The Journal of Immunology

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J Immunol 2005; 174:411-417; ;
doi: 10.4049/jimmunol.174.1.411
http://www.jimmunol.org/content/174/1/411

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Inhibition of Human Neutrophil IL-8 Production by Hydrogen Peroxide and Dysregulation in Chronic Granulomatous Disease[1]

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The innate immune response to bacterial infections includes neutrophil chemotaxis and activation, but regulation of inflammation is less well understood. Formyl peptides, byproducts of bacterial metabolism as well as mitochondrial protein biosynthesis, induce neutrophil chemotaxis, the generation of reactive oxygen intermediates (ROI), and the production of the neutrophil chemoattractant, IL-8. Patients with chronic granulomatous disease (CGD) exhibit deficient generation of ROI and hydrogen peroxide and susceptibility to bacterial and fungal pathogens, with associated dysregulated inflammation and widespread granuloma formation. We show in this study that in CGD cells, fMLF induces a 2- to 4-fold increase in IL-8 production and a sustained IL-8 mRNA response compared with normal neutrophils. Moreover, normal neutrophils treated with catalase (H_2O_2 scavenger) or diphenyleneiodonium chloride (NADPH oxidase inhibitor) exhibit IL-8 responses comparable to those of CGD neutrophils. Addition of hydrogen peroxide or an H_2O_2-generating system suppresses the sustained IL-8 mRNA and increased protein production observed in CGD neutrophils. These results indicate that effectors downstream of the activation of NADPH oxidase negatively regulate IL-8 mRNA in normal neutrophils, and their absence in CGD cells results in prolonged IL-8 mRNA elevation and enhanced IL-8 levels. ROI may play a critical role in regulating inflammation through this mechanism. ***The Journal of Immunology,*** **2005, 174: 411–417.**

Neutrophils are the principal circulating cellular mediators of innate immunity, responding to minute concentrations of exogenous and endogenous chemoattractants and migrating to sites of infection (1). Upon reaching infected tissues, neutrophils release toxic proteases, phagocytize pathogens, and generate reactive oxygen intermediates (ROI)[3] and hydrogen peroxide, promoting the destruction of invading organisms (1).

A neutrophilic response to inflammation is largely dictated by its initial receptor binding of chemoattractants. In addition to endogenous chemoattractants, including IL-8, C5a, and leukotriene B_4 (LTB_4) neutrophils detect and respond to the bacterial-derived formyl peptides (2–5). Formyl peptides are generated by bacterial endopeptidase cleavage of the first few amino acids, including the initial formyl-modified methionine group of bacterial proteins (6), and are also found in mitochondria (7). Neutrophils detect formyl peptides using either high affinity or low affinity formyl peptide receptors (FPR and FPRL1) (4). Detection of formyl peptides by these seven-transmembrane, G protein-coupled receptors elicits a repertoire of responses, including chemotaxis (4), generation of reactive oxygen intermediates (8), and degranulation (9).

Patients with chronic granulomatous disease of childhood (CGD) have genetic mutations in any of four components of the NADPH oxidase enzyme that is expressed in neutrophils and monocytes and is necessary for the generation of reactive oxygen intermediates (ROIs), such as superoxide anion, hydroxyl radical, and hydrogen peroxide (1). Their profound defect in innate immunity is reflected by their susceptibility to catalase-positive bacteria and fungi, including the *Aspergillus* species and *Staphylococcus aureus* (1). In addition to this severe immunodeficiency, CGD patients display signs of dysregulated inflammation, for which the underlying mechanism is unknown. For example, patients with CGD develop granulomas throughout their tissues, often resulting in urinary and gastrointestinal tract obstruction (10).

NADPH oxidase and its redox products induce transcriptional activation in some models (11–15); however, their potential to down-regulate the inflammatory response is not known. In this paper we show in a model of neutrophil activation by formyl peptides using CGD and normal cells that normal IL-8 production is regulated by hydrogen peroxide resulting from NADPH oxidase activation.

Materials and Methods

Materials

The following reagents were purchased from the indicated sources: fMLF, PMA, diphenyleneiodonium chloride (DPI), catalase, histidine, taurine, and superoxide dismutase were obtained from Sigma-Aldrich; the Ultraspec RNA isolation system was purchased from Biotecx Laboratories; [γ-^{32}P]dATP was obtained from NEN; 4–20% Tris-glycine gels and buffers were purchased from Invitrogen/NOVEX.

Isolation of peripheral blood neutrophils and stimulation

Normal volunteer and CGD patient blood samples were drawn under National Institutes of Health protocols 99-CC-0168 and 93-I-0119. Neutrophils were harvested by Ficoll-Paque Plus discontinuous gradient centrifugation, RBC sedimentation with dextran, and hypotonic lysis. Harvested neutrophils were diluted in HBSS (Cambrex) at 1–2 $\times$ 10^6 cells/ml and aliquoted into polypropylene tubes. Cells were incubated briefly for 10 min

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Received for publication June 10, 2004. Accepted for publication October 19, 2004.

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[1] The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsements by the U.S. government. This work was supported in whole or in part by federal funds from the National Cancer Institute, National Institutes of Health, under Contract NO1-CO-12400.

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[3] Abbreviations used in this paper: ROI, reactive oxygen intermediates; LTB_4, leukotriene B_4; CGD, chronic granulomatous disease; DPI, diphenyleneiodonium chloride; GRO-α; growth-related oncogene α.

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at 37°C, followed by stimulation with the indicated doses of fMLF and incubation at 37°C for 2–3 h. Experiments using reactive oxygen species scavengers or hydrogen peroxide were performed using neutrophils treated with the indicated mediator for 10 min before the addition of fMLF unless otherwise indicated. Reactive oxygen scavengers were used at the recommended concentrations (16).

Neutrophil cell protein harvest and chemokine/cytokine quantitation

Neutrophils were lysed in protein lysis buffer (0.1% Igepal CA 630 (Sigma-Aldrich), 50 mM Tris-HCl (pH 8.0), 0.15 M sodium chloride, 10 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, Complete inhibitor minitablet (Roche)/10 ml buffer, and 100 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride (Pefabloc; Roche)). After a 1-h incubation on ice, debris was pelleted, and supernatant proteins were stored at −80°C. Alternatively, neutrophils were solubilized in 0.2% Triton X-100 and IL-8, IL-1β, TNF-α, IL-6, IL-1 receptor antagonist, growth-related oncogene α (GRO-α), MIP-1α, and MCP-1 were quantitated using commercial ELISA kits (R&D Systems), according to the manufacturer's instructions (17).

Messenger RNA analysis

Neutrophil total RNA was extracted using the Ultraspec RNA isolation kit according to the manufacturer's instructions. After quantitation by spectrophotometer, RNA was separated by electrophoresis through a denaturing gel as previously described (18). RNA was transferred to a Nytran nylon membrane (Schleicher & Schuell), and hybridized to ^{32}P-dATP labeled IL-8 probe (R&D Systems human IL-8 probe mixture) as described previously (18), with subsequent washing and autoradiography. In addition, levels of IL-8, IL-1β, and GAPDH mRNA were determined using commercial Quantikine mRNA quantitation kits (R&D Systems) according to the manufacturer's instructions.

Hydrogen peroxide production

H_2O_2 was measured using the Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (Molecular Probes). Briefly, neutrophils (2×10^6 cells/ml HBSS) were incubated in a mixture containing 50 μM Amplex Red reagent and 0.1 U/ml HRP. Either fMLF (5×10^{-9} M) or PMA (100 ng/ml) was added, and the fluorescence was monitored using a fluorescence microplate reader (CytoFluor II; PerSeptive Biosystems) with a $\lambda_{excitation}$ of 530 nm and a $\lambda_{emission}$ of 590 nm. Unknowns were calculated from an H_2O_2 standard curve that ranged from 0.2–20 μM.

Statistical analysis

Data in this study were analyzed using standard univariate ANOVA, longitudinal repeated measures generalized least squares and linear effects mixed models, graphical techniques, and post-hoc tests (Tukey's, Dunnett's, and *t* tests). Longitudinal mixed models account for within-subject correlations over time. All tests were two-sided; a value of $p < 0.05$ was considered significant. In many cases, data were transformed to their common logarithm to satisfy homogeneity of variance requirements. The data presented represent the mean ± SEM. To determine the $t_{1/2}$ of IL-8 mRNA, the data point representing the peak response and the remainder of the points through the end of the incubation period were connected using a first-order exponential decay curve, and the decay constant, λ, was determined. The $t_{1/2}$ was calculated using the formula: $t_{1/2} = 0.693/\lambda$.

Results

Differential expression of neutrophil IL-8 in normal and CGD neutrophils

Previous observations of the effects of formyl peptide stimulation on peripheral blood neutrophils from normal subjects were confirmed using a chemotactic dose of fMLF (5×10^{-9} M) in vitro, resulting in an increase in neutrophil IL-8 production that was detected within 30 min and continued through 120 min (19) (Fig. 1, *top*). In contrast, treatment of neutrophils from CGD patients with fMLF resulted in an increase in neutrophil IL-8 production that was detected within 30 min and continued through 240 min. The response was independent of the CGD genotype (not shown) and was significantly greater than that seen in normal neutrophils ($p = 0.0382$).

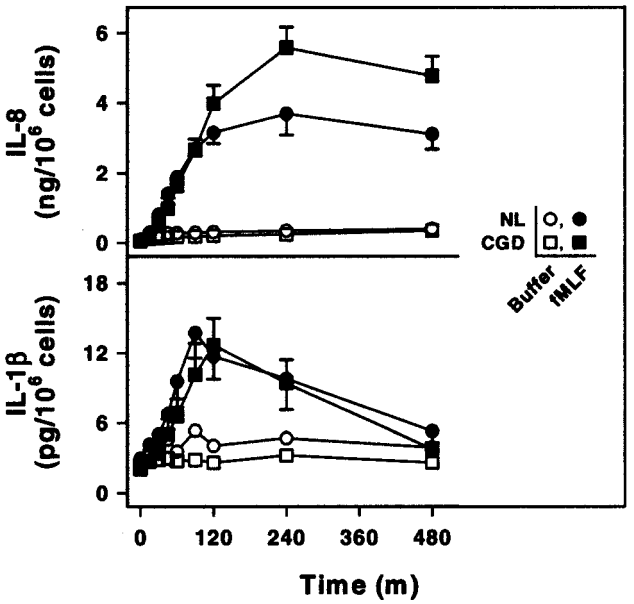

FIGURE 1. Differential expression of IL-8 and IL-1β in normal (NL) and CGD neutrophils. Neutrophils (1×10^6/0.5 ml HBSS with HEPES, pH 7.3) from either normal subjects or patients with CGD were incubated in the absence (○ and □) or the presence (● and ■) of fMLF (5×10^{-9} M). At the indicated times, the incubation was halted by the addition of cold 0.2% Triton X-100, and total IL-8 and IL-1β were determined as described in *Materials and Methods*. The data represent the mean ± SEM of neutrophils isolated from 13 normal subjects and 13 patients with CGD. The difference in IL-8 expression between normal and CGD neutrophils was significant ($p = 0.0382$).

The average rate of IL-8 accumulation in CGD and normal neutrophils did not differ through 90 min; subsequently, however, the average rate of IL-8 production in normal neutrophils dropped in the 90- to 120-min interval whereas IL-8 production in CGD neutrophils remained significantly elevated (0.016 ± 0.007 ng of IL-8/10^6/min in normal cells vs 0.049 ± 0.010 ng of IL-8/10^6/min in CGD cells; $p = 0.0119$). By 120–480 min, the average rate of IL-8 accumulation in CGD neutrophils dropped to the level observed in normal neutrophils (0.000 ± 0.001 ng of IL-8/10^6/min in normal cells vs 0.002 ± 0.001 ng of IL-8/10^6/min in CGD cells). The prolonged rate of production of IL-8 in CGD neutrophils was associated with a 50% or greater increase in IL-8 protein in CGD neutrophils compared with normal neutrophils, confirming our previous report (19).

The difference in IL-8 production in normal vs CGD neutrophils was not a general effect on all cytokine production, because no significant differences in the production of IL-1β were observed between normal and CGD neutrophils (Fig. 1, *bottom*). It should be noted that the level of IL-8 found in neutrophils was nearly 1000-fold greater than that of IL-1β. In addition, the IL-1β response was transient, suggesting that IL-1β is used and/or cleared comparably by normal and CGD neutrophils.

Neutrophils from both normal volunteers and CGD patients responded to fMLF stimulation in a dose-responsive manner, with significant increases ($p < 0.01$) in IL-8 production at doses of 5×10^{-9} and 1×10^{-7} M fMLF and with maximal stimulation at a dose of 5×10^{-9} M; however, IL-8 protein levels in CGD neutrophils were 2- to 4-fold higher than levels measured in normal volunteer neutrophils (Fig. 2). At higher doses of fMLF (0.1 μM), production of neutrophil IL-8 returned to baseline levels in both normal and CGD neutrophils (Fig. 2). The loss of IL-8 synthesis at higher doses of fMLF is not without precedent; neutrophil chemotaxis toward formyl peptides is also reduced at 1- to 5-μM doses

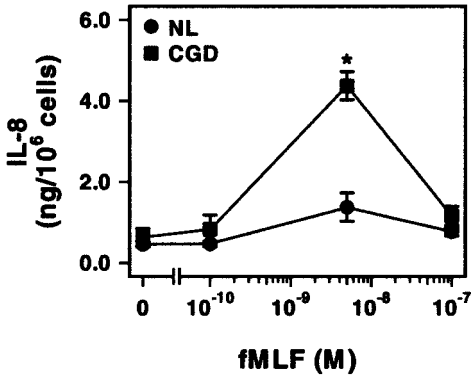

FIGURE 2. Dose response of IL-8 accumulation in normal (NL) and CGD neutrophils. Neutrophils were treated with increasing doses of fMLF as described in Fig. 1. The data represent the mean ± SEM of 12 normal subjects and four CGD patients. *, $p < 0.01$.

of fMLF and is attributed in part to the differing affinities of the two human formyl peptide receptors expressed on neutrophils (4).

Differential expression of IL-8 mRNA in normal and CGD cells

The increased levels of IL-8 protein detected in CGD neutrophils may be due to alterations in IL-8 mRNA synthesis or destruction or in IL-8 protein translation or release, or in some combination of these processes. To investigate the varied contributions of these events to our observations, IL-8 mRNA in normal and CGD neutrophils was examined and quantitated in response to both dose of formyl peptides and time of stimulation.

mRNA blotting of total RNA harvested from normal and CGD neutrophils after 3 h of stimulation with different doses of formyl peptide showed increased levels of IL-8 transcripts in CGD cells compared with normal cells (Fig. 3). Interestingly, an analysis of the effect of increasing concentrations of fMLF on IL-8 mRNA at 45 min after stimulation revealed similar responses in normal and CGD neutrophils (Fig. 4, *left panel*), suggesting that early after stimulation, IL-8 transcription was similar in normal and CGD

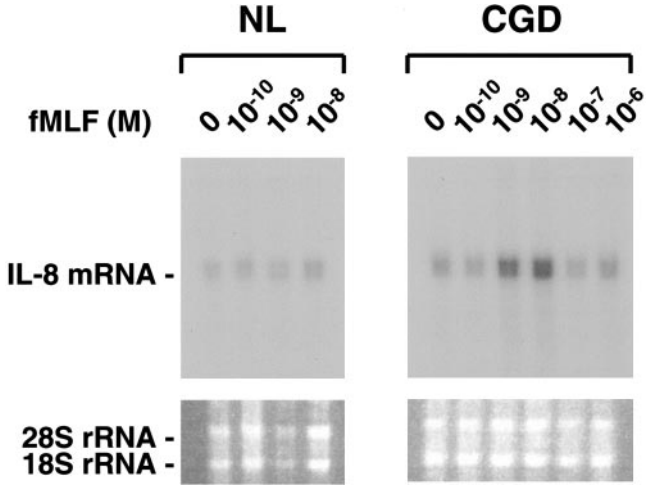

FIGURE 3. Northern blot analysis of prolonged expression of IL-8 mRNA in CGD neutrophils. The *top panels* represent Northern blot analysis of RNA isolated from both normal (NL; *left*) and CGD (*right*) neutrophils after 3-h incubation with the indicated doses of fMLF. The blots were hybridized with oligonucleotide probes encoding antisense IL-8. RNA loading was verified by ethidium staining of the membrane before hybridization (*bottom panels*).

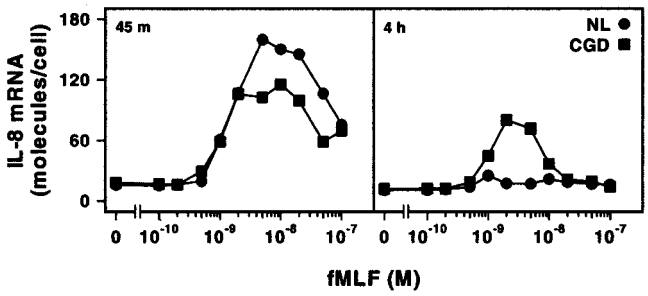

FIGURE 4. Prolonged expression of IL-8 mRNA in CGD neutrophils. Neutrophils isolated from two normal subjects (NL; ●) and a CGD patient (■) were incubated with the indicated doses of fMLF for 45 min (*left panel*) and 4 h (*right panel*). IL-8 mRNA was determined as described in Fig. 3.

cells. However, 4 h after fMLF stimulation, normal cells showed little or no increase in IL-8 mRNA, whereas CGD cells continued to show sustained IL-8 mRNA (Fig. 4, *right panel*).

Time-course analysis of IL-8 mRNA levels in normal and CGD neutrophils showed that the kinetics of IL-8 transcription in CGD cells were significantly altered compared with those in normal cells. In the first 90 min after fMLF stimulation, IL-8 mRNA levels in CGD cells and normal cells were very similar, corroborating the dose-response studies after 45 min of formyl peptide stimulation (Fig. 4). Subsequently, IL-8 mRNA levels observed in CGD cells from 90 min through 480 min were significantly elevated ($p < 0.01$; Fig. 5*A*) compared with those in normal cells. Integration of the areas under the curves revealed an average 2.5-fold increase in the IL-8 mRNA of CGD neutrophils compared with normal neutrophils ($p < 0.01$). Analysis of the mRNA decay curve (starting at the peak level through the return to basal level) yielded similar $t_{1/2}$ values for IL-8 mRNA in normal and CGD neutrophils ($t_{1/2}$ = 99 ± 7 min for normal cells vs 113 ± 5 min for CGD cells; $p = 0.127$), suggesting that the prolonged IL-8 mRNA response in CGD neutrophils was due to increased transcription and not to a change in mRNA stability. Additional studies using actinomycin D to block RNA synthesis indicated that the decay of synthesized IL-8 mRNA was not different in normal and CGD neutrophils treated with buffer or fMLF; therefore, the regulation of IL-8 mRNA in fMLF-treated neutrophils occurred at the level of mRNA synthesis, not at the level of RNA stability (our unpublished observations). Hence, it is likely that the elevated levels of IL-8 transcripts detected in CGD neutrophils were the result of prolonged transcription of the IL-8 gene in response to formyl peptide stimulation and not the attenuation of IL-8 transcript degradation.

In contrast to the processes regulating IL-8 synthesis, treatment of neutrophils with fMLF resulted in similar transient increases in IL-1β mRNA in normal and CGD neutrophils, in agreement with the IL-1β protein response (Fig. 5*B*). Interestingly, a small, but significant, increase in GAPDH mRNA was observed after treatment with fMLF, but these responses were not different in normal and CGD neutrophils (Fig. 5*C*).

Effects of scavengers and inhibitors of ROI in normal cells (Fig. 6)

The functional NADPH oxidase enzyme complex generates H_2O_2 in addition to other free radical oxygen molecules. In CGD cells, mutation or deletion of any one of four components of NADPH oxidase will result in a nonfunctional enzyme with little or no generation of ROI. NADPH oxidase catalyzes a single electron reduction of O_2 to superoxide anion, O_2^-, with subsequent conversion to H_2O_2 by superoxide dismutase. Neutrophil-derived myeloperoxidase, in turn, produces hypochlorous acid from H_2O_2 and

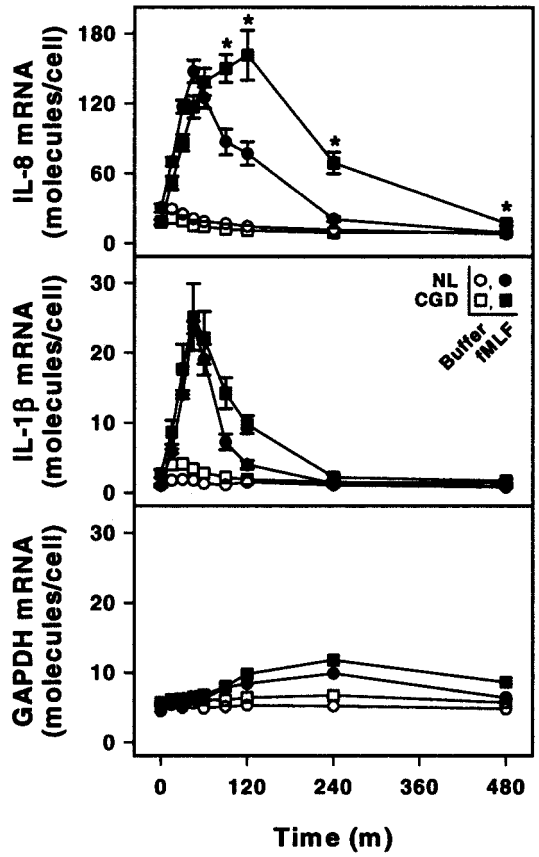

FIGURE 5. fMLF induces transient alterations in IL-8, IL-1β, and GAPDH mRNA. Neutrophils (2×10^6/ml) isolated from both 15 normal subjects (NL; ● and ○) and 14 CGD patients (■ and □) were treated with fMLF (5×10^{-9} M) for the indicated times, harvested by centrifugation, and dissolved in the cell lysis buffer. Levels of IL-8, IL-1β, and GAPDH mRNA were determined as described in *Materials and Methods*. Error bars not shown are buried within the symbol. *, $p < 0.01$.

chloride. DPI inhibits NADPH oxidase and other flavoenzymes by electron transfer and phenylation of the FAD component of the oxidase enzyme apparatus (20). Using scavengers and enzyme inhibitors of the ROI cascade, we examined the effects of individual components of the NADPH oxidase-driven respiratory burst on IL-8 production in normal volunteer neutrophils. Normal neutrophils treated with catalase (1000 U/ml), which degrades extracellular H_2O_2, produced significantly higher levels of IL-8 compared with PBS-treated cells (5.50 ± 0.82 vs 1.06 ± 0.31; $p < 0.001$). Addition of superoxide dismutase, which catalyzes the conversion of O_2^- to H_2O_2, had no effect on neutrophil IL-8 production compared with that by control PBS-treated cells. Other oxygen intermediate scavengers, such as histidine, taurine, and DMSO, which reduce levels of singlet oxygen ($^1O_2$), hypochlorous acid, and hydroxyl radical (· OH), respectively, had no effect on neutrophil IL-8 compared with PBS-treated cells. Thus, elimination of H_2O_2 from the buffer with catalase or prevention of its synthesis with DPI resulted in elevated IL-8 protein levels in normal neutrophils, analogous to observations made with CGD neutrophils, suggesting that H_2O_2 may be the causative agent in modulating IL-8 synthesis. We next examined the effects of adding H_2O_2 to normal neutrophils to determine whether formyl peptide-stimulated IL-8 production could be suppressed.

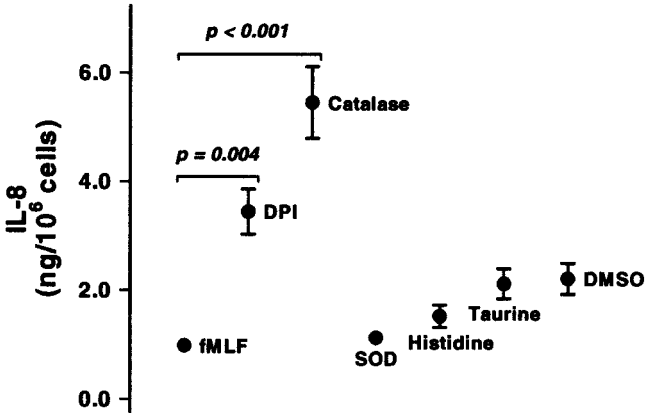

FIGURE 6. Effects of scavengers of ROI on IL-8 accumulation in normal neutrophils. Neutrophils (1×10^6/0.5 ml) were treated with DPI (2 μM), catalase (1000 U/ml), superoxide dismutase (SOD; 100 μg/ml), histidine (100 μM), taurine (10 mM), or DMSO (10 mM) for 10 min at 37°C before the addition of fMLF (5×10^{-9} M). Neutrophil IL-8 was determined as described in Fig. 1. The data represent the mean ± SEM of five experiments.

Effects of hydrogen peroxide and hypoxanthine plus xanthine oxidase

To test the hypothesis that exogenous H_2O_2 would suppress the synthesis of IL-8 in neutrophils, we examined the effects of the addition of H_2O_2 directly to cells in buffer as well as using the hypoxanthine/xanthine oxidase H_2O_2-generating system. Addition of physiologic doses of H_2O_2 (10–100 nmol/ml, levels comparable to those produced by 1×10^6 normal neutrophils activated with 100 ng/ml PMA) to normal neutrophils immediately before the addition of fMLF resulted in a dose-dependent inhibition of IL-8 production, but had no effect on untreated cells (Fig. 7, *top*). This inhibition was completely abrogated by the addition of catalase. Moreover, the addition of a H_2O_2-generating system, hypoxanthine plus xanthine oxidase, resulted in a dose-dependent inhibition of fMLF-induced IL-8 production, but had no effect on IL-8 production in untreated cells (Fig. 7, *bottom*). Maximum inhibition was achieved at a dose of 0.3 mU of xanthine oxidase activity/ml. This level of hypoxanthine plus xanthine oxidase activity resulted in the production of 10–15 nmol of H_2O_2 during a 2-h incubation compared with 100–150 nmol of H_2O_2 produced after treatment of normal neutrophils with 100 ng/ml phorbol ester. Interestingly, neutrophils appeared to be more sensitive to a lower level of H_2O_2 produced in a sustained fashion with the hypoxanthine plus xanthine oxidase-generating system than the same level of H_2O_2 (10^{-4} M; Fig. 7) given as a bolus. Heat treatment of the xanthine oxidase resulted in abrogation of its inhibitory effect, indicating that the activity of the enzyme was responsible for the inhibition.

Effect of ROI on cytokine production in normal neutrophils

To determine whether the enhancement of IL-8 levels with NADPH oxidase inhibition could be generalized to other cytokines and chemokines produced by neutrophils, immunoassays were conducted on normal neutrophils treated with buffer or fMLF (5×10^{-9} M) or with concurrent NADPH oxidase inhibition with either DPI (2 μM) or scavenging of hydrogen peroxide with catalase (1000 U/ml; Table I). Differences in chemokine production with NADPH oxidase inhibition reached statistical significance only with IL-8 and IL-β. Other cytokines and chemokines, including TNF-α, IL-6, IL-1R antagonist, GRO-α, MIP-1α, and MCP-1, showed no enhanced production with NADPH oxidase inhibition.

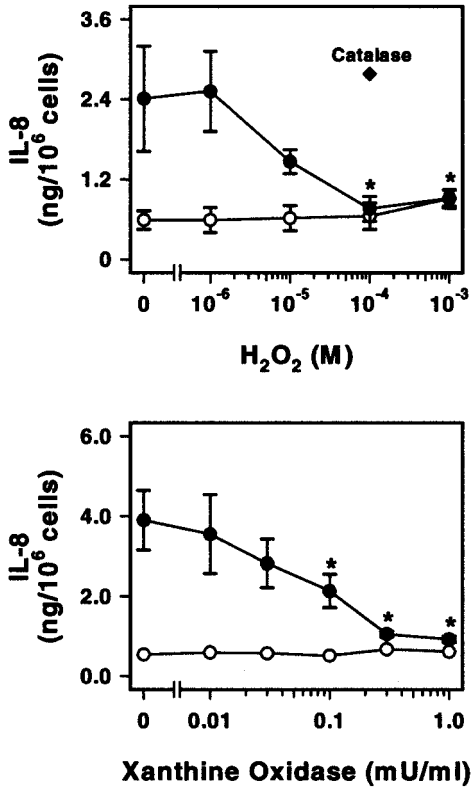

FIGURE 7. Effects of H_2O_2 and hypoxanthine/xanthine oxidase on IL-8 production in normal neutrophils treated with fMLF. Normal neutrophils were incubated in the presence of increasing doses of either H_2O_2 (*top panel*; n = 3) or xanthine oxidase plus 50 μM hypoxanthine (*bottom panel*; n = 10) in the absence (○) or the presence (●) of fMLF (5×10^{-9} M). IL-8 accumulation was determined as described in Fig. 1. Addition of catalase (1000 U/ml) to 10^{-4} M H_2O_2 (◆) reversed the effect of H_2O_2. *, $p < 0.05$, comparing cells treated with H_2O_2 or hypoxanthine plus xanthine oxidase with cells treated with buffer alone.

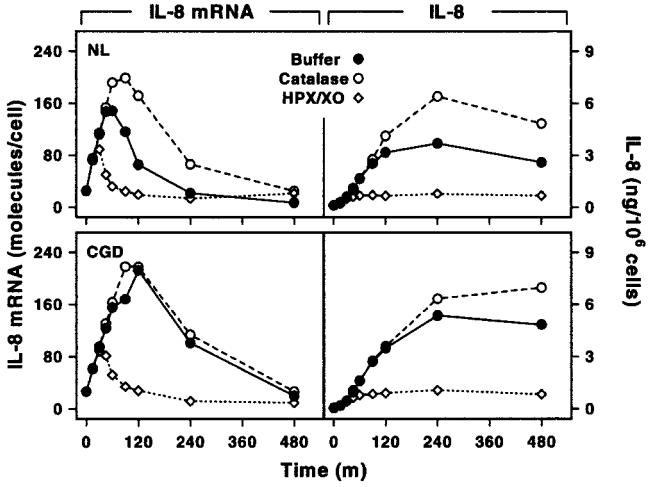

FIGURE 8. Effects of catalase and hypoxanthine/xanthine oxidase on normal (NL) and CGD IL-8 mRNA and protein synthesis. Neutrophils isolated from normal subjects (*top panel*; n = 3) and patients with CGD (*bottom panel*; n = 3) were treated with fMLF (5×10^{-9} M) in the presence of buffer, catalase (1000 U/ml), or hypoxanthine (50 μM)/xanthine oxidase (0.3 mU/ml). Neutrophil IL-8 mRNA and protein were determined as described in Figs. 1 and 3.

Effect of catalase and hypoxanthine/xanthine oxidase on normal and CGD IL-8 protein and mRNA (Fig. 8)

Because CGD neutrophils fail to produce ROI, it was postulated that ROI could regulate IL-8 mRNA. Addition of catalase (1000 U/ml; a scavenger of hydrogen peroxide) to normal neutrophils resulted in a prolonged IL-8 mRNA response and increased IL-8 protein ($p = 0.008$ and $p = 0.0465$, respectively, vs fMLF alone) comparable to the response observed in CGD neutrophils (Fig. 8). Catalase had no effect on the IL-8 mRNA and IL-8 protein response of CGD neutrophils treated with fMLF. In contrast, the addition of an O_2^-/H_2O_2-generating system, hypoxanthine/xanthine oxidase (0.3 mU/ml) inhibited the IL-8 mRNA and IL-8 protein response in both normal and CGD neutrophils ($p < 0.05$ vs fMLF alone). Control experiments using labeled recombinant human IL-8 protein or mRNA demonstrated that H_2O_2 (10^{-7}–10^{-3} M) did not degrade IL-8 protein or mRNA.

Discussion

Redox responsive transcriptional regulation has been shown in a number of studies to induce IL-8 expression in epithelial and endothelial cell lines (11–15). In human neutrophils, we show that inhibition of NADPH oxidase by a variety of chemical means and a genetic defect significantly increases IL-8 production in response to formyl peptide stimulation. Exposure of CGD neutrophils to an H_2O_2-generating system, hypoxanthine plus xanthine oxidase, under conditions that generate amounts of H_2O_2 comparable to those

Table I. *Cytokine specificity in normal neutrophils*[a]

Analyte	Buffer	fMLF (5×10^{-9} M)	+ DPI	+ Catalase
IL-8	340 ± 60	2030 ± 640[b]	2810 ± 330[c]	3270 ± 500[c]
IL-1β	0.34 ± 0.10	1.73 ± 0.18[b]	8.91 ± 3.35[c]	15.66 ± 7.66[c]
TNF-α	0.45 ± 0.23	2.98 ± 1.63	2.25 ± 1.26	1.73 ± 0.54
IL-6	0.04 ± 0.03	0.13 ± 0.09	0.12 ± 0.07	0.36 ± 0.14
IL-1R antagonist	1150 ± 270	1310 ± 360	1200 ± 340	1580 ± 410
GRO-α	70.6 ± 15.3	62.1 ± 13.2	78.8 ± 13.5	84.7 ± 15.0
MIP-1α	5.17 ± 1.49	7.45 ± 1.78	6.73 ± 1.34	17.38 ± 4.56
MCP-1	2.11 ± 1.60	2.04 ± 1.47	2.26 ± 1.31	3.65 ± 2.11

[a] Normal neutrophils (2×10^6/ml) were incubated for 10 min at 37°C with either DPI (2 μM) or catalase (1000 U/ml). fMLF (5×10^{-9} M) was then added, and the incubation was continued for 8 h. The incubation was terminated with the addition of ice-cold Triton X-100 (0.2%). The cellular lysates were analyzed for the indicated analytes. Values are expressed as picograms per 10^6 cells.

[b] $p < 0.05$ vs buffer.

[c] $p < 0.05$ vs fMLF.

produced by normal neutrophils (21), resulted in normalization of CGD IL-8 production to levels found in normal neutrophils. These results indicate a correlative relationship among neutrophil-associated hydrogen peroxide, NADPH oxidase activity, and generation of IL-8 mRNA and protein and suggest a chemical and biological mechanism related to activation of the NADPH oxidase apparatus that provides negative feedback to the transcription of IL-8 mRNA. Removal of the regulatory feedback by scavenging hydrogen peroxide with catalase results in the rapid (30 min) decline of IL-8 mRNA gene transcription in normal volunteer neutrophils. Loss of this regulatory signal in CGD patients results in sustained IL-8 transcription up to 4 h after neutrophil activation and a 2- to 4-fold increase in IL-8 protein production. Evidence in CGD animal models suggests that the elevated levels of chemokines homologous to IL-8 cause the generation of neutrophilic granuloma (22).

Although no significant differences in the IL-1β mRNA and protein responses to fMLF were observed in the neutrophils isolated from normal subjects and CGD patients, the primary focus of this report, there was some indication that the IL-1β response to fMLF could be modulated by ROI. In Table I and data not shown, the addition of catalase enhanced the IL-1β protein response, whereas the addition of xanthine oxidase suppressed the response. The transience of the IL-1β protein response to fMLF suggested that IL-1β was being internalized and/or degraded during the incubation. In a single experiment, treatment of normal neutrophils with catalase had little measurable effect on the IL-1β mRNA response to fMLF, suggesting that the effect of catalase on the IL-1β protein response was more likely attributable to either stabilization of the protein through inhibition of internalization and/or degradation or other complex mechanisms and was not an effect on mRNA.

Patients with CGD display a profound loss of their innate immune response as a consequence of their lack of peroxide production in response to microbial agents. Excessive granuloma formation, resulting in gastrointestinal and genitourinary obstructions, also occurs frequently in CGD patients; however, the underlying mechanism is not known (10). Similarly, neutrophil migration and accumulation in CGD patients measured with the Rebuck skin window technique are increased (23). The $p47^{phox}$ and $gp91^{phox}$ murine knockout models of CGD both display evidence of excessive inflammatory responses (24–26). For example, $p47^{phox}$- and $p91^{phox}$-deficient mice show significantly increased neutrophil egress into the peritoneal cavity with the sterile inflammatory stimulus, thioglycolate (24, 25). However, X-linked, $gp91^{phox}$-deficient mice develop a profuse granulomatous inflammatory reaction in response to pulmonary inoculation with sterile *Aspergillus fumigatus* hyphae not seen in control mice (26). The level of keratinocyte-derived cytokine mRNA in total lung RNA extracts is significantly elevated in $gp91^{phox}$ knockout mice compared with that in wild-type mice 24 h to 1 wk after inoculation (26). Keratinocyte-derived cytokine, the murine GRO-α homologue that binds the murine IL-8 orphan receptor, is a potent chemoattractant for mouse neutrophils and is the likely inflammatory signal inducing neutrophil migration and the sterile neutrophilic pneumonitis seen in this model (27).

It has been reported previously that in vitro CGD neutrophils accumulate the chemoattractant LTB_4 due to reduced LTB_4 degradation by reactive oxygen species (28). Our findings with increased IL-8 cannot be explained by this mechanism and reveal a new regulatory mechanism to modulate and abate cellular recruitment of the innate immune response. The transcription factors AP-1 and NF-κB, both of which bind to regulatory sites in the IL-8 gene, are thought to exhibit differential redox regulation: reductants favor AP-1 activity, whereas oxidants dramatically enhance NF-κB activity (29). However, DNA binding studies that we have performed failed to show any effect of catalase or hypoxanthine plus xanthine oxidase on normal neutrophil AP-1 and NF-κB activities. Therefore, the mechanism for hydrogen peroxide regulation of IL-8 synthesis remains to be elucidated. Coupling successful neutrophil migration and activation with the release of oxidants to the signals that diminish these responses represents a pivotal regulatory mechanism in inflammation and a potential site for pharmacological intervention in the outcome of inflammatory responses.

Acknowledgments

We thank Debra Long Priel, Rhonda DaSilva, and Danielle Fink for their excellent technical assistance. We are also grateful to Dr. Mariusz Lubomirski for his contributions to the statistical analyses.

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