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Research Article

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Disparate Effects of Interferon-γ and Tumor Necrosis Factor-α on Early Neutrophil Respiratory Burst and Fungicidal Responses to *Candida albicans* Hyphae In Vitro

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Abstract

We examined effects of priming with recombinant human interferon-γ (IFN) or tumor necrosis factor-α (TNF) on neutrophil responses to *Candida albicans* hyphae. Both cytokines increased early superoxide generation after hyphal stimulation. The more pronounced effects of TNF were accompanied by an augmented surface membrane depolarization rate and were insensitive to both pertussis toxin and calcium ion chelation, but were negated by concomitant incubation with puromycin or cycloheximide during priming. IFN augmented hyphal killing despite its only minor enhancement of early respiratory burst responses, but TNF reduced neutrophil fungicidal activity to nearly 40% below those by unprimed control cells even though it enhanced early superoxide responses more dramatically. Though TNF-primed neutrophils killed hyphae at normal initial rates, IFN-primed or even unprimed cells manifested more fungicidal sustained activity. These disparate consequences of cytokine priming on hyphal destruction were paralleled by differences in late generation of potentially candidacidal oxidants, hydrogen peroxide, and hypochlorous acid. IFN added during priming failed to correct TNF-associated functional defects in neutrophil anti-*Candida* responses. Thus, augmentation of early respiratory burst responses to oxidant-sensitive organisms need not necessarily reflect concomitant salutary effects on microbicidal activity. (*J. Clin. Invest.* 1991. 87:711–720.)
Key words: *Candida albicans* • neutrophil • cytokines • fungicidal

Introduction

Candidiasis represents a major cause of morbidity and mortality in a broad range of immunocompromised patients. *Candida albicans,* the most common causative species, exists in lesions as yeastlike blastoconidia and larger pseudohyphae or hyphae (1, 2). Since polymorphonuclear leukocytes (PMN) are the most critical host effector mechanism against dissemination of candidiasis (1–5), it is important to define mechanisms controlling their fungicidal capability. Our early work (3–5) established that the larger, uningestible forms of *C. albicans* are among the many organisms requiring activation of PMN respiratory burst and degranulation responses for generation of microbicidal activity (6–9). Hyphae are almost always present within lesions (10), and thus must be cleared by host defenses if candidiasis is to be eliminated and dissemination prevented.

Among the many natural mediators capable of modulating PMN function both in vitro and in vivo, the cytokines, interferon-γ (IFN) or tumor necrosis factor-α (TNF),[1] share diverse effects on host defense mechanisms that may be highly consequential. For example, incubation in vitro with recombinant human IFN or TNF primes PMN for augmented stimulation of multiple responses including adherence (11), surface receptor number and/or affinity (11–13), phagocytosis (11, 14), oxidant release (12, 14–20), degranulation (15, 17, 20, 21), antibody-dependent cell-mediated cytotoxicity (11–13, 14, 16), and intracellular antimicrobial activity (20–26). The likely significance of these effects is supported by their occurrence at concentrations documented within inflammatory foci in vivo (28–31), as well as the enhancement of PMN function after systemic administration to humans or experimental animals (27, 30–35). However, two cytokines may have disparate effects on any one PMN functional parameter (16, 19, 32), different microorganisms may elicit dissimilar responses from PMN primed with one particular cytokine (26, 30), and induced alterations may be detrimental rather than beneficial to host defense mechanisms (36–38). Definitive explanations for this diversity of cytokine effects and specificity of responses to different microbial stimuli await more complete definition of activation mechanisms by such primed PMN.

Thus far, those basic mechanisms partially defined for cytokine-induced modifications in PMN function derive primarily from experiments using formylmethionylleucylphenylalanine (FMLP), phorbol myristate acetate (PMA), immunoglobulin (Ig) G–coated particles (11–19). However, our previous comparisons of responses to different stimuli by unprimed PMN indicate that *C. albicans* hyphae may elicit disparate patterns of early cellular events linked to PMN respiratory burst activation (39–42). Since cytokine effects on microbicidal responses

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1. *Abbreviations used in this paper:* BAPTA/AM, [1,2]-bis[*O*-aminophenoxy]ethane-*N,N,N',N'*-tetraacetic acid acetoxymethyl ester; DCF, 2'7'-dichlorofluorescein; DCFH, 2'7'-dichlorofluorescein; diS-C_3[5], [3,3']-dipropylthiodicarbocyanine iodide; DTP, sodium dithiodipropionate; indo-1/AM, indo-1 acetoxymethyl ester; LDH, lactate dehydrogenase; MHBSS, calcium- and magnesium-free modified HBSS, pH 7.4; NBT, nitroblue tetrazolium; PT, pertussis toxin; TNF, tumor necrosis factor-α.

may be organism specific (26, 30), we evaluated the effects of IFN and TNF priming on PMN responses to hyphae. We found that priming with IFN or TNF augmented the early rise in cytosolic calcium and initial respiratory burst responses, though TNF effects on the latter appeared to be calcium independent. Despite the more marked increment in early O_2^- generation after TNF priming, total hyphal killing was reduced, even though the initial rate of the latter process had appeared normal. In contrast, IFN enhanced PMN killing. These cytokine-induced alterations in fungicidal activity were associated with parallel changes in late release of oxidants, H_2O_2 and HOCl, by PMN responding to hyphae.

Methods

Reagents. Dr. Michael Shepard, Genentech, Inc., San Francisco, CA, kindly provided recombinant human IFN (4×10^7 U/mg) and TNF (5×10^7 U/mg), each containing < 0.125 endotoxin units per milliliter by the *Limulus* amebocyte assay, and supplied affinity-purified polyclonal rabbit anti-human TNF and murine anti-human IFN monoclonal antibodies. We purchased the fluorescent probes [3,3']-dipropylthiodicarbocyanine iodide (diS-C_3[5]), indo-1 acetoxymethyl ester (indo-1/AM), [1,2]-bis[o-aminophenoxy]ethane-*N,N,N',N'*-tetraacetic acid acetoxymethyl ester (BAPTA/AM), and (DCFH) diacetate from Molecular Probes, Inc., Junction City, OR. We purchased reagent grade type VI horse ferricytochrome *c*, bovine erythrocyte superoxide dismutase (SOD), horseradish peroxidase, homovanillic acid, catalase, [5,5']dithio-bis-[2-]nitrobenzoic acid, sodium dithiodiproprionate (DTP), dimethyl sulfoxide (DMSO), taurine, mannitol, methionine, sodium benzoate, EDTA, EGTA, trypan blue, nitroblue tetrazolium (NBT), [*N,N'*]-dimethylformamide, phenolphthalein glucuronic acid, phenolphthalein, glucuronic acid, glycine, sodium pyruvate, NADH, lactate dehydrogenase (LDH), polymixin B, Triton X-100 and Ficoll-Hypaque (sodium diatrizoate) from Sigma Chemical Co., St. Louis, MO; dextran T500 from Pharmacia Fine Chemicals, Uppsala, Sweden; pertussis toxin (PT) from List Biological Laboratories, Campbell, CA; and RPMI-1640, Dulbecco's PBS, HBSS, and its modified Ca^{++}- and Mg^{++}-free form (MHBSS), pH 7.4, from Gibco Laboratories, Grand Island, NY. All reagents and buffers were endotoxin free, and were checked for contamination at intervals using the *Limulus* amebocyte assay (sensitivity < 10 pg/mg protein).

Isolation, pretreatment, and priming of PMN. We sedimented heparinized venous blood from healthy volunteers in 3% dextran, then centrifuged the supernatant on Ficoll-Hypaque and hypotonically lysed contaminating erythrocytes (39). After separation, we suspended cells (98.5–100% PMN as determined by microscopic examination of sampled unstained cells) in MHBSS and stored on ice until use. For selected experiments, we treated PMN with PT before experiments, as in our previous studies (40), by tumbling 1×10^7/ml PMN in MHBSS plus 0.1% BSA at 37°C for 2 h with or without 500 ng/ml PT. To initiate experiments, we rotated plastic tubes with PMN in HBSS end over end at 6 rpm at 37°C for 30–90 min with cytokines or buffer alone, then washed the PMN before incubation with fungi or other agonists.

Organisms. We maintained a previously described clinical isolate of *C. albicans* as blastoconidia on modified Sabouraud's dextrose agar and germinated into hyphal forms as previously described (39). To determine whether or not results obtained using this isolate were strain specific, we also performed selected experiments using three other isolates that had been originally obtained from patients with disseminated candidiasis. Briefly, we subcultured blastoconidia for 48 h, incubated them in RPMI-1640 medium on a gyratory shaker for 4 h at 37°C, centrifuged hyphae at 2,000 *g* for 10 min, resuspended them in HBSS, opsonized them by incubation in pooled normal human sera for 30 min at 37°C, washed them twice, then resuspended them in HBSS, and kept them on ice until use.

Membrane depolarization. We monitored changes continuously with a thermostatically controlled spectrofluorometer (model 650/10, Perkin-Elmer Corp., Garden Grove, CA) using the lipophilic, cationic fluorescent probe, diS-C_3[5], as previously described (39). After suspending PMN in HBSS (2×10^6/ml final concentration) in reaction cuvettes 2 min before stimulation, we equilibrated them with 1.5 μM diS-C_3[5] with continuous stirring, and then added hyphae (1.4×10^6/ml). For comparisons between stimuli, relative rates of PMN membrane depolarization correspond reproducibly to the slopes of tangents drawn to initial increases in continuous tracings of fluorescence, while subsequent decreasing fluorescence values indicate degradation of the probe by oxidants released during the PMN response (39). After stimulation, we determined the initial rate of increasing fluorescence at 670 nm (excitation at 620 nm) in this fashion. Results are expressed and compared as mean relative rates of increased or decreased fluorescence for all experiments.

Calcium chelation and cytosolic calcium flux. We loaded PT-treated or control cytokine-primed or unprimed PMN with indo-1 in MHBSS by incubation for 30 min at 37°C with 1 μM of cell permeant indo-1/AM, which is hydrolyzed in the cytoplasm to the impermeant (trapped) form. In experiments requiring chelation of intracellular Ca^{++}, as in our prior studies (42), we simultaneously loaded 1 μM BAPTA/AM, the cell-permeant ester of a nonfluorescent organic Ca^{++} chelator (43). After washing cells by equilibration in MHBSS for 15 min at 25°C, we resuspended them in HBSS or MHBSS and stored them on ice until use. For chelation of extracellular Ca^{++}, as in past experiments (42), we added 5 mM EGTA to cell suspensions immediately before stimulation as described by Tsien et al. (44), to prevent depletion of intracellular Ca^{++} stores. We continuously monitored intracellular indo-1 fluorescence of 2×10^6 PMN stimulated in suspension with 2×10^6 hyphae in a thermostatically controlled cuvette with stirring using dual emissions of 405 and 485 nm and excitation of 355 nm as previously outlined (41–42).

PMN adherence. As previously described (39), we tumbled 2×10^6/ml PMN and hyphae together at 37°C for 15 min, then fixed them with 1% paraformaldehyde. To assure blinded conditions, observers who were unaware of original prestimulation treatments and incubation conditions counted the numbers of PMN attached to each of 100 hyphae.

Initial PMN respiratory burst responses. We determined O_2^- release by PMN by quantitative reduction of cytochrome *c* (45) as in our past studies (39–42). After adding 100 μM cytochrome *c* and 2×10^6/ml of hyphae in 1 ml total volume to PMN (2×10^6/ml) warmed to 37°C, we stopped reactions after 10 min at 37°C by immediate immersion of reaction tubes in ice, followed by centrifugation at 4°C, and reading of supernatants at 550 nm (spectrophotometer model 576ST, Perkin-Elmer Corp.). Addition of catalase to incubation tubes did not significantly change cytochrome *c* reduction under the conditions used with these stimuli. Duplicate samples containing 100 μg/ml of SOD were analyzed in parallel. Results are expressed as nanomoles of SOD-inhibitible ferricytochrome *c* reduced per 2×10^6 stimulated PMN per 10 min, using the previously described extinction coefficient of 21.1 M^{-1} cm^{-1} (46), followed by subtraction of calculated values for corresponding SOD and unstimulated control PMN measured simultaneously. The latter ranged from 0.03 to 0.18 nmol/2×10^6 PMN per 10 min. Controls containing stimuli without PMN did not release detectable O_2^-.

In selected experiments, we determined visual patterns of early oxidant generation by PMN. For continuous observation after stimulation, we incubated the diacetate ester of the oxidant-sensitive, minimally fluorescent dye, DCFH (1–10 μM in MHBSS containing 0.1% ethanol), in the dark with 4×10^6/ml PMN at 37°C for 15 min, washed, resuspended in HBSS, then incubated with hyphae (1:1 final ratio) in siliconized Mackaness chambers on a heated microscope stage. Using alternate observation by phase contrast and fluorescence (excitation at 490 ± 5 nm, emission 510–550 nm) we then visualized the timing and localization after PMN–hyphal contact of intracellular DCFH oxidation to fluorescent 2',7'-dichlorofluorescein (DCF), as modified slightly from the procedure originally outlined by Salata et al. (47). Alternatively, after incubation with hyphae for timed intervals, we smeared PMN on cover slips and stained with NBT (30 min at 37°C, pH 7.0) for

localization of oxidant production as originally described by Briggs et al. (48).

Late release of oxidants by PMN. As in our previous studies (49), we determined generation of H_2O_2 by measuring the H_2O_2-dependent, horseradish peroxidase-mediated oxidation of homovanillic acid to the fluorescent dimer: we incubated 2,2'-dihydroxy-3,3'-dimethoxydiphenyl 5,5' diacetic acid, PMN (1×10^6) and hyphae (5×10^6) in 1 ml of Dulbecco's PBS containing 100 μM homovanillic acid and 1 U horseradish peroxidase in a 37°C shaking water bath. We stopped reactions after 30–60 min by adding 0.25 ml of 0.1 M glycine-NaOH (pH 12) containing 25 mM EDTA. After centrifugation (1,000 *g* for 10 min), we determined fluorescence in supernatants (excitation at 312 nm and emission at 420 nm), then calculated H_2O_2 generation by comparison with a standard linear curve generated using known concentrations of H_2O_2, as described by Ruch et al. (50). We also measured HOCl generation as before (49) by trapping with taurine and quantitating the resulting taurine chloramine produced, as modified slightly from the method originally outlined by Weiss et al. (51). This involved incubating 2.5×10^6 PMN/ml at 37°C for 60 min in a shaking water bath with 5×10^6 hyphae/ml in Dulbecco's PBS containing 15 mM taurine. We then added 1 ml 5-thio-2-nitrobenzoic acid (prepared from 5,5'-dithio-bis[2-nitrobenzoic acid]; reference 52), determined absorbance of supernatants at 412 nm, and calculated HOCl generation by comparison with standards (51).

Degranulation. As in prior studies (53), we incubated 10^7 PMN and hyphae for 10 min, placed them on ice, centrifuged at 4°C, and added 0.3 ml of supernatant to 0.8 ml of acetate buffer (pH 4.5) plus 0.1 ml 10 mM phenolphthalein glucuronic acid. After incubating 4 h at 37°C, we added 1.8 ml of cold glycine buffer (pH 10.5) to terminate reactions, measured optical densities at 550 nm, determined concentration of β-glucuronidase from standard curves as originally described by Talalay et al. (54), and related it to total cell activity determined after lysis and solubilization of 10^7 PMN with 0.1% Triton X-100. After calculating percentage release (amount in supernatant divided by [total amount in supernatant + cell pellet] × 100), we calculated specific β-glucuronidase release by subtracting values for LDH release, a widely used, standard indicator of nonspecific loss of cytoplasmic contents. We determined LDH in cell lysates and supernatants spectrophotometrically as before (53) after incubation of samples with 30 mM pyruvate and 6.6 mM NADH in PBS (pH 7.0).

PMN fungicidal activity. An assay modified slightly from the procedure originally defined by Schaffner et al., referred to by its creators and in subsequent work by others as the "limiting dilution" method (55, 56), measures the ability of large numbers of PMN to sterilize microwells in plastic plates (Costar Corp., Cambridge, MA) containing minimal numbers of hyphae. We opsonized hyphae (one to three per well) with 50% pooled human serum after germination in situ in RPMI at 37°C, then washed, and added 2×10^5 PMN in HBSS to each well in a 100 μl final volume. At timed intervals, we stopped reactions by adding distilled water, then washed wells again with distilled water, overlaid them with modified Sabouraud's agar (2% dextrose, pH 7.0) and cultured 72 h at 25°C. Growth in wells in control plates were used to determine the hyphal inoculum. Controls were prepared and handled exactly as described above, except that distilled water was added to each well immediately after the PMN. With this technique, wells on control plates that remain sterile after culture for 72 h presumably indicate those that received no hyphae at the time when an estimated one to three organisms per well were originally dispensed. The number of culture positive control wells then corresponds to the initial inoculum. It is assumed that identical numbers of wells received no organisms on the experimental plates used for PMN incubations. Therefore, as in other microbicidal assays, the percentage killing is calculated from: (the inoculum, represented by the number of culture positive wells on control plates) minus (organisms surviving after incubation with PMN, represented by the number of wells on experimental plates) divided by (the inoculum, or numbers of culture-positive control wells) × 100. We also performed confirmatory assays in selected experimental conditions using a more cumbersome, time-consuming microcolony counting assay modified slightly from our previously published procedure (57). This involved allowing blastoconidia in RPMI (2×10^3 per microwell) to settle evenly and adhere to microwell surfaces, then germinate in RPMI to form hyphae, followed by opsonization, washing, and addition of PMN as above. After PMN lysis with distilled water at timed intervals and overlaying with cooled (45°C) Sabouraud's agar, we counted microcolonies developing around individual hyphae at 25°C after 6–8 h, and again at 18–24 h to verify that no further increment in colonies had occurred. When we determined percentages killed by comparison with controls as outlined above, we obtained closely parallel, highly correlated results in four experiments using both assays to measure effects of priming with each cytokine ($R^2 = 0.89$), and thus we routinely used the less time-intensive limiting dilution assay thereafter.

Statistical analysis. To normalize data presented as percentages, we transformed for analysis by means of the arcsine of the square root of the percentage in keeping with standard procedures recommended for use of parametric statistical analysis. Experimental groups were compared using two-tailed *t* tests for paired or unpaired samples where appropriate (58).

Results

Cytokine effects on the early PMN respiratory burst response. Depending upon the specific agonist and experimental circumstances, cytokine priming may enhance early respiratory burst responses by variable degrees (14, 17–22, 27). Therefore, we first defined the optimum conditions for priming of PMN O_2^- generation elicited by opsonized *C. albicans* hyphae. Under some conditions, cytokines by themselves may activate respiratory burst responses by adherent PMN without additional stimuli (14, 30). However, we primed and stimulated PMN in suspension as in the past (3–5, 39–42), a setting that facilitated PMN activation only after addition of hyphae. Our previous studies with unprimed PMN had established that hyphae elicited low absolute levels of O_2^- release when compared to respiratory burst responses evoked by standard, nonviable stimuli, including soluble FMLP and particulate opsonized zymosan (39–42). Nevertheless, responses to hyphae were in ranges reported for levels of respiratory burst activation by other types of microorganisms (59, 60) and were of sufficient magnitude to mediate fungicidal activity (3–5). Our previous studies defined ratios of hyphae to PMN supporting optimal respiratory burst stimulation (39). We noted small increments in this response after priming PMN by incubation at 37°C for 30 min with as little as 1–10 U/ml IFN or 0.1–1.0 U/ml TNF. However, the ability to reproduce uniform, maximal effects in every experiment required 60–90 min incubation with higher concentrations of cytokines. Using IFN for priming in five preliminary experiments, as little as 1–10 U/ml augmented responses in some cases, but 1,000 U/ml was the lowest IFN concentration to induce significant augmentation of O_2^- responses by PMN from all five donors. With two- to fourfold lower ratios of hyphae to PMN, baseline O_2^- responses fell and augmentation to 123–139% of unstimulated controls by 10–100 U/ml IFN became apparent. This is consistent with suggestions that optimum stimulus concentrations can reduce or eliminate evidence of PMN respiratory burst enhancement by IFN (17–19, 21, 27, 32). In contrast, priming with lower concentrations of TNF increased O_2^- responses to opsonized hyphae even with optimal conditions for PMN stimulation by hyphae (Table I). Mean maximum increments occurred after priming with 100 U/ml TNF. Therefore, we used 1,000 U of IFN and 100 U of TNF in all subsequent experiments to assure peak effects of cytokine priming. Though others have used lower cytokine concentrations to amplify PMN responses to various stimuli,

Table I. Dependence of Superoxide Responses to Candida Hyphae on TNF Concentration during Priming of PMN before Stimulation

TNF	O_2^- release	Percent release by control buffer-primed PMN
U/ml	*nmol/2 × 10^6 PMN per 10 min*	
0	5.29±0.65	100.0
0.1	5.92±0.82	111.9
1.0	6.17±0.49	116.6
10	7.22±0.25	136.4*
100	7.69±0.26	145.4
1000	7.06±0.37	133.4*

Mean ± SEM of 10 separate triplicate experiments determining responses after preincubation of PMN at 37°C for 90 min with TNF or buffer alone, followed by washing and stimulation with hyphae.
* $P < 0.05$.
$P < 0.01$.

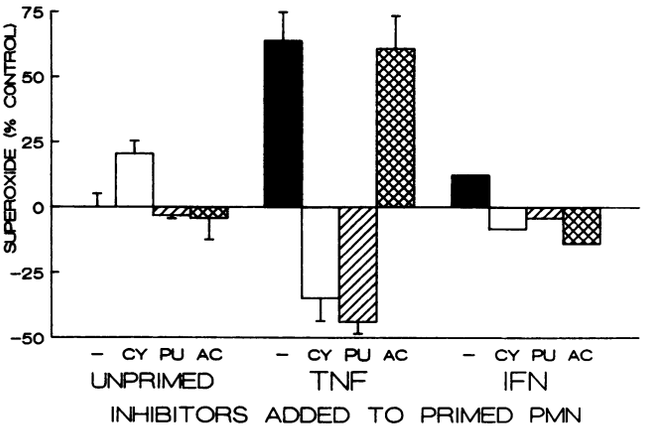

Figure 1. Effects of protein synthesis inhibitors on cytokine-induced augmentation of PMN superoxide responses to opsonized hyphae. Before stimulation, PMN were incubated for 90 min at 37°C with TNF, IFN, or buffer alone, with and without addition of one of the following: buffer alone (-), 20 μg/ml puromycin (*PU*), 50 μg/ml cycloheximide (*CY*), or 5 μg/ml actinomycin D (*AC*). Cells were washed free of cytokines and inhibitors before addition of hyphae. Bars and lines represent mean and SE of percentage positive or negative change compared to unprimed PMN incubated before stimulation for 90 min in buffer containing neither cytokines nor inhibitors. Values for IFN depict means of two separate experiments, each performed in triplicate (no error bars shown), while other data derived from three to five separate experiments.

numerous investigators have reported maximal PMN priming effects using IFN and TNF concentrations equivalent to those determined for our experimental system (11, 16, 18, 19, 20, 23, 61). Priming with either cytokine under these ideal conditions amplified the early respiratory burst response, as determined in 18 separate triplicate experiments. However, TNF consistently augmented effects more dramatically, to 138.6% of responses by unprimed control PMN ($P < 0.001$) compared to only 115.1% of controls with IFN ($P < 0.05$). Addition of IFN to TNF during priming did not further increase effects of the latter. Though cytokines may enhance adherence under some conditions, direct counts indicated that cytokine-induced increments in O_2^- release were not associated with increased numbers of PMN attaching to hyphae. Addition of progressively higher titers of corresponding specific anti-cytokine antibodies during priming caused dose-related inhibition of respiratory burst augmentation, but 0.5 μg/ml polymixin B had no effects and the factor causing enhancement was heat labile, making it unlikely that endotoxin contamination was responsible for the primed PMN responses. It also seemed doubtful that the 0–1.5% monocytes contaminating PMN preparations could have accounted for these cytokine effects. To confirm this definitively, we tested responses of cytokine-primed or unprimed monocytes. The mononuclear cell preparations used contained 31–38% monocytes, with lymphocytes comprising the balance. In each assay, we added sufficient numbers of cytokine-primed or unprimed mononuclear cells to provide monocyte concentrations corresponding to 5% of the PMN used in the experiments summarized above. When stimulated with hyphae, even these excess numbers of monocytes neither produced significant responses by themselves nor altered responses by PMN.

Recently, it has become evident that cytokines can alter gene expression or protein synthesis by human monocytes and even PMN, thereby inducing prolonged effects on oxidative microbicidal function (34, 35, 62, 63). We therefore performed preliminary experiments utilizing inhibitors of new cellular protein and DNA-dependent RNA synthesis. We first determined the maximum concentrations of these compounds that neither reduced responses by unprimed PMN nor altered hyphal–PMN interactions. The usable concentrations equaled or exceeded those previously shown by others to inhibit cytokine-induced protein synthesis (17, 21). As shown in Fig. 1, incubation with these maximum usable concentrations of inhibitors comparably reversed the small increment in PMN O_2^- generation attributable to IFN priming, though only effects of actinomycin D and cycloheximide were significant in each of two separate triplicate experiments. None of these inhibitors significantly reduced respiratory burst responses of unprimed PMN. More striking effects were noted when PMN were primed with TNF in the presence of either protein synthesis inhibitor, puromycin or cycloheximide. Stimulatory effects not only were ablated, but O_2^- release was diminished below levels obtained upon stimulation of unprimed control PMN. In contrast, actinomycin D had no significant effect on respiratory burst activation by TNF-primed PMN. When we added these inhibitors to IFN during priming, all reversed the small degree of augmentation of seen using these optimal conditions for hyphal stimulation of PMN. However, only effects of actinomycin D and cycloheximide, not puromycin, were significant in each of two triplicate experiments. These data suggested the possibility that these cytokines, particularly TNF, might induce sustained functional alterations in PMN, though perhaps mediated by different mechanisms.

Early events in the PMN activation response: membrane depolarization and cytosolic Ca^{++} rise. Since we could not attribute differences in O_2^- generation to augmented PMN adherence, we next examined other early events in PMN activation responses to identify alternate cytokine-specific changes in signal transduction. Fig. 2 depicts the effects of cytokine priming on PMN membrane depolarization after hyphal stimulation. While TNF markedly increased the rate of depolarization as determined by the initial increase in dye fluorescence, effects of IFN were minimal and insignificant ($P > 0.2$ by paired sample Student t test comparing rates in centimeters per minute). Subsequent destruction of the fluorescent probe, a quantitative indicator of early PMN oxidant release (39), was also increased to

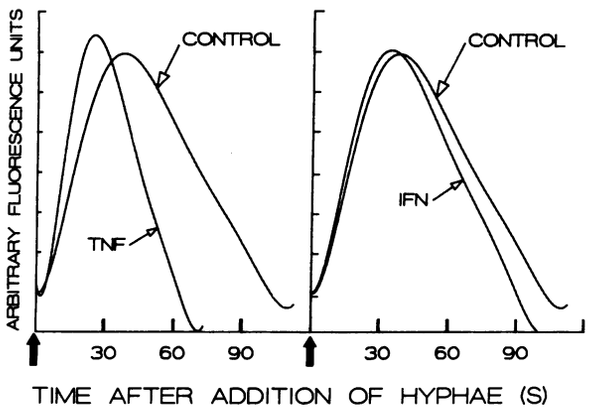

Figure 2. Effects of cytokine-priming on the time course of changes in PMN transmembrane potential elicited by opsonized hyphae. PMN were primed and preloaded with diS-$C_3(5)$ at 37°C before stimulation at zero time (*solid arrows*). The initial slope in increasing fluorescence reflects the depolarization rate; the later decreasing fluorescence indicates dye destruction attributable to PMN oxidant release (42). Tracings are from a single experiment that is representative of three separate determinations.

a greater degree by TNF and IFN. Compared to unprimed control PMN, priming with TNF and IFN respectively induced 168.6% vs. 123.3% increases in oxidant release. These data confirmed the relative effects of these cytokines on early respiratory burst responses noted above using direct assays of O_2^- release.

We then evaluated cytokine effects on the early, transient rise in free cytosolic calcium ion concentration $[Ca^{++}]_i$, since this ion flux is postulated to act as a "second messenger" in the PMN activation response (39–44). As shown in Fig. 3, both TNF and IFN increased the rate as well as magnitude of $[Ca^{++}]_i$ after hyphal stimulation.

Our previous studies had indicated that hyphal stimulation of unprimed PMN differed from effects elicited by the soluble agonist, FMLP. The latter is thought to proceed totally via pathways requiring both the $[Ca^{++}]_i$ and PT-sensitive guanine

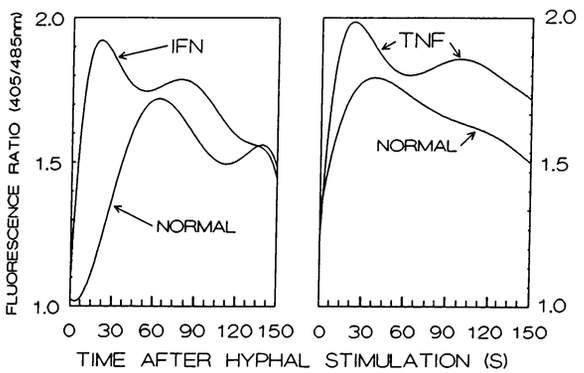

Figure 3. Effects of cytokine priming on the time course of changes in PMN cytosolic Ca^{++} elicited by opsonized hyphae. Primed or unprimed PMN were loaded with 1 μg/ml indo-1/AM before stimulation, and change in the ratio of fluorescence emitted at 405/485 nm was recorded continuously, after addition of hyphae at time zero. Tracings are from a single experiment that is representative of three and five separate determinations for IFN and TNF-primed PMN, respectively.

nucleotide-binding regulatory proteins (40, 42). Accordingly, we examined the Ca^{++} dependency and PT-susceptibility of cytokine-induced enhancement of respiratory burst activation by opsonized hyphae.

As in our previous studies (40), PT-treatment of unprimed PMN completely eliminated the $[Ca^{++}]_i$ after stimulation by either FMLP or opsonized hyphae. Whereas this likewise totally ablated the O_2^- response to FMLP, the hyphae nevertheless evoked nearly half the magnitude of respiratory bursts manifested by comparably stimulated, untreated PMN. IFN priming of PT-treated PMN augmented the $[Ca^{++}]_i$ only minimally (data not shown). However, effects of TNF on such cells differed qualitatively.

When stimulated by opsonized hyphae, TNF-primed, PT-treated PMN exhibited $[Ca^{++}]_i$ rises approaching patterns manifested by similarly stimulated unprimed, untreated PMN (Fig. 4). To further delineate the separate necessity for a $[Ca^{++}]_i$ in cytokine-mediated respiratory burst augmentation, we also loaded PMN with the nonfluorescent organic Ca^{++} chelator, BAPTA. These conditions totally eliminated the $[Ca^{++}]_i$ after stimulation by FMLP or hyphae, and addition of EGTA in some experiments further modified the $[Ca^{++}]_i$ by eradicating the late effects of extracellular Ca^{++} influx (42). Separate or combined PT treatment with or without intracellular and/or extracellular Ca^{++} chelation variably inhibited respiratory burst responses of unprimed PMN to opsonized hyphae (Fig. 5), as in our previous studies (42). These different treatments also variably affected the small augmentation of PMN O_2^- responses after IFN priming. In contrast, none of these treatments, separately or in combination, had major effects on the absolute amount of respiratory burst augmentation resulting from TNF priming. Even though PT treatment combined with intracellular and extracellular Ca^{++} chelation almost totally eliminated O_2^- generation by unprimed control PMN, augmentation of respiratory burst responses by TNF priming remained significant under these conditions ($P < 0.01$). In fact, TNF-priming induced remarkably consistent absolute increments in the burst, ranging from 1.6 to 2.4 nmol $O_2^-/2 \times 10^6$ PMN per 10 min. Thus, augmentation by TNF occurred regardless of the presence or absence of PT treatment or chelation, and the degree to which these various additives inhibited responses to hyphae by unprimed PMN (Fig. 5). This suggested independence of TNF effects on O_2^- generation from both pertussis

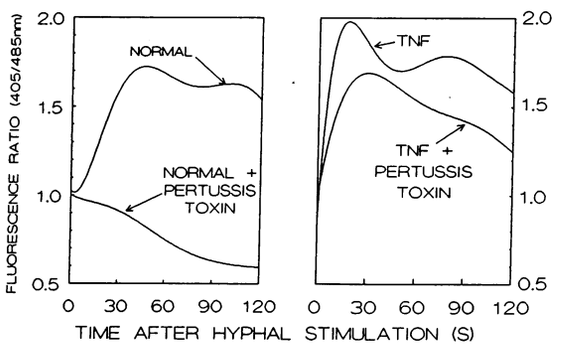

Figure 4. Effects of TNF-priming on the cytosolic Ca^{++} response to opsonized hyphae by PT-treated PMN which had been loaded with indo-1. Tracings were drawn from calculated mean values in two separate experiments which continuously recorded changes in ratios of fluorescence emitted at 405/485 nm upon addition of hyphae at time zero.

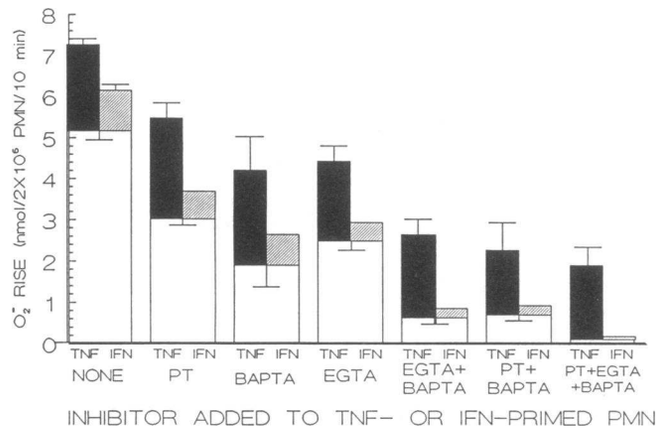

Figure 5. Effects of *PT* treatment (*PT*) and/or Ca^{++} chelation (intracellular with BAPTA, extracellular with EDTA) on augmentation of PMN superoxide responses to opsonized hyphae. Wide, open bars (and downward lines) represent mean (and SE) superoxide release by unprimed PMN under each set of conditions; stacked solid and hatched bars (and upward lines) depict mean (and SE) augmentation of responses attributable to priming with TNF or IFN, respectively. Data depicted represent three to eight separate experiments, each performed in triplicate, except for values shown for effects of PT and chelators on IFN-primed PMN, which derive from two separate experiments, each performed in triplicate (error bars not shown).

toxin-sensitive guanine nucleotide regulatory proteins and the early rise in $[Ca^{++}]_i$.

Effects on fungicidal mechanisms. Considering that there were several differences in cytokine effects on initiation of PMN activation, we next examined alterations in killing of hyphae by PMN. Based on our previous studies, this process requires early azurophil degranulation in addition to a sustained respiratory burst, but proceeds relatively slowly, achieving maximum results only after 30–60 min (3–5). Therefore, we next determined the effects of cytokine priming of PMN on killing of hyphae after 60 min incubation in 13 separate triplicate experiments. Priming of PMN with IFN slightly but significantly augmented killing of opsonized hyphae, as mean killing increased to 119.5±2.1% of values for unprimed controls (to 63.8% with TNF vs. 53.4% in unprimed controls, $P < 0.001$). In surprising contrast, TNF-treated PMN evidenced impaired maximum fungicidal activity compared to both unprimed and IFN-exposed cells. Only 33.4% of hyphal inocula were killed by TNF-primed PMN, a 37.4±5.1% reduction compared to killing by unprimed control PMN ($P < 0.00001$). Moreover, addition of IFN to TNF during the priming process did not totally correct this apparent TNF-induced defect, inasmuch as killing of hyphae remained 27.8% below fungicidal responses by unprimed control PMN in three separate triplicate experiments ($P < 0.01$).

Since these results of TNF priming on PMN fungicidal responses were unexpected, we sought to determine whether differential cytokine effects were limited to activity against a single isolate. Effects of cytokine priming on PMN responses to hyphae from three other *C. albicans* clinical isolates paralleled those noted with our standard isolate in three to five separate triplicate experiments. In particular, with two of the three additional isolates, TNF priming of PMN reduced killing even more dramatically than with standard isolate hyphae (14.3% and 25.6% more inhibition). Killing of the third isolate was altered insignificantly by TNF priming of PMN (4.2% above killing of these hyphae by unprimed PMN). In any case, TNF priming appeared to have generalized effects on PMN antihyphal fungicidal activity that diverged from the consistent augmentation of early PMN respiratory burst responses by this cytokine, and also from the ability of other cytokines such as IFN to improve the efficiency of hyphal killing by PMN.

Since our assay system utilized extremely high ratios of leukocytes to hyphae, it seemed possible that these disparate cytokine effects might have been due to small numbers of contaminating monocytes. Therefore, we tested sufficient numbers of cytokine-primed or unprimed mononuclear cells to exceed any levels that might conceivably have contaminated our PMN preparations. In four separate experiments, each microwell contained $\sim 3 \times 10^4$ total cytokine-primed or unprimed mononuclear cells. In each assay, we adjusted total cells added so as to deliver 1×10^4 monocytes, the number equivalent to 5% of the PMN used to obtain the data noted above. At most, monocyte priming had only minimal effects on hyphal killing in every case. For example, killing by TNF-primed monocytes was marginal, 12.4±2.0% compared with 9.2±3.1% by unprimed control monocytes and 90.5±2.8% by PMN from these same 4 donors. In contrast, TNF-priming of these PMN reduced killing by 20.6±7.2%, an effect unchanged by supplementation with 5% additional monocytes, as killing remained diminished by 19.5±5.6%. Similarly, when corresponding numbers of TNF-primed monocytes were added to unprimed PMN, killing rose insignificantly, by 6.0±4.3%. IFN-priming of monocytes had no significant effects under these conditions. Since these data reflected effects of more than threefold the numbers of monocytes present in any of the PMN preparations used in our experiments, we could attribute contrasting effects on fungicidal responses to cytokine-priming effects on PMN. These data do not address the reasonable possibility that monocytes, lymphocytes, or other cell types still may be intermediaries or indirectly contribute to the PMN priming process.

Inhibitors of protein synthesis further reduced hyphal killing by TNF-primed PMN comparably to effects on the early respiratory burst response noted above and in Fig. 1. Incubation with 20 μg/ml puromycin further inhibited killing by 58.8% (mean of two separate experiments each performed in triplicate, each $P < 0.05$ compared to activity of TNF-primed PMN without puromycin). Similarly, 50 μg/ml cycloheximide reduced hyphal killing by 47.9% compared to activity of TNF-primed PMN in the absence of that inhibitor. These data suggest that TNF priming might induce distinct, potentially persisting alterations in PMN fungicidal responses. Our data cannot address the analogous potential for persistent IFN-priming effects since these inhibitors had far less striking, insignificant effects on killing by IFN-primed or unprimed PMN.

Because PMN killing of hyphae occurred slowly, cytokines might differentially affect either initiation or sustaining of responses required for optimal fungicidal activity. Therefore, we next compared the time course of killing by cytokine-primed and unprimed PMN. Though TNF-treated PMN exhibited active early fungicidal effects, responses did not seem to be sustained as well as those by unprimed or IFN-exposed cells (Fig. 6). These findings raised the possibility that disparate effects of cytokines might relate primarily to alterations in the relative abilities of PMN to sustain responses rather than to the magnitude of initial stimulatory effects on PMN activation.

We next examined possible alternative explanations for these observed disparities between effects of the cytokines on

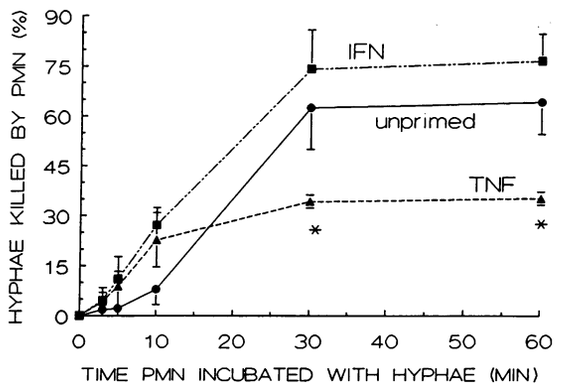

Figure 6. Effects of priming PMN with IFN (■), TNF (▲), or buffer alone (unprimed, ●) on time course of hyphal killing determined by limiting dilution assay. TNF-associated reduction was significant at 30 and 60 min (* indicates $P < 0.05$ compared with unprimed control or IFN-treated PMN in three separate experiments, each performed in triplicate).

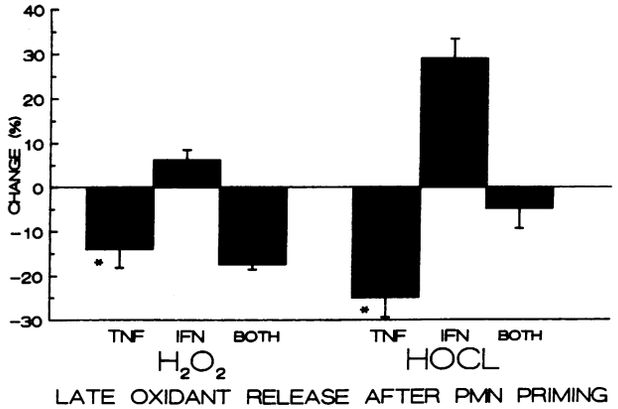

Figure 7. Effects of cytokine priming on stimulation by hyphae of PMN oxidant release: H_2O_2 (after 30 min) and HOCl (after 60 min) in three and five separate experiments respectively, each performed in triplicate (* indicates $P < 0.05$ comparing effects of TNF priming with unprimed controls and $P < 0.01$ with effects of IFN priming). HOCl production by PMN primed with both IFN and TNF was below levels released by cells primed with IFN alone ($P < 0.05$) but not significantly less than by unprimed PMN.

initiation of the PMN respiratory burst and subsequent fungicidal responses. Our prior studies of hyphal clearance from endothelial cell monolayers had shown remarkably localized delivery of fungicidal, potentially cytotoxic PMN products, supporting specific microbicidal activity without concomitant damage of host cells (64). Given the early, more pronounced respiratory burst response after TNF priming, it appeared possible that nonspecifically directed oxidant release might decrease fungicidal efficiency and/or damage effector cells themselves. However, both trypan blue staining and LDH release indicated that viability of PMN remained 97.0–98.5% after incubation with hyphae irrespective of priming. Moreover, cytokine-priming did not alter microscopic patterns of oxidant generation, as judged by both staining of PMN either for oxidant-induced local precipitation of NBT-formazan (48) and observation of PMN loaded with nonfluorescent DCFH for discrete areas of fluorescence due to oxidant-dependent conversion to DCF (47). Since optimum hyphal killing requires azurophil granule contents (3–5), disparate cytokine effects on the burst and early azurophil degranulation responses provided another potential explanation for the unexpectedly observed differences in fungicidal responses. As in our previous studies (4, 53), hyphae induced low but significant levels of specific release of the azurophil granule marker, β-glucuronidase, in ranges comparable to typically reported responses to particulate stimuli by PMN in the absence of cytochalasin B. Neither priming with TNF nor IFN affected this response, as specific β-glucuronidase release by such PMN was nearly identical (6.4% and 6.3%, respectively, mean of four separate experiments, each performed in triplicate).

Since the above data suggested differences in the mechanisms of action of TNF and IFN on initiation of PMN responses, it appeared possible that they also have disparate effects on the regulation of ongoing, late release of oxidants presumably required for optimum fungicidal activity. This proved to be the case, as PMN primed with TNF generated significantly less of the potentially critical oxidant products, H_2O_2 and HOCl, than did IFN-primed cells (Fig. 7). After incubation with hyphae, 2.5×10^6 unprimed PMN released means of 18.8 nmol H_2O_2/30 min and 37.9 nmol HOCl (chloramines produced)/h. IFN increased H_2O_2 and HOCl respectively to 24.3 and 48.8 nmol, in contrast to only 15.9 and 28.5 nmol with TNF ($P < 0.01$). Concomitant priming with both TNF and IFN did not correct this defect in H_2O_2 production. While HOCl generation increased only to levels approaching those by unprimed PMN, it remained below responses by PMN primed with IFN alone ($P < 0.05$). Neither mannitol (40 nM) nor DMSO (14 mM) specifically affected hyphal killing by primed PMN. However, DTP (10 mM), which prevents accumulation of chloramines (65), induced 32.5% inhibition of killing by IFN-primed PMN ($P < 0.05$), but reduced fungicidal activity by TNF-primed PMN insignificantly, only by 10.4% ($P > 0.10$). Use of scavengers and inhibitors was limited by the necessity to eliminate concentrations of agents which altered viability or function of PMN or fungi, as in our previous studies (5, 6).

Discussion

PMN constitute the major defense against disseminated candidiasis by virtue of their capacity to kill not only small *Candida* blastoconidia, but also large pseudohyphae and hyphae that are characteristically present within lesions (1–6). Thus, cytokine effects on responses by these host cells are of potentially critical significance in this infection. We noted here that cytokines primed PMN for an augmented early PMN generation of oxidants in response to stimulation by *C. albicans* hyphae. By themselves, these data are comparable to findings previously reported by several investigators measuring cytokine effects on PMN responses to a variety of standard agonists, including soluble FMLP or phorbol esters, as well as particulate opsonized zymosan, heterologous erythrocytes, or *Candida* blastoconidia (14–23, 25). In our studies, we could not attribute enhancement of functional responses to hyphal stimulation to the presence of low levels of contaminating lipopolysaccharides (LPS), which were below levels of detectability by the limulus lysate assay. Effects were reversed by anti–cytokine antibodies or heating but not polymixin B, which blocks LPS but not cytokine priming (16). Moreover, TNF activity was largely Ca^{++} independent, in contrast to requirements for LPS modification of PMN function (66). Because our past work had

established the necessity for PMN oxidant generation in mediation of hyphal killing (3–5), we expected that increased early respiratory burst responses would augment fungicidal activity concomitantly. Instead, we observed a striking disparity in cytokine effects on these PMN functions. IFN-priming did induce a predicted, albeit small, parallel enhancement of both early respiratory burst activation and fungicidal activity. However, TNF reduced hyphal killing far below levels achieved by unprimed PMN, despite marked amplification of early oxidant responses to hyphal stimulation. Controls eliminated the possibility that cytokine effects on contaminating monocytes contributed significantly to these results.

Though multiple published reports confirm the abilities of both TNF and IFN to augment PMN respiratory burst responses to a variety of stimuli, important differences are apparent in effects of these cytokines on other functional parameters as well as in their mechanisms of action (12, 13, 18, 22). Given the differing requirements for effective microbicidal responses against disparate microorganisms (7–9), it is not surprising that effects of a particular cytokine on PMN killing reportedly have varied depending upon the target and experimental conditions (23–25, 27, 31, 35). Our previous data likewise indicate that initiation of PMN activation may differ depending upon the stimulus, as patterns of early responses may vary if evoked by opsonized or unopsonized hyphae, or by other agonists (39–42). Thus, though particular cytokines presumably mediate effects on PMN by defined mechanisms (11–38, 61–63), specific stimuli might provoke divergent sequences and magnitudes of functional responses, so that diverse outcomes ultimately may transpire. In any case, our data now suggest that there are important differences in the mechanisms by which TNF and IFN prime PMN for functional responses to *Candida* hyphae. For example, we noted that priming of PMN with TNF, as opposed to IFN, resulted in more rapid and pronounced PMN membrane depolarization and enhanced cytosolic Ca^{++} rise that was relatively resistant to PT. These latter, early cellular events were associated with a large increment in the initial respiratory burst response to hyphae. This augmented primary phase of oxidant release evoked by hyphae appeared to be independent of Ca^{++} and insensitive to pertussis toxin. Though TNF effects on HL-60 promyelocytic cells have been associated with a pertussis toxin-sensitive GTP-binding protein (67), data reported by Berkow and Dodson (61) established that TNF priming of mature human PMN can occur without participation of PT-sensitive guanine nucleotide regulatory proteins (61). This toxin had no effects on O_2^- responses of primed or unprimed PMN to phorbol myristate acetate, a direct activator of protein kinase C. However, respiratory burst and other responses by both primed and unprimed PMN were almost totally ablated upon stimulation with FMLP (61), an agonist known to activate PMN entirely via receptors linked to PT-sensitive GTP-binding proteins. In contrast, respiratory burst responses to *Candida* hyphae by unprimed PMN appeared to be only partially dependent upon pertussis toxin-sensitive guanine nucleotide regulatory proteins (40). Thus, agonists that can activate PMN respiratory bursts via PT-insensitive mechanisms appear to reveal the potential independence of at least some TNF-priming effects from PT-inhibitible guanine nucleotide regulatory proteins. However, an active respiratory burst response is not the sole requirement for killing of *C. albicans* hyphae by PMN, as degranulation appears to be necessary as well (3–5). While our own as well as previously published data suggest that Ca^{++} mobilization is not required for augmentation of O_2^- release by TNF (68), its effects on degranulation have been noted to be at least Ca^{++} dependent (13, 15). Cytokine effects on ion fluxes might then affect coordination of the responses comprising fungicidal activity.

We also noted somewhat unexpected effects of protein synthesis inhibitors on TNF-dependent alterations in PMN responses to hyphae. In previously published experiments delineating mechanisms by which cytokines augmented antibody-dependent cell-mediated cytotoxicity against mammalian target cells, TNF-priming had immediate but short-lived effects. In contrast, IFN effects were far more prolonged, though required several hours for induction of high-affinity receptors for monomeric IgG-Fc (18). Our preliminary studies using structurally unrelated inhibitors of protein synthesis during briefer periods of priming raise the possibility that TNF-mediated alterations in PMN responses to opsonized *C. albicans* hyphae likewise may require new protein synthesis. Since cytokines appear to affect several potentially pivotal steps in the PMN activation sequence, it will be important to determine definitively in future experiments to what degree our observations are specific primarily for the stimulus, the priming cytokine, or for particular interactions between both of these variables in combination.

Augmentation of PMN responses by cytokines need not necessarily have beneficial consequences, as evidenced by experimental models causally linking TNF to localized, inflammatory tissue damage in vivo (29, 37). Among its effects on PMN and other cells, TNF has been reported to induce synthesis and release of platelet activating factor and augmented, post-stimulation production of leukotriene B_4 (69, 70), both with the potential to mediate nonspecific inflammation. Our own previous studies indicated that unprimed PMN could clear *Candida* hyphae from infected endothelial cell monolayers with high specificity, as organisms were killed while immediately adjacent endothelial cells were preserved, apparently undamaged despite the vigorous inflammatory response (64). It therefore appeared possible that TNF-priming might foster enhanced, but perhaps less specifically localized release of toxic PMN products, possibly having even the potential to damage effector cells themselves. However, we noted neither loss of viability following stimulation of TNF-primed cells nor alteration in apparent patterns of localization of respiratory burst oxidant production.

The time course of cytokine effects on PMN fungicidal activity was noteworthy in our experiments. Though cytokine-priming augmented early respiratory burst responses, IFN and TNF induced disparate alterations in late generation of potentially antimicrobial oxidants, H_2O_2 and HOCl, changes that paralleled cytokine effects on the relatively slow process of hyphal killing. Apparent initial differences in effects of TNF and IFN priming on PMN signaling events occurring early after hyphal stimulation may then relate to subsequent modulation or termination of respiratory burst responses (71, 72). In contrast to oxidant production, PMN degranulation was unaffected by cytokines. In view of the necessity for oxidants in hyphal killing established in our previous work (3–5), our observed differences in H_2O_2 and HOCl generation after cytokine priming may represent the primary basis for altered fungicidal activity, though evidence that this is the case remains incomplete. Regulation of the respiratory burst as well as activity of its potentially microbicidal products is highly complex (7–9). Moreover, even with unprimed PMN, only indirect evidence by ourselves and others supports previously published identifi-

cations of specific oxidative products as putative mediators of hyphal killing (5, 73). By itself, H_2O_2 is unlikely to accumulate in sufficient concentrations to be fungicidal, though it can cross biological membranes, is produced both in vitro and in vivo, and can interact with other leukocyte products to produce more potent end-products. For instance, with myeloperoxidase release from PMN granules, chlorinating agents such as HOCl and chloramines are produced (51, 65, 73–75). These account for 28% or more of the oxygen consumed after stimulation (74), and have been suggested to mediate PMN killing of filamentous *Candida* forms (73). Several factors may influence microbicidal efficacy of these compounds. For instance, HOCl rapidly reacts with amines to form a complex mixture of nitrogen-chlorine derivatives which remain stable for hours, but the products formed and their relative activities depend upon reactive compounds in the milieu (8, 51, 75). Formation of monochloramines (such as taurine chloramine) which do not penetrate biological membranes may actually protect cells against toxic damage by more permeant, lipophilic products, and factors such as local pH or presence of other reactive substances may alter rates of production and degradation (65, 73, 75). In addition, cytotoxic and antibacterial efficacy of products of this system depends upon availability and accessibility of susceptible sites in target organisms or sensitivity to indirect deprivation of crucial nutrient sources (8, 51, 65, 75). The bases for fungicidal effects of these substances so far is undetermined, though the slow rate of hyphal death is incompatible with the almost immediate lethal effects of HOCl itself (51, 65, 75). Besides the well-known incomplete specificity of available scavengers, optimum concentrations of most such compounds appear to have at least some effects either on fungal viability or surface features involved in leukocyte activation. Thus, extensive additional data will be required to verify the specific PMN products mediating hyphal killing by both normal and cytokine-primed PMN.

Nevertheless, our data suggest that at least for responses to hyphae, IFN and TNF can differentially affect initiation and subsequent sustaining of processes mediating PMN microbicidal activity. Other investigators have documented that priming of PMN with either TNF or IFN can similarly inhibit growth (25) or enhance killing (18, 23) of *C. albicans* blastoconidia. However, our previous data had indicated that PMN responses elicited by hyphae may differ from those elicited by yeastlike forms (39–42). Our results reported here suggest that these disparities between PMN responses to hyphae and blastoconidia may extend to effects of cytokines as well. Since hyphae are characteristically present within invasive lesions of candidiasis, the relative effects of different cytokines may prove to be critically important as potentially opposing local modulators of host defenses against disseminated candidiasis.

Acknowledgments

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