobilization of intracellular pools of CD11 leukocyte adhesion molecules. The results did not exclude mediation of the response by a short-lived rTNFα-induced molecule, or the involvement of adhesion molecules. Finally, the lag period did not appear to represent the time taken for either rTNFα or the state of adherence to act as a priming agent in preparing the cells for rapid triggering by the other stimulus. By definition, a priming stimulus acts before a triggering stimulus. In contrast, the rTNFα response required that adherence be coterminous with exposure to rTNFα. It thus appears likely that rTNFα is indeed a triggering stimulus, but has an exceptionally prolonged period of signal transduction, during which cell adherence is required. This property may afford a unique opportunity for temporal resolution of the transients in membrane potential, intracellular H^+ and Ca^{++} concentrations, phosphoinositide turnover, and kinase activity that are suspected to play a role in activating the oxidase.

Cytochalasins are widely used to augment the respiratory burst of PMN in response to soluble stimuli. In contrast, cytochalasins abolished the secretion of H_2O_2 by adherent PMN in

3. Though azide was added to the PMN to inhibit myeloperoxidase and catalase, it was not considered a metabolic poison in this cell type, which is deficient in mitochondria.

response to rTNFα. This was only evident if cytochalasins were added during the lag period. An inhibitory effect of cytochalasins on ROI secretion in response to rTNFα was also observed by Tsujimoto et al. (18), but not by Larrick et al. (19). Tsujimoto et al. speculated that microfilaments may be needed for internalization of rTNFα-receptor complexes, and that internalization of these complexes is necessary for initiating ROI secretion (18). However, additional hypotheses should be considered, including the transmission via microfilaments of a signal from adherence receptors that alters the function of TNF receptors (49), increased mobilization of TNF receptors to the plasma membrane in response to microfilament-dependent cell spreading, or capacitation of the triggering capacity of TNF receptors by association with the cytoskeleton. This last hypothesis would stand in contrast to the inhibitory effect of cytoskeletal association on the triggering capacity of FMLP receptors (50). Indeed, as shown in Fig. 8, cytochalasins had diametrically opposite effects on the responses of adherent PMN to rTNFα and FMLP. Only further study will settle whether assembly of microfilaments contributes to a change in the number, affinity, clustering, internalization, and/or signalling capacity of TNF receptors.

In the host, the requirement for prolonged adherence to a surface during exposure to TNF may constitute a control mechanism. Normal vascular endothelium is strongly antagonistic to the adherence of PMN (35), and hence probably to their ability to respond to TNF. Thus, if TNF produced in a local inflammatory site should leak into the circulation, where rTNFα has a $t_{1/2}$ of only 6 min (47), systemic activation of PMN would be avoided. On the other hand, in a local inflammatory site into which PMN have migrated, the elaboration of TNFα or TNFβ by macrophages and lymphocytes may recruit the full extracellular cytotoxic potential of the PMN respiratory burst, without the need for each individual PMN to ingest a large number of microbes. Perhaps this contributes to abscess formation.

There are several other situations in which PMN may come in prolonged contact with abnormally adhesive endothelium or subendothelial structures, and simultaneously be exposed to TNF. The ability of LPS to elicit TNF (47) and the ability of LPS (51) and TNF (36) to render endothelium adhesive for PMN suggest how the PMN respiratory burst might be triggered in capillary beds during endotoxemia, as in the acute respiratory distress syndrome (1), or during prolonged systemic infusion of rTNFα (52). In the Schwartzman reaction, the local priming dose of LPS leads to capture of PMN at the site (53). The systemic challenge dose presumably elicits release of endogenous TNF (47). The resultant triggering of adherent PMN may add to the local endothelial damage caused by LPS itself (54) and thus promote hemorrhagic necrosis of the site where the first injection was made. Similarly, given that tumor capillary endothelium is often discontinuous (55) and may in some cases be particularly sensitive to induction of adhesiveness by TNF, it is possible that activation of adherent PMN in tumor vessels contributes to hemorrhagic necrosis induced by TNF (56). Finally, the ability of LPS to induce hemorrhagic necrosis of tumors that are immunogenic (57) may be due to local induction of TNF from a preexistent infiltrate of MNL. This, in turn, may activate PMN in tumor vessels.

The same reasoning suggests that there might be a therapeutic advantage to localizing PMN in tumor beds, such as with radiotherapy or complement-fixing monoclonal antibodies, and then infusing small quantities of rTNFα over 15–90 min. Drugs that sensitize tumor and endothelial cells to oxidant injury by inhibiting antioxidant defenses would be expected to increase the resultant damage (58–61).

It will be important to learn if other PMN-secretory products with the potential to damage tissues, such as elastase and cationic proteins (4), are released under the conditions described here. It also remains to be seen if eosinophils respond like neutrophils. There are additional cytokines besides TNFα, IL-1, and IFNα that are released by macrophages in response to LPS, whose effects in this system would be of interest (62). Finally, it should be determined if other cells at the site of inflammation, such as endothelium, share the ability of macrophages and lymphocytes to commandeer the arsenal of adherent PMN through release of cytokines.

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