

# Lactoferrin Release and Interleukin-1, Interleukin-6, and Tumor Necrosis Factor Production by Human Polymorphonuclear Cells Stimulated by Various Lipopolysaccharides: Relationship to Growth Inhibition of *Candida albicans*

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Lipopolysaccharides (LPSs) from *Escherichia coli*, *Serratia marcescens*, and *Salmonella typhimurium*, at doses from 1 to 100 ng/ml, strongly enhanced growth inhibition of *Candida albicans* by human polymorphonuclear leukocytes (PMN) in vitro. Flow cytometry analysis demonstrated that LPS markedly augmented phagocytosis of *Candida* cells by increasing the number of yeasts ingested per neutrophil as well as the number of neutrophils capable of ingesting fungal cells. LPS activation caused augmented release of lactoferrin, an iron-binding protein which itself could inhibit the growth of *C. albicans* in vitro. Antibodies against lactoferrin effectively and specifically reduced the anti-*C. albicans* activity of both LPS-stimulated and unstimulated PMN. Northern (RNA blot) analysis showed enhanced production of mRNAs for interleukin-1  $\beta$ , tumor necrosis factor alpha, and interleukin-6 and in neutrophils within 1 h of stimulation with LPS. The cytokines were also detected in the supernatant of the activated PMN, and their synthesis was prevented by pretreatment of LPS-stimulated PMN with protein synthesis inhibitors, such as emetine and cycloheximide. These inhibitors, however, did not block either lactoferrin release or the anti-*Candida* activity of LPS-stimulated PMN. These results demonstrate the ability of various bacterial LPSs to augment neutrophil function against *C. albicans* and suggest that the release of a candidastatic, iron-binding protein, lactoferrin, may contribute to the antifungal effect of PMN. Moreover, the ability to produce cytokines upon stimulation by ubiquitous microbial products such as the endotoxins points to an extraphagocytic, immunomodulatory role of PMN during infection.

Lipopolysaccharides (LPS; endotoxins) are among the most powerful bacterial products, endowed with proinflammatory and immunomodulatory properties (31, 39). In particular, LPS induce a variety of responses in monocytes and polymorphonuclear cells (PMN; neutrophils) both in vitro and in vivo (5, 8, 22), of which some may be related to the mechanism of endotoxic shock, a severe clinical entity (29, 43), as well as to other manifestations of LPS activity in the pathophysiology of infections (31, 46). Among the neutrophil responses primed by endotoxin are the enhanced expression of adherence-related membrane glycoproteins (such as the CD11-CD18 family [24, 38]) and secretion of oxygen metabolites, inflammatory factors, hydrolases, and lactoferrin (18, 20, 21, 25). Increases in microbial killing and migration are also commonly observed in LPS-stimulated PMN (6, 7), and augmented production of platelet activation factor and leukotrienes has been reported (14, 44).

Although neutrophils have long been considered terminally differentiated cells incapable of synthesizing or secreting proteins, recent but conclusive data have shown that these phagocytic cells are able to synthesize and secrete interleukin-1 (IL-1) and tumor necrosis factor (TNF) (12, 15, 26, 28). These cytokines have multiple effects on neutrophil functions, including increased migration, priming for en-

hanced oxidative metabolism, and sustained phagocytosis and killing of fungal cells (9, 16, 36, 37).

In view of these observations, and because of our interest in the mechanisms of immune response to the dimorphic opportunistic fungus *Candida albicans*, we have investigated here the effect of LPS on the anti-*Candida* activity of neutrophils from normal human donors, with the aim of understanding the mechanisms underlying LPS activation of PMN candidastatic and/or candidacidal activity. Three different bacterial sources of LPS were used to confirm their uniform stimulating effect on neutrophil function. In addition, we investigated whether this stimulation could be correlated with the ability of PMN to produce cytokines and secrete lactoferrin, an iron-binding antimicrobial protein (23, 32, 35).

## MATERIALS AND METHODS

**Preparation of human PMN.** Leukocyte buffy coats, obtained from normal volunteers at the South West Florida blood bank (Tampa, Fla.), were diluted 1:2 in phosphate-buffered saline (PBS), and 35 to 40 ml was layered on 10 ml of Ficoll-Hypaque solution (Pharmacia Fine Chemicals, Piscataway, N.J.). After centrifugation for 30 min at 400  $\times$  g at room temperature, the leukocyte layer lying on the surface of the erythrocyte pellet was collected, and the erythrocytes were lysed by hypotonic shock with sterile distilled water (30 s at room temperature). The cells were washed twice in

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PBS before being readjusted to the desired cell density. Cyto-centrifuged preparations of PMN stained with Giemsa were found, by morphology, to contain greater than 99% PMN.

**Bacterial LPS and other reagents.** Chromatographically purified LPS from *Escherichia coli* (serotype O111:B4), *Salmonella typhimurium*, and *Serratia marcescens* were purchased from Sigma Chemical Co., St. Louis, Mo. (catalog no. L4391, L5143, and L4766, respectively). According to the manufacturer's specifications, LPS were obtained by phenolic extraction of whole bacterial cells, followed by gel filtration and sterilization with gamma rays. Lactoferrin from human milk (approx. 90% pure, as determined by sodium dodecyl sulfate [SDS] gel electrophoresis), human transferrin (approx. 98% pure), rabbit anti-human lactoferrin antibodies, goat anti-human transferrin antibodies, emetine, and cycloheximide were also obtained from Sigma Chemical Co. Unless otherwise specified, the LPS used was that from *E. coli*.

**Culture of *C. albicans*.** The *C. albicans* strain used in this study was a clinical isolate from a patient with chronic mucocutaneous candidiasis and was identified by the taxonomic criteria established by Lodder (27). Stock cultures were maintained by weekly transfers onto fresh Sabouraud agar plates and incubation at 28°C.

**Assay for neutrophil anti-*Candida* activity.** Anti-*Candida* activity was assayed by a rapid radiolabel microassay to measure the growth inhibition of *C. albicans* by PMN in vitro (11). The assay determines both candidastatic activity and candidacidal activity and is equally effective in monitoring yeast and hyphal growth inhibition (10; unpublished data). Briefly, neutrophils were diluted to  $6 \times 10^5$ ,  $3 \times 10^5$ , and  $1.5 \times 10^5$  per ml in RPMI 1640 containing 2% heat-inactivated (65°C, 30 min) fetal bovine serum (FBS) with 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 2 mM L-glutamine, 100 U of penicillin per ml, and 100 µg of streptomycin per ml (FBS medium), and 50 µl of each dilution was added to triplicate wells of a 96-well flat-bottomed microplate. For activation of PMN, each kind of LPS was serially diluted and added to neutrophils in the wells at a final concentration of 1 to 1,000 ng/ml. The cells were incubated for 30 min at 37°C before 500 cells of *C. albicans*, resuspended in 25 µl of FBS medium, were added to each well. *C. albicans* was also added to empty wells to serve as controls. The cell mixtures were incubated at 37°C for 18 to 20 h before 50 µl of sterile water containing 10 µCi of [<sup>3</sup>H]glucose (glucose D-5; 6-<sup>3</sup>H; specific activity, 66.9 Ci/mmol; DuPont de Nemours & Co. NEN Products, Boston, Mass.) per ml was added. After an additional 3 h of incubation at 37°C, the proliferating fungi that had incorporated the radiolabel were harvested by first adding 50 µl of bleach to all wells and then processing through a harvester (Skatron, Oslo, Norway) with distilled water onto glass fiber filters. The incorporated label was counted on a beta counter. The mean of the triplicate wells was determined, and the standard error of the mean (SEM) was usually within 5% of the mean. The percent growth inhibition of *C. albicans* was calculated as follows: % growth inhibition = [(cpm with *Candida* cells alone - cpm with effector + *Candida* cells)/cpm with *Candida* cells alone] × 100. In some cases, the growth inhibition at the various effector-to-target cell (E:T) ratios was used to calculate inhibition units (IU), 1 IU being defined as 20% growth inhibition of 500 yeast cells. The data were then expressed as IU per 10<sup>7</sup> effector cells.

**Inhibition of *Candida* growth by lactoferrin and its reversal by antilactoferrin antibodies.** Human lactoferrin was diluted

in FBS medium (100 to 1 µg/ml). Then, 50 µl of each dilution was added to triplicate wells of a 96-well flat-bottomed microplate, after which 50 µl of FBS medium containing 500 *C. albicans* was added. Other wells received transferrin (100 µg/ml) instead of lactoferrin or anti-human lactoferrin or antitransferrin serum, at various dilutions, before the addition of fungal cells. *Candida* cells were also added to triplicate wells with FBS medium only to serve as a control. The plate was then incubated at 37°C for 18 to 20 h before being labeled with [<sup>3</sup>H]glucose, as described above.

**Inhibition of neutrophil anti-*Candida* activity by anti-human lactoferrin antibodies.** Neutrophils in FBS medium were stimulated with 100 ng of LPS per ml. Anti-human lactoferrin, diluted to a final concentration of 1:1,000, was added to the wells and incubated for 30 min before *Candida* cells were added to the mixture. The neutrophil/*Candida* cell mixtures were then incubated for a further 18 to 20 h at 37°C before labeling with [<sup>3</sup>H]glucose, as described above.

**FITC labeling of *C. albicans*.** Heat-killed *C. albicans* cells ( $2 \times 10^8$ /ml) were labeled with 1 mg of fluorescein isothiocyanate (FITC; Sigma Chemical Co.) per ml in 50 mM sodium carbonate-100 mM sodium chloride solution, pH 9.2, at room temperature for 1 h. This procedure was modified from one used for mycobacteria (2). Unconjugated FITC was removed by washing the cells five times in PBS. More than 99% of the cells were made fluorescent by this procedure, as checked microscopically under the fluorescence microscope.

**Phagocytosis.** To measure the phagocytic activity of neutrophils, a flow cytometric assay based on the uptake of FITC-labeled *C. albicans* was used as modified from a previously established procedure to quantitate fluorescent-particle phagocytosis by monocytes (42). Briefly, 10<sup>6</sup> neutrophils in a final volume of 150 µl of FBS medium were untreated or treated with 100 ng of LPS per ml for 30 min at 37°C. Then, 20 µl of FITC-labeled *C. albicans* at  $5 \times 10^8$ /ml was added to each sample, and the mixture was vortexed and incubated for 30 min at 37°C. Finally, 1 ml of warm PBS was added to each tube before the fluorescence of ingested yeasts within neutrophils was read with a FACScan (Becton Dickinson, Mountain View, Calif.). The yeast-PMN mixture was gated to exclude free FITC-labeled *C. albicans* from the measurement. A solution of trypan blue (1%, wt/vol) was added to the samples in order to quench the fluorescence of possible *C. albicans* yeasts adhered to the outside of the PMN. It was found that 20 µl of 1% trypan blue was optimal for quenching the fluorescence of FITC-labeled *C. albicans* alone in 1 ml of FBS medium. Others have used crystal violet for FITC quenching, but trypan blue proved to be at least equally effective (3). Data were analyzed in terms of light scatter and fluorescence intensity so as to distinguish PMN with internalized fluorescent cells from nonphagocytizing PMN, as well as the degree of phagocytosis per single PMN, as indicated by the average fluorescence intensity. An aliquot of the same cell samples was cyto-centrifuged and stained with Giemsa to check visually for phagocytosis under the light microscope.

**Detection of lactoferrin in supernatants of PMN.** For release of lactoferrin,  $3 \times 10^6$  PMN per ml were cultured in FBS medium with or without 100 ng of various LPS per ml for 1, 3, 6, and 24 h. The supernatants were collected and spun free of cells and debris. Lactoferrin levels were determined by a previously established sandwich enzyme-linked immunosorbent assay (ELISA) (1). Briefly, flat-bottomed, 96-well microtiter plates were coated overnight with rabbit anti-human lactoferrin serum diluted 1:400 in bicarbonate

buffer (pH 9.6). The plates were washed with PBS supplemented with 0.05% Tween 20 (Sigma Chemical Co.). The test supernatants, diluted 1:10 or 1:30 in PBS supplemented with 0.1% bovine serum albumin, were applied in triplicate and left overnight at 4°C. Serial dilutions of human lactoferrin from 1,000 to 1 ng/ml were applied in triplicate to fit a standard curve. After extensive washing, alkaline phosphatase-conjugated, affinity-purified rabbit anti-human lactoferrin (Jackson Immuno Research Laboratories Inc., West Grove, Pa.) was added to each well. After incubation at 37°C for 90 min and washing, a phosphatase substrate (Sigma) was added, and the developed color was read at 405 nm on an ELISA reader. Results were calculated from sigmoidal standard curves by using the spline curve-fitting method of the Riacalc program (Pharmacia Wallac Oy, Turku, Finland). This ELISA detected up to less than 10 ng of lactoferrin per ml. It did not detect transferrin, and no interference was seen with the constituents of FBS medium, which was used as a blank in the assay.

**TNF assay.** TNF was titrated by measuring the lysis of the TNF-sensitive murine cell line WEHI-164 (9). Cells from an exponentially growing culture of WEHI-164 cells were pelleted in a 5-ml sterile plastic tube, and 100  $\mu$ Ci of radiolabeled sodium chromate ( $\text{Na}^{51}\text{Cr}\text{O}_4$ ; Amersham, Arlington Heights, Ill.) was added for 1 h at 37°C. The cells were then washed three times and adjusted to  $5 \times 10^4$  cells per ml. Then, 100  $\mu$ l of the labeled WEHI-164 tumor cells was added to 100  $\mu$ l of the serial dilution of TNF-containing culture supernatants, and after 18 h at 37°C, the  $^{51}\text{Cr}$  released in the supernatants from lysed target cells was harvested and counted in a gamma counter. Maximum isotope incorporation was determined by counting the radioactivity in target cells alone, and spontaneous release was measured by counting the radioactivity in supernatants of target cells incubated with medium alone.

The spontaneous release in the 18-h assay was always in the range of 18 to 25%. Serial dilutions of a standard human recombinant TNF- $\alpha$  (Genentech Corp., South San Francisco, Calif.) were included as a control, and the concentration of TNF in the samples was calculated from the standard curve.

**IL-1 $\beta$  and IL-6.** To quantitate the production of interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-6 by PMN, a commercially available ELISA (Quantikine Research and Diagnostic System, Minneapolis, Minn.) was used. The data were analyzed as instructed by the developer of the ELISA and calculated by using the spline curve-fitting method of the Riacalc program (see above).

**Treatment with emetine and cycloheximide.** Neutrophils suspended to  $10^6$ /ml in FBS medium were treated with emetine or cycloheximide for 1 h at 37°C, at concentrations ( $10^{-5}$  M and 200  $\mu$ g/ml, respectively) sufficient to inhibit all protein synthesis (13, 18). The cells were washed twice in warm PBS, evaluated for viability by trypan blue exclusion, and resuspended to the desired number in FBS medium to assay for anti-*Candida* activity and release of lactoferrin or TNF in their supernatants, as described above.

**Preparation of RNA.** Neutrophils were cultured in FBS medium at  $3 \times 10^6$  cells per ml at 37°C for 1 or 3 h with 100 ng of LPS per ml. Neutrophils cultured for 1 h without LPS served as the control. Total RNA was prepared by the method of Chomzynski and Sacchi (4) by acid guanidinium thiocyanate-phenol-chloroform extraction.

**Northern (RNA blot) analysis.** For each sample, 20  $\mu$ g of total RNA was subjected to gel electrophoresis with 0.8% agarose (Bethesda Research Laboratories, Rockville, Md.).

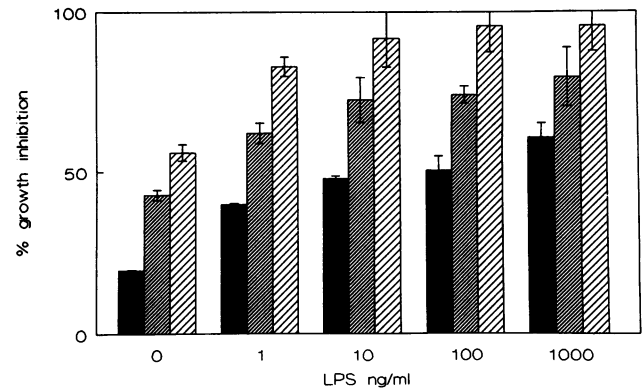


FIG. 1. Dose-response of LPS enhancement of neutrophil anti-*Candida* activity. Neutrophils were incubated for 30 min at 37°C with *E. coli* LPS in triplicate wells at the indicated doses before *C. albicans* was added, yielding E:T ratios of 60:1 (widely hatched bars), 30:1 (closely hatched bars), and 15:1 (solid bars). The percent growth inhibition  $\pm$  SEM is shown.

The RNA was transferred to 0.45- $\mu$ m Nytran filters (Schleicher & Schuell Inc., Keene, N.H.) and fixed by shortwave UV radiation. Membranes were prehybridized for at least 2 h at 45°C in 5 $\times$  Denhardt's solution (0.5% SDS, 200  $\mu$ g of salmon sperm DNA per ml, 50% formamide, 10 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 6.5], 10 mM EDTA, 0.1% sodium pyrophosphate, 5 $\times$  SSC [1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate]). Hybridization was performed in an identical solution supplemented with  $2 \times 10^6$  cpm of a  $^{32}\text{P}$ -labeled TNF- $\alpha$  cDNA probe (Genentech Corp.), IL-1 $\beta$  cDNA probe, or IL-6 cDNA probe (kindly provided by Steven Clark, Genetics Institute, Cambridge, Mass.) per ml at 45°C overnight. The probes were labeled with [ $^{32}\text{P}$ ]dCTP (specific activity, 3,000 Ci/mmol; New England Nuclear, Boston, Mass.) with a Random Primed DNA labeling kit (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.). The membranes were washed twice in 1 $\times$  SSC at 60°C for 30 min and exposed to Kodak X Omat-Ar films with an intensifying screen for 3 days for the TNF- $\alpha$  and IL-6 probes and for 4 h for the IL-1 $\beta$  probe.

**Statistical analysis.** Statistical analysis of the data was done with Student's *t* test or the Mann-Whitney *U* test, as appropriate.

## RESULTS

### Effect of various LPS on neutrophil anti-*Candida* activity.

We tested the effect of three different LPS isolated from the cell walls of *E. coli*, *S. marcescens*, and *S. typhimurium*. Graded concentrations of each LPS were added to neutrophils before addition of the target cells (*C. albicans*) to yield E:T ratios of 60:1, 30:1, and 15:1. For all donor cells tested, LPS in a dose range from 1 to 1,000 ng/ml strongly enhanced the anti-*Candida* activity of human neutrophils. For most PMN donors, optimal stimulation was achieved at an LPS concentration of 10 ng/ml, and there were no statistically significant differences, at least at the highest E:T ratio, in the growth inhibition measured at 10, 100, and 1,000 ng of LPS per ml. For instance, for the particular donor cells shown in Fig. 1, at E:T ratios of 15:1 and 60:1, LPS at 10 ng/ml increased the anti-*Candida* activity to about 50 and 90% growth inhibition from 20 and 60%, respectively. Over the same range of doses (1 to 1,000 ng/ml) and at all E:T ratios

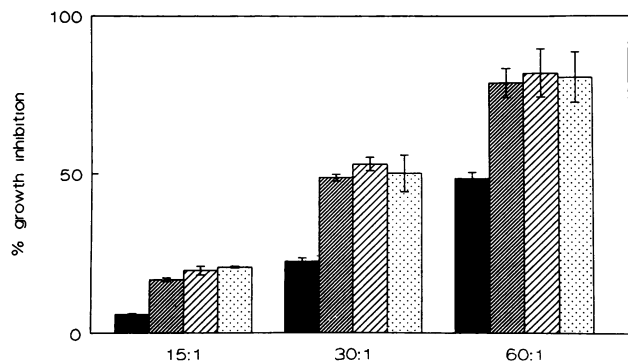


FIG. 2. Effect of various bacterial LPS on neutrophil anti-*Candida* activity. Neutrophils were incubated for 30 min at 37°C in triplicate wells with medium alone (solid bars) or 100 ng of LPS from *E. coli* (closely hatched bars), *S. marcescens* (widely hatched bars), or *S. typhimurium* (stippled bars) per ml. *C. albicans* was then added to yield E:T ratios of 60:1, 30:1, and 15:1. The percent growth inhibition  $\pm$  SEM is shown.

used, LPS extracted from all the above-mentioned bacteria gave similar enhancement of neutrophil ability to inhibit growth of *C. albicans* (Fig. 2). LPS itself had no direct effect on *Candida* growth at any concentration used (data not shown).

Overall, PMN from 13 donors were tested with 100 ng of *E. coli* LPS per ml for stimulation of PMN anti-*Candida* activity; the mean  $\pm$  SEM was  $1,035.6 \pm 152.0$  IU, compared with  $403 \pm 47.9$  IU for unstimulated control PMN. The difference between the means was highly significant ( $P < 0.005$  by both the pooled Student's *t* test and the Mann-Whitney *U* test), demonstrating that LPS stimulation was not biased by the high variability in the anti-*Candida* activity of PMN from different donors.

**Effect of LPS on phagocytic activity.** By using a flow cytometric assay based on the uptake of FITC-labeled *C. albicans* by neutrophils (42), as well as direct microscopic observations of Giemsa-stained cytocentrifuge preparations, it was possible to show that LPS enhanced neutrophil phagocytosis by increasing both the percentage of cells able to ingest *C. albicans* and the number of yeasts ingested per neutrophil. Figure 3 shows the data from three experiments (three donors) with the FACScan. Both the percent phagocytosing neutrophils (Fig. 3B) and the fluorescence intensity representative of the average number of *Candida* cells ingested per neutrophil (Fig. 3A) were derived from the relevant parameters of flow cytometric measurements. The presence of a quencher (trypan blue [1%, wt/vol]) did not change the fluorescence units of LPS-stimulated PMN (data not shown), supporting the notion that the fungal cells were indeed ingested by, rather than adherent to, the neutrophils.

**Release of lactoferrin.** To obtain some insight into the mechanism of *Candida* growth inhibition by neutrophils and its enhancement by LPS, we investigated the release of secondary granules as measured by lactoferrin secretion. As shown in Table 1, an appreciable amount of lactoferrin was released in cultures of unstimulated neutrophils in FBS medium, and the peak of lactoferrin release occurred at 24 h. LPS promoted a rapid augmentation of lactoferrin release by PMN, as demonstrated by the marked difference between unstimulated and LPS (100 ng/ml)-stimulated neutrophils within 1 h of stimulation ( $320 \pm 22$  versus  $2,669 \pm 24$  ng of lactoferrin per ml, respectively). In addition, the amount of

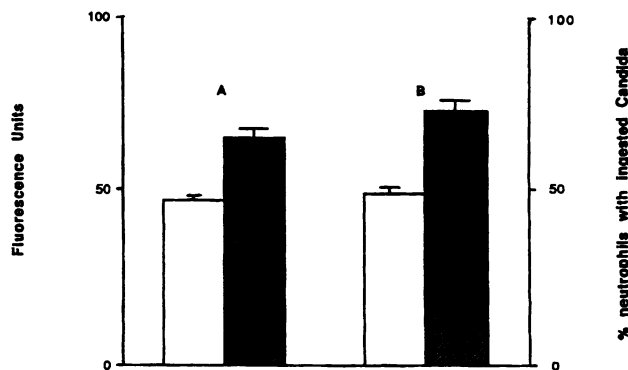


FIG. 3. Effect of LPS on neutrophil phagocytosis, determined by flow cytometry. Neutrophils were incubated for 30 min at 37°C with medium (open bars) or 100 ng of *E. coli* LPS per ml (shaded bars). Then, FITC-labeled *C. albicans* was added and incubated for another 30 min at 37°C before phagocytosis was assessed by flow cytometry. (A) Fluorescence units indicating the number of FITC-labeled yeasts ingested per neutrophil. (B) Percentage of neutrophils with ingested *Candida* cells. The data are presented as means  $\pm$  standard deviation for three different experiments performed with PMN from three separate donors.

lactoferrin released after 1 h closely approached the total amount detected in unstimulated controls after 24 h. Overall, the amount of lactoferrin detected in cultures of neutrophils stimulated by LPS was always significantly higher, at every time point tested, than that in unstimulated cultures and increased in a time-dependent manner. The LPS from *S. marcescens* and *S. typhimurium* were also able to promote lactoferrin release from secondary granules (data not shown).

**Ability of lactoferrin itself to inhibit *Candida* growth and its importance in the anticandidal activity of neutrophils.** Since lactoferrin acts as a bacteriostatic agent (23–32), and, as shown by the results in the preceding sections, it was released in appreciable amounts during LPS stimulation, we examined whether lactoferrin had a direct effect on the growth of *C. albicans*. By the same rapid [ $^3$ H]glucose uptake assay used to assay lysis of PMN, we found that this iron-binding protein was able to inhibit *C. albicans* growth in a dose-dependent manner over a range from 1 to 100  $\mu$ g/ml. At the highest lactoferrin dose, fungal growth was inhibited by about 70%. The ability of lactoferrin to inhibit *Candida* growth at any dose tested was completely abolished by using antilactoferrin serum at a 1:100 dilution. Transferrin and antitransferrin serum were also used in the same range of doses, but transferrin showed no effect on *Candida* growth, and antitransferrin antibodies were unable to reverse the growth-inhibitory effect of lactoferrin (Table 2). Consequently, we used rabbit anti-human lactoferrin antibodies in the anti-*Candida* PMN assay to ascertain whether these antibodies could also inhibit the neutrophil anti-*Candida* activity stimulated by LPS. In these experiments, a 1:1,000 dilution of antilactoferrin serum was used, which could neutralize the inhibitory effects on *Candida* growth obtained with 10  $\mu$ g of lactoferrin (Table 2). Figure 4 shows the results from one of two experiments performed with similar results. The antilactoferrin antibodies decreased the anti-*Candida* activity of unstimulated neutrophils as well as that of neutrophils stimulated by LPS (100 ng/ml), especially at the lowest E:T ratio, where growth inhibition was reduced from  $78.4\% \pm 5\%$  to  $23.5\% \pm 0.4\%$ . In these experiments, the

TABLE 1. Kinetics of lactoferrin release from neutrophils

Stimulation of neutrophils	Mean lactoferrin released (ng/ml) $\pm$ SEM (n = 3)			
	1 h	3 h	6 h	24 h
None	320.1 $\pm$ 22.8	1,330.5 $\pm$ 30.1	2,200.0 $\pm$ 51.0	3,387.2 $\pm$ 89.3
<i>E. coli</i> LPS (100 ng/ml)	2,669.2 $\pm$ 24.5	4,742.0 $\pm$ 52.4	6,823.4 $\pm$ 262.0	7,391.4 $\pm$ 276.5

antitransferrin serum, used at the same dilution (1:50) as in the experiments reported in Table 2, was unable to reverse the ability of PMN to inhibit the growth of *C. albicans* (data not shown: see also the results in Table 2).

**Synthesis of cytokines by neutrophils.** In view of the recently discovered ability of neutrophils to synthesize various cytokines with potential antimicrobial activity (12, 15, 26, 28), we examined whether PMN were able to produce cytokines such as IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 in response to LPS under the conditions of our anti-*Candida* assay. All three cytokines were readily detected in the supernatants of PMN activated with LPS (100 ng/ml). Figure 5 indicated a peak of TNF- $\alpha$  production at about 6 h, while IL-1 $\beta$  and IL-6 were still on the rise at 24 h. Similarly, Northern blot analysis (Fig. 6) revealed the efficient ability of neutrophils to synthesize mRNAs for the three cytokines. Unstimulated PMN produced some cytokine-specific mRNA, but the degree of cytokine gene transcription was greatly augmented by LPS stimulation. Peak mRNA transcription was detected after 1 h of LPS stimulation. In keeping with the Northern blot data, unstimulated neutrophils constitutively produced a background level of TNF, IL-1 $\beta$ , and IL-6. Dose-response experiments showed that PMN released maximal TNF after stimulation with 100 to 1,000 ng of LPS per ml (data not shown). LPS isolated from other sources also stimulated the production of TNF. In a typical experiment, LPS from *E. coli*, *S. marcescens*, and *S. typhimurium*, used at 100 ng/ml, stimulated the production of 33  $\pm$  0.3, 30  $\pm$  0.7, and 20  $\pm$  1.0 (mean  $\pm$  SEM) pg of TNF per ml, respectively, from PMN.

**Effect of inhibitors of protein synthesis on neutrophil function.** The data in the previous section demonstrate that PMN are biosynthetically active during the expression of anti-

*Candida* activity in vitro and that this biosynthetic activity is stimulated by LPS. In this line, it was of interest to examine whether inhibition of protein synthesis, presumably blocking cytokine production, could affect one or more aspects of PMN activation by LPS. Thus, neutrophils were incubated for 1 h at 37°C in FBS medium with or without emetine or cycloheximide, washed twice, and then stimulated or not with LPS (100 ng/ml) before anti-*Candida* activity, lactoferrin release, and TNF production were assessed. Figure 7 shows that neither drug significantly inhibited the anti-*Candida* activity of either unstimulated or LPS-stimulated PMN, nor did they reduce the release of lactoferrin. As expected, however, these drugs almost totally abrogated TNF production (Fig. 6). Similar results were obtained in other two experiments with different PMN donors, and the inhibitors of protein synthesis did not affect the viability of PMN per se (data not shown).

## DISCUSSION

The goal of the present study was to unravel the various effects exerted by LPS on human PMN in vitro, in terms of both anti-*Candida* activity and stimulation of cytokine production. We have shown that indeed three kinds of LPS isolated from different bacteria were able to consistently potentiate the anti-*Candida* activity of human neutrophils. The activation by LPS was seen to occur at doses ranging from 1 to 1,000 ng/ml, but with PMN from most donors, 10 ng of LPS per ml was sufficient to give maximal (or nearly so) PMN activation. Remarkable differences between unstimulated and LPS-stimulated cells were found in the release of lactoferrin after 60 min. We have also demonstrated that LPS induced the transcription of mRNAs for TNF- $\alpha$ ,

TABLE 2. *Candida* growth inhibition by lactoferrin and its reversal by specific antilactoferrin antibodies<sup>a</sup>

Condition	Lactoferrin concn ( $\mu$ g/ml)	% Inhibition of <i>C. albicans</i> growth (mean $\pm$ SEM)	
Medium alone	100	70.2 $\pm$ 6.6	
	10	29.2 $\pm$ 4.5	
	1	16.2 $\pm$ 3.2	
Antilactoferrin serum	100	1.1 $\pm$ 0.1	
	1:50	10	4.8 $\pm$ 0.05
		1	3.9 $\pm$ 0.13
	1:1,000	100	48.8 $\pm$ 1.8
		10	0.2 $\pm$ 0.0
		1	0.0 $\pm$ 0.0
Antitransferrin serum, 1:50	100	79.4 $\pm$ 2.0	
	10	19.2 $\pm$ 0.4	
	1	18.5 $\pm$ 0.2	
Transferrin (100 $\mu$ g/ml)	1	6.9 $\pm$ 0.3	

<sup>a</sup> Five hundred *C. albicans* yeasts were cultured in triplicate wells at 37°C in FBS medium for 18 to 20 h in the presence of human lactoferrin or human transferrin, as indicated. Dilutions of antilactoferrin or antitransferrin serum were added to the wells just before lactoferrin addition. The values are expressed as means  $\pm$  SEM for triplicate wells.

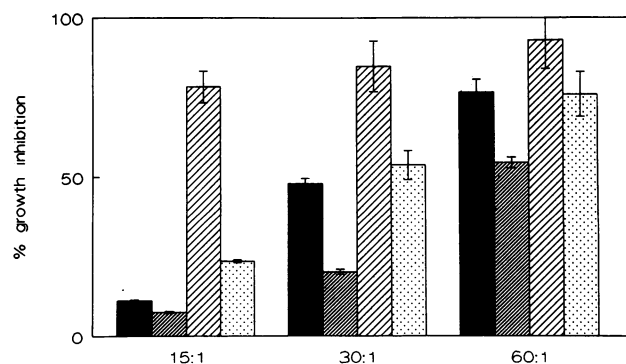


FIG. 4. Effect of antilactoferrin antibodies on neutrophil anti-*Candida* activity. Neutrophils, unstimulated or stimulated with *E. coli* LPS (100 ng/ml), were incubated for 30 min at room temperature in triplicate wells with medium (unstimulated, solid bars; stimulated, widely hatched bars) or rabbit anti-human lactoferrin (unstimulated plus antibody, closely hatched bars; stimulated plus antibody, stippled bars). *C. albicans* was then added to yield the indicated E:T ratios. The data are presented as means of triplicate wells  $\pm$  SEM.

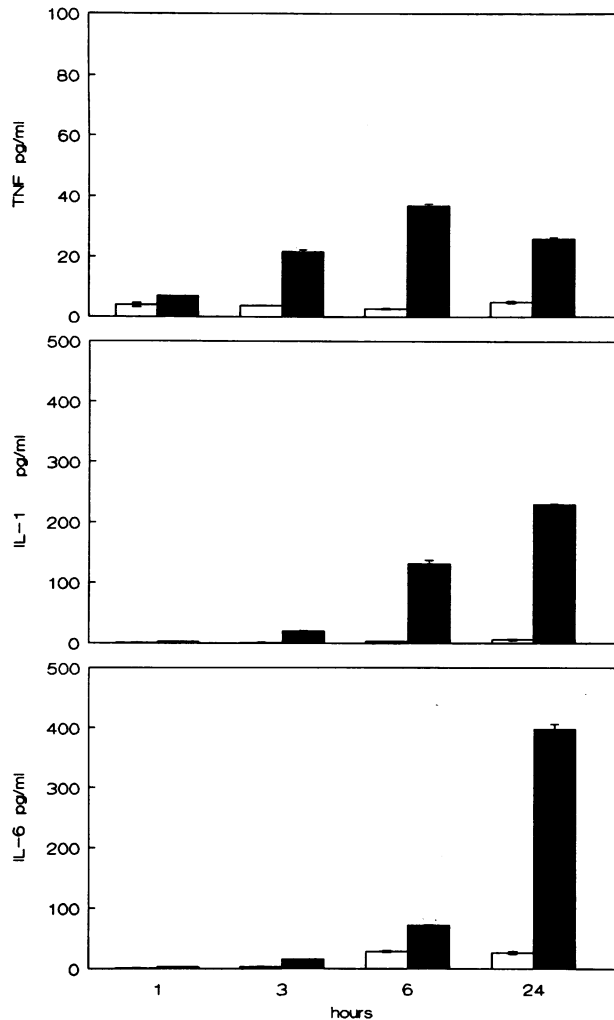


FIG. 5. Neutrophil production of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in response to LPS. Neutrophils were cultured with medium alone (open bars) or with 100 ng of *E. coli* LPS (solid bars) per ml for 3, 6, or 24 h at 37°C prior to collection of supernatants for assessment of the presence of cytokines with a specific ELISA kit. The data are presented as means  $\pm$  standard deviation for two different experiments.

IL-1 $\beta$ , and IL-6, which peaked at 1 h. Accordingly TNF, IL-1 $\beta$ , and IL-6 were indeed detected in the neutrophil supernatant, with maximum production of TNF found at 6 h and of IL-1 $\beta$  and IL-6 after 24 h. These effects demonstrate that PMN are being rapidly and intensely activated by LPS, in keeping with their recognized role in first-line antimicrobial activity.

Because of recurrent *Candida* infections in neutropenic and immunocompromised patients, including those infected by human immunodeficiency virus (30), we focused our attention on candidastatic mechanisms used by neutrophils. Our data strongly suggest that both intracellular and extracellular anti-*Candida* mechanisms are being activated by LPS. Although limited by the use of heat-inactivated cells, the data obtained by flow cytometry of PMN with FITC-labeled *Candida* cells with or without LPS stimulation indicate an intracellular fungistatic activity that is activated by LPS. Oxygen radicals produced in the respiratory burst

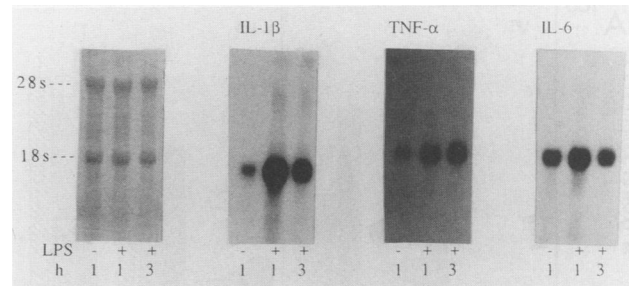


FIG. 6. Northern blot analysis of LPS induction of mRNAs for IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 in PMN. Neutrophils were cultured in medium for 1 h (lanes -) or stimulated with 100 ng of LPS per ml (lanes +) for 1 or 3 h. The same filter was hybridized sequentially with cDNA probes for IL-1 $\beta$ , TNF- $\alpha$ , and IL-6. The positions of 18S and 28S rRNAs are indicated. The leftmost panel shows the electrophoretic pattern of total RNA transblotted onto a Nytran filter and stained with 0.04% methylene blue in 0.5 M sodium acetate.

and release of enzymatic granules into the phagocytic vacuole are probably responsible for *Candida* killing. The extracellular mechanism seems to be related, at least in part, to lactoferrin release. Although the bacteriostatic role of this iron-binding protein is known (23, 32, 35), its effect on *C. albicans* remains uncertain (33). Kirkpatrick et al. showed the capacity of iron-poor milk to limit colonial growth of *C. albicans* and attributed this inhibition to lactoferrin (23). Sohnle and Collins-Lech (41) have recently reported that disruption of neutrophils causes the release of a cytoplasmic protein which can inhibit *C. albicans* growth, yet the mechanism involved did not appear to depend upon iron binding. We have demonstrated here that lactoferrin itself can inhibit, although not totally, the growth of *C. albicans*, and antibodies against human lactoferrin were able to completely reverse this inhibition. In addition, it is unlikely that the augmented anti-*Candida* activity observed after stimulation with LPS is due to enhanced PMN disruption or death, as these cells transcribed and translated cytokine genes efficiently during LPS activation, in a process that was totally inhibited by inhibitors of protein synthesis.

The sensitivity of *C. albicans* to lactoferrin gains more biological importance when the presence of this protein in biological fluids, including breast milk, plasma, saliva, tears, and pancreatic juice, is considered. Lactoferrin levels in plasma are known to increase during inflammation, accompanied by a decrease in the lactoferrin content of PMN (19, 45). Lactoferrin is stored in secondary granules, and several cytokines as well as LPS are able to induce its release (1, 25). Further studies are needed to know whether other proteins released by PMN could potentiate and act in concert with lactoferrin in inhibiting *Candida* growth and whether this activity relies only on the depletion of iron in the medium.

Because neutrophils are able to synthesize TNF and IL-1 (12, 15, 26, 28), we investigated the possibility that PMN could produce other cytokines. The finding that neutrophils are able to produce IL-6 may be particularly relevant for the "immunomodulatory" properties of PMN, because these cells, through the production of IL-6, may be able to participate in the modulation of several B- and T-lymphocyte responses (17, 40). We were particularly interested in examining whether the ability of LPS-primed PMN to produce IL-1, TNF- $\alpha$ , and IL-6 correlated with their increased candidastatic potential and/or release of lactoferrin. We observed that the expected blockade of TNF production by

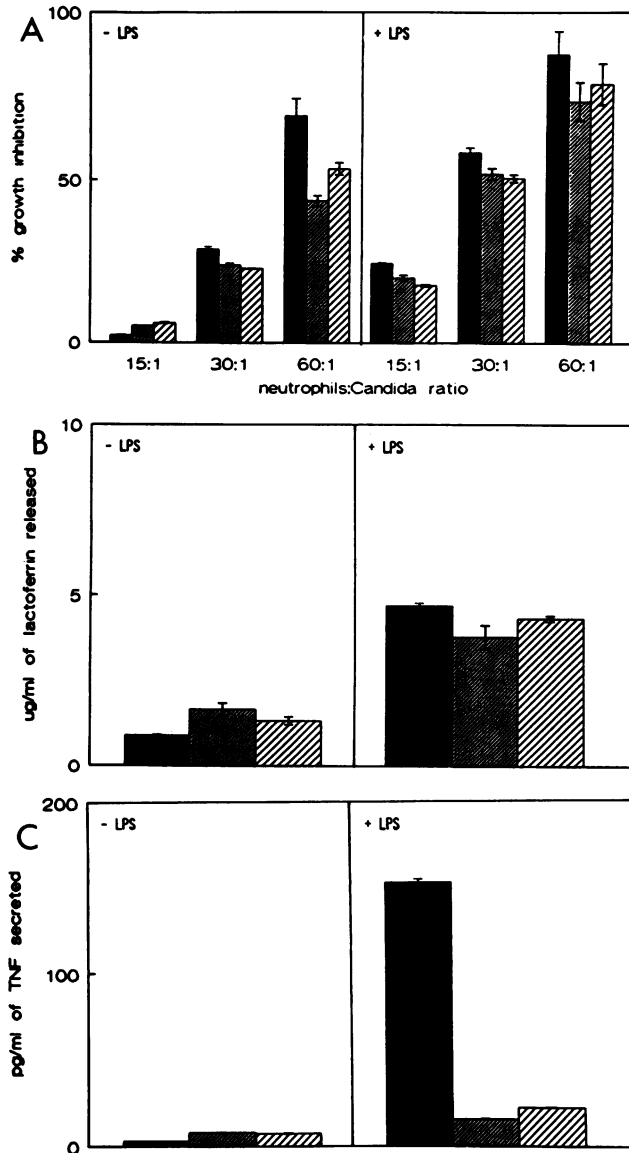


FIG. 7. Effect of inhibition of protein synthesis on neutrophil functions. Neutrophils were cultured with medium alone (solid bars) or with  $10^{-5}$  M emetine (closely hatched bars) or 200  $\mu$ g of cycloheximide per ml (widely hatched bars) for 1 h at 37°C, washed twice, stimulated with LPS (100 ng/ml) or not stimulated, and then assayed for (A) anti-*Candida* activity, (B) lactoferrin release, and (C) TNF production. The data are presented as means of triplicate wells  $\pm$  SEM.

inhibitors of protein synthesis was not paralleled by a proportional, commensurate loss of the ability to inhibit the growth of *C. albicans*, whether the PMN were stimulated by LPS or not. Similarly, lactoferrin release was not appreciably affected by the protein synthesis inhibitors. The lack of correlation between lactoferrin release and cytokine production during inhibition of protein synthesis is a strong argument against the possibility that lactoferrin was released under the stimulating activity of PMN-derived TNF.

Nonetheless, the lack of correlation between production of cytokines and anti-*Candida* activity in our *in vitro* assay does not diminish the significance of their possible secretion

*in vivo*. In fact, TNF, IL-1, and IL-6 could mobilize and activate neighboring neutrophils, besides interacting with monocytes, natural killer cells, and lymphocytes. Evidence for this autocrine-paracrine regulation of PMN anti-*Candida* activity through cytokines has recently been obtained (37a).

The rapid antimicrobial response of neutrophils and the synthesis of cytokines could be a first process in preventing microbial invasion and a rapid way to recruit other inflammatory cells. Overall, it is worth emphasizing that neutrophils can produce the same cytokines attributed to monocytes, but because of their prompt delivery to the site of inflammation, PMN responses *in vivo* could be far more rapid than those of monocytes. All this further stresses the main role that is currently attributed to PMN in the control of tissue invasion by *C. albicans* and the critical importance of neutropenia in the development of deep-seated candidiasis (34).

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