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Enzymatically Inactive Eosinophil Peroxidase Inhibits Proinflammatory Cytokine Transcription and Secretion by Macrophages

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The present investigators have reported previously that macrophages (Mϕ) can bind either myeloperoxidase (MPO) or eosinophil peroxidase (EPO) resulting in enhanced cytotoxicity to *Candida albicans.* Since MPO was shown to be immunomodulatory, the present study was initiated to determine whether either EPO or partially fragmented EPO (fgEPO) also modulated cytokine secretion. Murine peritoneal Mϕ simultaneously stimulated with fgEPO and one of the following, (1) LPS, (2) mannosylated bovine serum albumin (mBSA), (3) interferon-γ (IFN-γ), or (4) Poly I:C, demonstrated both dose- and time-dependent decreases in TNF-α and IL-6 and a dose-dependent decrease in IFN-α/β. The mRNA levels of Mϕ exposed to fgEPO and mBSA demonstrated that fgEPO modulated Mϕ cytokine function by decreasing TNF-α and IL-6 mRNA transcripts without altering transcription of TGF-β or GM-CSF. These results demonstrate a possible interaction between the Mϕ and eosinophil that could result in reduction of inflammation.

INTRODUCTION

It has been reported that eosinophils, neutrophils (PMN), and macrophages (Mϕ) are abundant in inflammatory responses associated with disease states such as asthma and allergy (1–4). However, the function of the Mϕ in the above diseases is largely unknown. Classically, in these diseases, Mϕ were thought to have a minimal role in the clearance of antigens from the infection site and a major role in the recruitment of other cells, such as the PMN (1, 5). Within the context of inflammation, it is known that Mϕ contribute to the inflammatory process by numerous pathways including the secretion of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6). All of these cytokines are considered to be proinflammatory.

In addition to Mϕ, neutrophils and eosinophils are frequently the host's first line of defense against invading pathogens (6). In particular, eosinophilia is associated with certain diseases such as asthma. Although a high eosinophil count has been associated with this disease, the number of Mϕ and PMN was thought to dramatically decrease in the latter stages of asthma or allergy (5). Recently, however, researchers have demonstrated that in addition to eosinophils, both Mϕ and PMN are present throughout the course of these diseases and may play a more active role in the return of the immune system to homeostasis (2, 4, 5, 7).

Although the incidence of asthma and allergy has been increasing in the general population for the past 2 decades (7–10), the role eosinophils play in asthma has not been fully elucidated. Several lines of evidence support the concept that eosinophils play a pivotal role in this disease. Besides their increased presence in the circulation, bronchial secretions, and lung parenchyma, eosinophils generate various substances that are injurious to the lung. Within the granules of eosinophils, numerous mediators are found including eosinophil peroxidase (EPO) (11–13). This enzyme participates in a cytotoxic triad similar to that formed utilizing myeloperoxidase (MPO). Like MPO, EPO is a highly cationic, mannosylated protein that shares 68% amino acid sequence homology with MPO (14). Although it is known that EPO is bound and internalized by neutrophils (15), its effect on Mϕ has not been investigated extensively. With respect to neutrophils and MPO, alveolar Mϕ ingest MPO from the microenvironment and utilize the enzyme in cytocidal activities (16, 17). Therefore, Mϕ at a site inflammation would be exposed to *both* MPO and EPO as well as their degradation products, i.e., enzymatically inactive MPO (iMPO) and partially fragmented EPO (fgEPO).

Previous work in our laboratory has demonstrated that MPO modulates Mϕ functions. These studies have shown that either recombinant MPO or recombinant iMPO could alter the following: (1) phagocytosis and killing of both *Escherichia coli* and *Candida albicans* (18, 19), (2) release of reactive oxygen intermediates (ROI) (18, 20), and (3) cytokine secretion (unpublished

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data). In the latter studies both MPO and iMPO were employed and iMPO was consistently more potent in inducing Mϕ cytokine secretion than MPO. Therefore, these data suggest that the Mϕ can bind both enzymatically active and inactive carbohydrate-rich peroxidases resulting in altered immune function.

Because of the similarity of the two enzymes, our original hypothesis was that EPO would modulate Mϕ activities similar to MPO. The purpose of this study was to determine the effects of EPO on Mϕ cytokine secretion. Modulation of Mϕ cytokine production or secretion by either EPO or fgEPO would elucidate a possible unrecognized interaction between the eosinophil and Mϕ and would further demonstrate a function for the Mϕ in the regulation of inflammation associated with various disease states.

MATERIALS AND METHODS

Animals

Age-matched, C57BL/6 mice, 18–22 g of either sex were purchased from The Jackson Laboratories (Bar Harbor, ME). All animals were maintained in facilities that abided by federal guidelines for animal care.

Materials

Three preparations of EPO were utilized in this study: (1) enzymatically active purified porcine EPO (pEPO), a generous gift from EOE, Inc. (Little Rock, AR), (2) fgEPO generated by the papain digestion of pEPO (fgEPO(P)), and (3) partially fragmented human EPO (fgEPO(H)) obtained from patients, which was generously provided by Dr. Gerald Gleich (Mayo Clinic, Rochester, MN). In this manuscript, fgEPO refers to both fgEPO(P) and fgEPO(H). Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO (Long Island, NY). Mannosylated bovine serum albumin (mBSA) was purchased from EY Laboratories (San Mateo, CA) and interferon-γ (IFN-γ) was purchased from Endogen (Woburn, MA). Phosphate-buffered saline, pH 7.2 (PBS), was prepared as needed. The following were purchased from Sigma (St. Louis, MO): Poly I:C, gentamicin sulfate, lipopolysaccharide (LPS), and Hepes. Fetal bovine serum (FBS) was obtained from Intergen (Purchase, NY). Other commercially purchased reagents were methylcellulose (Fisher Scientific, Pittsburgh, PA) and a modified Lowry protein determination assay (Biorad, Hercules, CA). All reagents were tested for endotoxin contamination using the Limulus amoebocyte lysate test (Associates of Cape Cod, Woods Hole, MA). All preparations of EPO employed in these studies contained $\leq$0.01 ng/ml of endotoxin. This level of endotoxin did not stimulate Mϕ cytokine secretion.

Macrophage Collection

Murine resident and thioglycollate-induced peritoneal Mϕ (TG-Mϕ) were collected as described previously (21). Briefly, mice were sacrificed by cervical dislocation and Mϕ collected by peritoneal lavage using PBS at 4°C. Cells were washed and resuspended in DMEM supplemented with gentamycin and 25 mM Hepes. The Mϕ cell number was adjusted to 1.2×10^6 Mϕ/ml and 100 μl of the cell suspension was added to each well of a 96-well Costar microtiter plate (Fisher Scientific). Following incubation for 2 h at 37°C under 5% CO_2, cell monolayers were washed to remove nonadherent cells. The Mϕ population was $\geq$99% as determined by microscopy.

Protease Digestion of EPO

Agarose beads containing covalently bound papain were purchased from Pierce (Rockford, IL). Porcine EPO, bovine serum albumin (BSA), or PBS was incubated for 4 h with papain-coated agarose beads at 37°C under 10% CO_2. Following incubation, the beads were removed by centrifugation. Digested EPO or BSA was stored in aliquots at $-$70°C until needed. Samples were shown to have similar protein concentrations before and after protease digestion, as determined by a modified Lowry protein determination assay. The enzymatic activity of pEPO and fgEPO incubated with the beads was determined using *o*-dianisidine as a substrate. Since there was no protein noted in PBS controls, papain did not appear to leach from the beads. Also, following exposure to the beads, increase in protein was noted for either BSA or fg(EPO). There was, however, a marked reduction in enzymatic activity of the fgEPO ($>$99%).

Enzyme-Linked Immunosorbent Assay (ELISA)

Sandwich ELISA minikits were purchased from the following manufacturers: (1) TNF-α kit (Genzyme, Cambridge, MA), (2) IL-6 kit (Endogen, Cambridge, MA), and (3) transforming growth factor-β (TGF-β) kit (Promega, Madison, WI). All ELISA experiments utilized optimized procedures provided by the manufacturer. Briefly, Maxisorb 96-well microtiter plates (Nunc Inc., Naperville, IL) were coated with monoclonal antibody specific for the cytokine of interest. Wells were washed using a wash bottle, blocked with medium containing bovine serum albumin, and incubated with sample. Following incubation, wells were washed again and horseradish peroxidase (HRP)-labeled, polyclonal antibody specific for the cytokine of interest added. After incubation, wells were washed and the amount of HRP-labeled antibody was detected with tetramethylbenzidine. Absorbances were read at a wavelength of 450 nm on a 96-well microtiter plate reader (Dynatec Laboratories, Chantilly, VA).

Ribonuclease Protection Assay (RPA)

A semi-quantitative RPA was used to measure levels of RNA. Total RNA utilized in RPA was obtained from 1.2×10^6 TG-Mϕ using TRIzol reagent (Gibco BRL, Gaithersburg, MD) as described by the manufacturer. Isolated RNA samples were reconstituted in DEPC-treated H_2O at 1 mg/ml as determined by UV-spectrophotometric analysis. A RiboQuant multiprobe nonradioactive RPA, containing TNF-α, IL-1α, IL-1β, TGF-β, and granulocyte–Mϕ colony stimulating factor cytokine probe templates, was purchased from Pharmingen (San Diego, CA). All experiments performed utilized an optimized procedure provided by the manufacturer. Briefly, 5 μl of sample, containing 5 μg RNA, was placed into tubes (Marsh Biomedicals, Rochester, NY) and dried in an Eppendorf vacuum evaporator centrifuge (Fisher Scientific). Samples were reconstituted in hybridization buffer and allowed to hybridize with excess biotinylated-RNA probe sets, thereby generating dsRNA. Samples were subsequently treated with RNase to degrade unhybridized ssRNA and hybridized RNA extracted using phenol:chloroform. Hybridized dsRNA was precipitated and reconstituted in loading buffer that contained tracking dyes. Samples were separated on a 5% acrylamide/8 M urea gel and transferred, via electrophoresis (CBS Scientific, Del Mar, CA), to Hybond (+) charged nylon membrane (Amersham, Arlington Heights, IL). The membrane was subsequently dried and RNA cross-linked in an 80°C oven for 2 h. The membrane was blocked in a casein-containing solution (Boehringer Mannheim, Indianapolis, IN) and treated with Avidx-AP (Tropix, Bedford, MA), an avidin-conjugated alkaline phosphatase. The sealed membrane was exposed to CDP-Star substrate (Tropix) to initiate chemiluminescence. Subsequently, the membrane was exposed to CL-Hyperfilm (Amersham) for 1.5 h and developed.

Interferon Bioassay

Thioglycollate-induced peritoneal Mϕ were cultured with various concentrations of fgEPO and 0.625 μg/ml of Poly I:C for 2 h. After incubation, cells were washed to remove residual fgEPO and poly I:C and incubated with fresh medium for 6 h. Culture media were subsequently collected and assayed for presence of interferon-α/β (IFN-α/β) using the method described by Mone and Lefkowitz (22). Briefly, undiluted samples were added to monolayers of L929 cells in a 96-well plate and a 1:2 serial dilution was performed in the wells. After a 12-h incubation, culture media were removed and cells were infected with 40 plaque-forming units of vesicular stomatitis virus for 1 h prior to the addition of 0.5% methylcellulose in DMEM 2% FBS. Once plaques had formed, cells were fixed and stained with crystal violet that was dissolved in methanol. The number of plaques was enumerated and IFN-α/β units were calculated using the formula previously described by Epstein (23).

TABLE 1
Comparison between pEPO and fgEPO

Treatment	Protein concentration (μg/ml)	Activity (U/ml)
pEPO	1026.2 ± 34	1820 ± 150
fgEPO(P)	1064.5 ± 15	3 ± 3**
fgEPO(H)	1430 ± 37	0**

Note. The concentration of protein present in all treatments utilized in this study was determined using a modified Lowry assay. Tubes containing sample plus working reagent were incubated for 15 min at 24°C. After incubation, the absorbance at 550 nm was measured and the concentration was determined relative to an albumin standard curve. In addition, enzyme activity of all treatments in this study was determined using *o*-dianisidine as a substrate. Cuvettes containing sample plus *o*-dianisidine substrate were incubated at 24°C for 1 min. After incubation, the absorbance at 460 nm was measured and converted to units of enzyme activity per milliliter. Values represent the mean ± SEM of duplicate readings. All experiments were repeated at least three times.

**$P \leq 0.001$.

Statistical Analysis of Data

One-way analysis of variance and Student–Newman–Keuls multiple comparison tests were performed to determine significance levels among the different treatment groups and controls. Nontransformed means are illustrated in the appropriate figures. Data are presented as the mean ± SEM. All experiments were repeated at least three times.

RESULTS

Polyacrylamide Comparison of Various fgEPO

It has been speculated that a cysteine protease such as cathepsin L, which may be present in eosinophilic granules, could cause proteolytic degradation of EPO *in vivo* (Gleich, personal communication). Because of this, digestion was done *in vitro* using papain, a well-characterized cysteine protease similar to cathepsin L. Treatment of enzymatically active pEPO with papain resulted in a significant reduction in enzymatic activity as measured by *o*-dianisidine (Table 1). Digestion of pEPO with papain resulted in almost complete loss of enzymatic activity similar to the fragmented enzyme obtained *in vivo* (Table 1). To compare fgEPO isolated *in vivo* with that obtained *in vitro,* both preparations were separated on nondenaturing and denaturing polyacrylamide gels (Figs. 1A and 1B). All lanes received 10 μg protein as determined by a modified Lowry assay. Both fgEPO(H) and fgEPO(P) demonstrated similar banding patterns with molecular weight fractions ranging from 14 to 65 kDa.

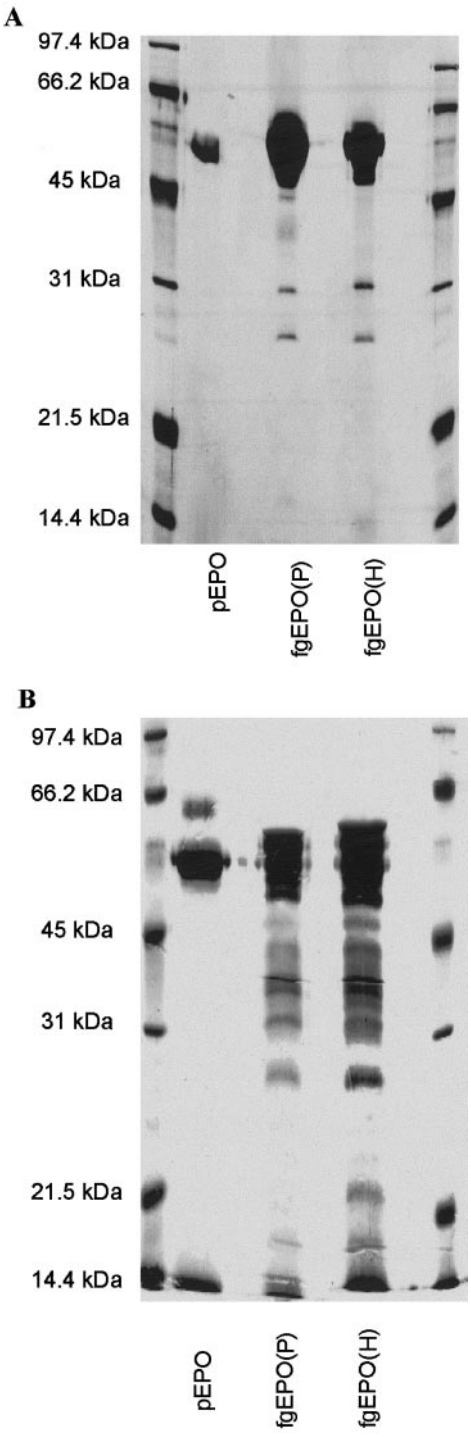

FIG. 1. (A) Nondenaturing PAGE. Ten micrograms of the indicated proteins was loaded, under nondenaturing conditions, into each lane of an acrylamide gel. The gel was subsequently stained with Coomassie brilliant blue and bands were visualized. The degree of fragmentation in digested samples [(fgEPO(P) and fgEPO(H)] can be compared to undigested pEPO. (B) SDS–PAGE. Ten micrograms of the indicated proteins was loaded, under denaturing conditions, into each lane of an acrylamide gel. The gel was subsequently stained with Coomassie brilliant blue and bands were visualized. The degree of fragmentation in digested samples [fgEPO(P) and fgEPO(H)] can be compared to undigested pEPO.

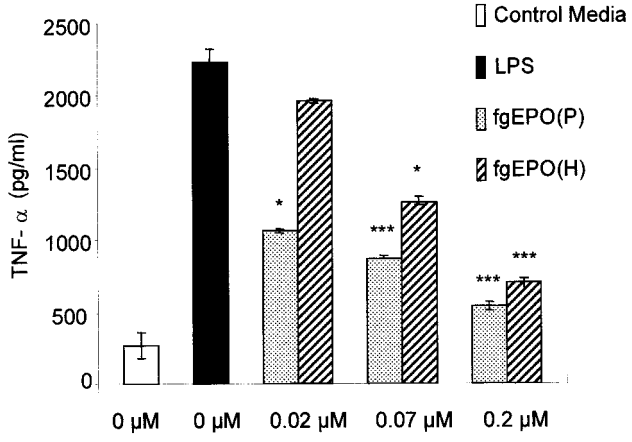

FIG. 2. Dose effect of fgEPO on the secretion of TNF-α by LPS-induced Mϕ. One hundred microliters of TG-Mϕ, at 1.2×10^6 cells/ml, was added to each well of a microtiter plate. After a 2-h attachment, monolayers were washed and treated as follows: (1) media alone, (2) media containing 0.06 ng/ml LPS, or (3) media containing 0.06 ng/ml LPS with various concentrations of fgEPO. After 6 h of incubation, supernatants were removed and assayed for TNF-α via ELISA. Values represent the means $\pm$ SEM of duplicate wells. All experiments were repeated at least three times. $*P \leq 0.05$, $***P \leq 0.001$.

Modulation of Mϕ Cytokine Secretion by fgEPO

Initial cytokine studies were undertaken to determine the effect of pEPO on Mϕ cytokine secretion. Macrophages exposed to various concentrations of the intact enzyme did not exhibit increased TNF-α secretion (data not shown). Other studies were done which utilized Mϕ simultaneously exposed to fgEPO and one of the following: (1) 0.06 ng/ml LPS, (2) 12.5 μg/ml mBSA, (3) 10 U/ml IFN-γ, (4) 0.625 μg/ml Poly I:C. (These concentrations were determined to be stimulatory in previous studies.) In preliminary experiments, TG-Mϕ were stimulated simultaneously with LPS and various concentrations of fgEPO for 6 h. Following 6 h of incubation, supernatants were removed and assayed for TNF-α via ELISA. LPS-stimulated Mϕ exposed to fgEPO demonstrated a dose-dependent decrease in the levels of secreted TNF-α (Fig. 2). Since EPO is known to directly bind LPS, which could inhibit stimulation of Mϕ, other studies were conducted utilizing mBSA as the stimulant. Macrophages simultaneously exposed to fgEPO and mBSA also demonstrated a dose-dependent decrease in TNF-α secretion (Fig. 3). At the lowest concentration utilized, 0.02 μM fgEPO, TNF-α titers were markedly less than that of Mϕ cultures exposed to mBSA alone. The greatest decrease in TNF-α secretion was obtained using 0.2 μM fgEPO.

To demonstrate that fgEPO could modulate Mϕ TNF-α secretion in a time-dependent manner, TG-Mϕ were treated with (1) medium alone, (2) medium containing 12.5 μg/ml mBSA, or (3) medium containing 12.5 μg/ml mBSA and 0.2 μM fgEPO. At various time

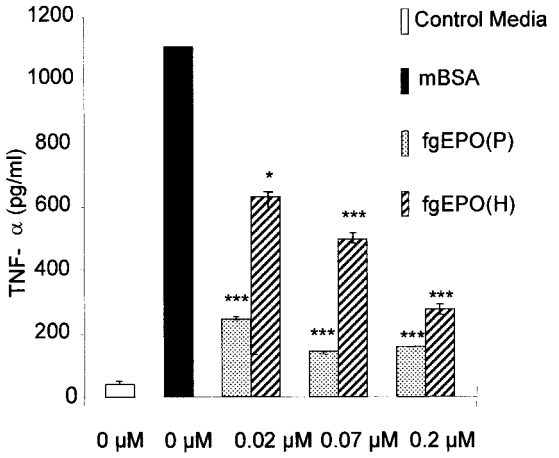

FIG. 3. Dose effect of fgEPO on the secretion of TNF-α by mBSA-induced Mϕ. One hundred microliters of TG-Mϕ, at 1.2×10^6 cells/ml, was added to each well of a microtiter plate. After a 2-h attachment, monolayers were washed and treated as follows: (1) media alone, (2) media containing 12.5 μg/ml mBSA, or (3) media containing 12.5 μg/ml mBSA with various concentrations of fgEPO. After 6 h of incubation, supernatants were removed and assayed for TNF-α by ELISA. Values represent the means $\pm$ SEM of duplicate wells. All experiments were repeated at least three times. $^*P \leq 0.05$, $^{***}P \leq 0.001$.

intervals, supernatants were removed and assayed via ELISA (Fig. 4). Supernatants removed after 1 h of incubation with mBSA and fgEPO displayed no differences in cytokine secretion compared to mBSA-stimulated controls. However, by 3 h there was greater than a 50% decrease in TNF-α titer from cultures treated with fgEPO(P) and greater than 25% decrease in TNF-α titer from fgEPO(H)-treated cultures. After 12 h of incubation, fgEPO(P)-treated cultures demonstrated greater than 67% decrease in TNF-α while fgEPO(H)-treated cultures demonstrated greater than 33% decrease in TNF-α titers (Fig. 4). Similar results were obtained with resident Mϕ (data not shown).

To determine if other "proinflammatory" cytokines (i.e., IL-6) were modulated similarly, resident peritoneal Mϕ were stimulated as described above and assayed for IL-6. Again, the data demonstrated that Mϕ treated with both mBSA and fgEPO demonstrated a time-dependent decrease in IL-6 secretion relative to those treated with mBSA alone (Fig. 5). Macrophages exposed to fgEPO(H) and mBSA for 3 h demonstrated only a 10% decrease in secreted IL-6 compared to mBSA-stimulated cultures. However, by 9 h, when maximal IL-6 secretion occurred, treated cells demonstrated greater than 33% decrease in IL-6. Cytokine levels in Mϕ exposed to fgEPO(P) and mBSA were dramatically decreased (>50%) within 3 h and remained about 75% below mBSA-alone-stimulated cultures by 9 h.

Macrophages can secrete cytokines that can either enhance or diminish inflammation. Transforming growth factor-β is considered to be among the latter. To determine the effects of fgEPO on the secretion of TGF-β, TG-Mϕ were treated with fgEPO alone or fgEPO and mBSA. Supernatants were subsequently removed at various time intervals and assayed for the presence of TGF-β. Cultures exposed simultaneously to both fgEPO and mBSA failed to demonstrate altered TGF-β titers relative to mBSA controls (Fig. 6).

Since mBSA could stimulate Mϕ cytokine secretion by binding to the macrophage mannose receptor (MMR) (24, 25), attempts were made to determine whether inhibition of cytokine secretion by fgEPO was a phenomenon restricted to only mannosylated stimuli. Therefore, Mϕ cytokine inducers, such as IFN-γ and Poly I:C, were utilized as stimulants. Thioglycollate-induced Mϕ treated with medium containing Poly I:C and fgEPO demonstrated both a dose- and time-dependent decrease in TNF-α secretion (Figs. 7 and 8). Additionally, TG-Mϕ stimulated with Poly I:C and fgEPO demonstrated similar results to those obtained with mBSA and fgEPO, i.e., no differences in secreted levels of TGF-β (data not shown). Similar results to that obtained with Poly I:C were noted when IFN-γ was employed as the stimulus (data not shown).

Poly I:C is known to induce Mϕ to secrete substantial quantities of IFN-α/β (26). Experiments were performed in which TG-Mϕ were stimulated for 2 h with one of the following: (1) medium alone, (2) medium containing 0.625 μg/ml Poly I:C, (3) medium contain-

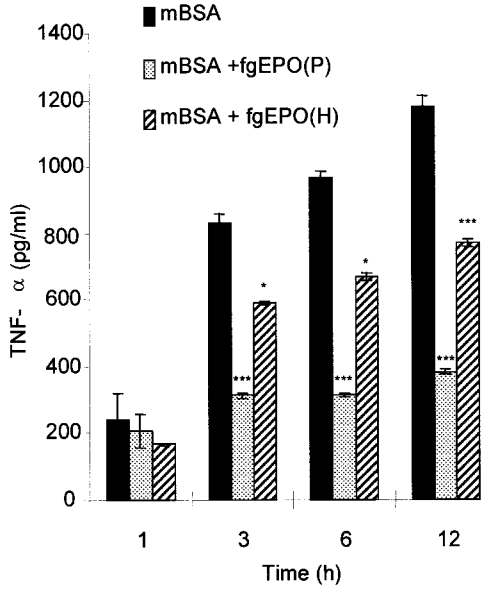

FIG. 4. Kinetics of TNF-α secretion by Mϕ exposed to fgEPO and mBSA. One hundred microliters of TG-Mϕ, at 1.2×10^6 cells/ml, was added to the wells of a microtiter plate. After a 2-h attachment, monolayers were washed and treated with (1) media containing 12.5 μg/ml mBSA or (2) media containing 12.5 μg/ml mBSA with 0.2 μM fgEPO. Supernatants were removed at various time periods and assayed for TNF-α via ELISA. Values represent the means $\pm$ SEM of duplicate wells. All experiments were repeated at least three times. $^*P \leq 0.05$, $^{***}P \leq 0.001$.

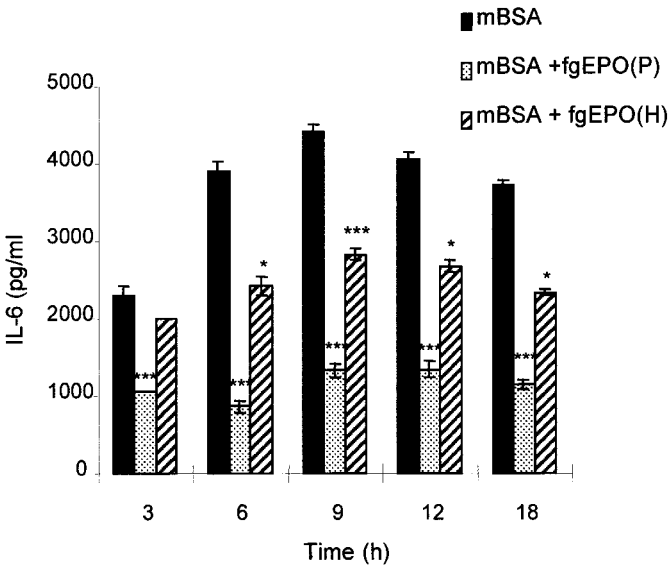

FIG. 5. Kinetics of IL-6 secretion by Mϕ exposed to fgEPO and mBSA. One hundred microliters of resident peritoneal macrophages, at 1.2×10^6 cells/ml, was added to each well of a microtiter plate. After a 2-h attachment, monolayers were washed and treated with (1) media containing 12.5 μg/ml mBSA or (2) media containing 12.5 μg/ml mBSA with 0.2 μM fgEPO. Supernatants were removed at various time periods and assayed for IL-6 via ELISA. Values represent the means ± SEM of duplicate wells. All experiments were repeated at least three times. *$P \leq 0.05$, ***$P \leq 0.001$.

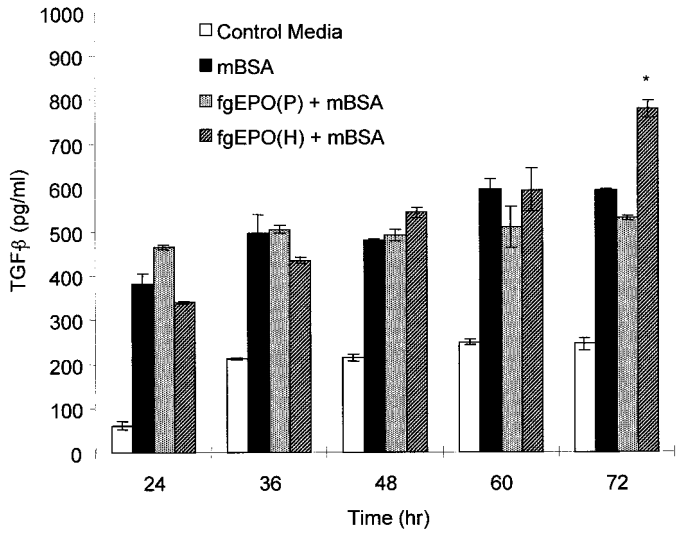

FIG. 6. Kinetics of TGF-β secretion by Mϕ exposed to fgEPO and mBSA. One hundred microliters of resident peritoneal macrophages, at 1.2×10^6 cells/ml, was added to each well of a microtiter plate. After a 2-h attachment, monolayers were washed and treated with (1) media containing 12.5 μg/ml mBSA or (2) media containing 12.5 μg/ml mBSA with 0.2 μM fgEPO. Supernatants were removed at various time periods and assayed for TGF-β via ELISA. Values represent the means ± SEM of duplicate wells. All experiments were repeated at least three times. mBSA vs fgEPO (H) + mBSA. *$P \leq 0.05$.

ing 0.625 μg/ml Poly I:C and various concentrations of fgEPO. Following incubation with the various treatments, supernatants were removed and analyzed for IFN-α/β by bioassay. Results demonstrated that fgEPO reduced the Poly-I:C-stimulated secretion of IFN-α/β by Mϕ in a dose-dependent manner (Fig. 9).

The figures utilized in this manuscript are representative of multiple experiments with each stimulus. Although the magnitude of cytokine levels appears different between fgEPO samples utilized with mBSA, LPS, and Poly I:C stimulants, slight variations in the potency of fgEPO during repeat experiments was observed. However, this variability among experiments was not significantly different.

Modulation of Mϕ Cytokine mRNA Levels by fgEPO

An RNase protection assay was employed to determine whether the reduction in proinflammatory cytokine secretion (TNF-α, IL-1α, IL-1β) described above was the result of a decrease in the transcription or translation of cytokine mRNA. The labeled cytokine RNA probe set utilized contained messenger for IL-1α, IL-1β, and TNF-α. The internal standards glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and L32, constitutive mRNA for maintenance genes, were also employed in all probe sets. As with other studies, TG-Mϕ were exposed to either medium alone or various concentrations of fgEPO and 12.5 μg/ml mBSA. After 2 h of incubation, Mϕ were lysed in TRIzol and total RNA was extracted. Samples were subsequently hybridized and resolved on an acrylamide/urea gel as

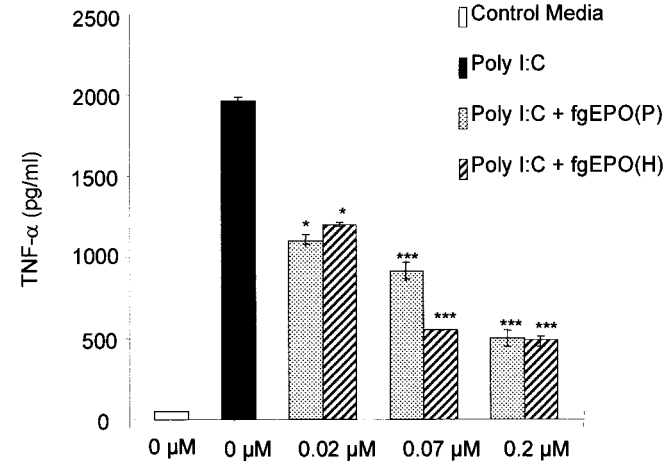

FIG. 7. Dose effect of fgEPO on the secretion of TNF-α by Poly I:C-induced Mϕ. One hundred microliters of TG-Mϕ, at 1.2×10^6 cells/ml, was added to the wells of a microtiter plate. After a 2-h attachment, monolayers were washed and treated as follows: (1) media containing 0.625 μg/ml Poly I:C or (2) media containing 0.625 μg/ml Poly I:C with 0.2 μM concentration of fgEPO. After 6 h of incubation, supernatants were removed and assayed for TNF-α via ELISA. Values represent the means ± SEM of duplicate wells. All experiments were repeated at least three times. *$P \leq 0.05$, ***$P \leq 0.001$.

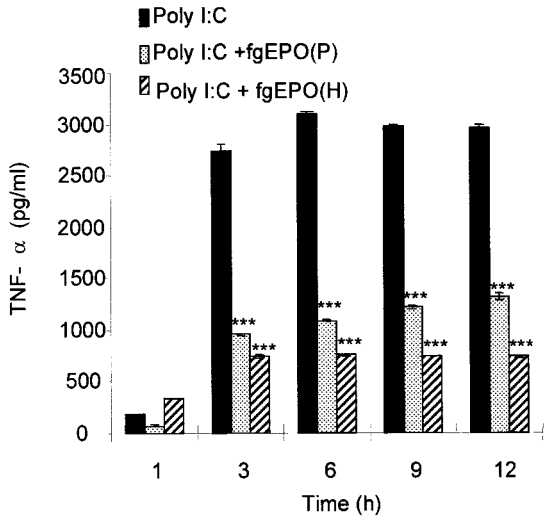

FIG. 8. Kinetics of TNF-α secretion by Mϕ exposed to fgEPO and Poly I:C. One hundred microliters of TG-Mϕ, at 1.2×10^6 cells/ml, was added to each well of a microtiter plate. After a 2-h attachment, monolayers were washed and treated as follows: (1) media containing 0.625 μg/ml Poly I:C or (2) media containing 0.625 μg/ml Poly I:C with 0.2 μM fgEPO. Supernatants were removed at various time periods and assayed for TNF-α via ELISA. Values represent the means ± SEM of duplicate wells. All experiments were repeated at least three times. $*P \leq 0.05$, $***P \leq 0.001$.

described previously. Macrophages exposed to mBSA and fgEPO demonstrated an observed dose-dependent decrease in TNF-α, IL-1α, and IL-1β mRNA. Messenger RNA levels for both the constitutive proteins, GAPDH and L32, appeared the same for all treatments (Fig. 10).

As stated previously, there were no differences measured in the secretion of TGF-β from TG-Mϕ exposed to fgEPO and mBSA. However, it is possible that fgEPO could either enhance or inhibit GM-CSF or TGF-β cytokine mRNA transcription without subsequent modulations in cytokine secretion. Therefore, total RNA from TG-Mϕ, treated as described above, were also hybridized with the appropriate cytokine probe set. The data demonstrate that fgEPO did not appear to affect the transcription of either TGF-β or GM-CSF (Fig. 11).

DISCUSSION

In vitro studies by the present investigators demonstrated that enzymatically active pEPO enhanced Mϕ-mediated phagocytosis and killing of *C. albicans* along with the production of various cytotoxic compounds such as ROI (27). Because *in vivo* one could find both intact EPO and fgEPO, the present study utilized both forms of this enzyme. Since activated Mϕ are more likely to be present during the later stages of asthma, allergy, or helminth infections than resident tissue Mϕ, TG-induced Mϕ were used as models of inflammatory Mϕ (7, 28). Moreover, it is during the later stages of these diseases that eosinophils are reported to be abundant; therefore, most experiments in this study utilized TG-Mϕ rather than resident Mϕ. The exception to this involves assays for IL-6. Because it has been reported previously and confirmed in this laboratory that resident peritoneal Mϕ but not TG-Mϕ secrete IL-6 (29), resident Mϕ were employed for studies involving this cytokine.

All previous experiments with pEPO involved short-term exposure of Mϕ to this enzyme (10 min or less). Cytokine induction experiments involved much longer exposure times; therefore, initial experiments were performed to determine whether exposure of Mϕ to pEPO for more than 1 h would be cytotoxic. The relative amount of cytotoxicity was measured by trypan blue dye exclusion. Intact pEPO was found to be highly toxic to Mϕ if the exposure time was greater than 30 min. The observed cytotoxicity could be due, in part, to the formation of toxic compounds such as hypohalous acids and the enhanced ROI production reported previously (27). Even at noncytotoxic concentrations (less than 0.07 μM), Mϕ that were exposed to pEPO did not demonstrate enhanced TNF-α secretion over that of the controls (data not shown).

As stated previously, it has been suggested that a protease, believed to be cathepsin L, is present within the eosinophil granule (unpublished data) (Dr. Gerald Gleich, personal communication). Cathepsin L, a cys-

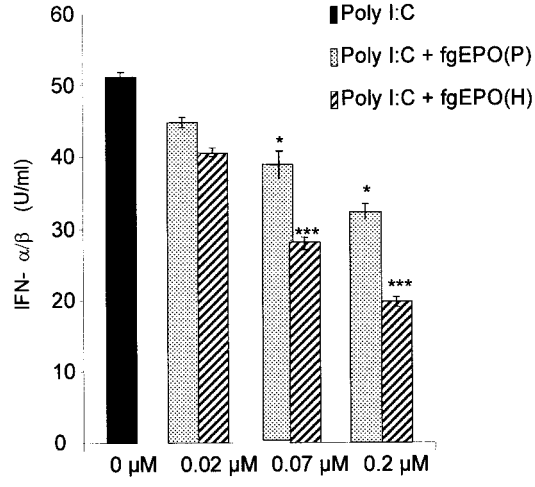

FIG. 9. Dose effect of fgEPO on the secretion of IFN-α/β by Poly I:C-induced Mϕ. One hundred microliters of TG-Mϕ, at 1.2×10^6 cells/ml, was added to each well of a microtiter plate. After a 2-h attachment, monolayers were washed and treated as follows: (1) media containing 0.625 μg/ml Poly I:C or (2) media containing 0.625 μg/ml Poly I:C with various concentrations of fgEPO. After an additional 2-h incubation with treatments, cells were washed vigorously to remove excess Poly I:C and incubated with fresh medium for 6 h. Supernatants were subsequently removed and assayed for IFN-α/β via bioassay. Values represent the means ± SEM of six replicate wells. All experiments were repeated at least three times. $*P \leq 0.05$, $***P \leq 0.001$.

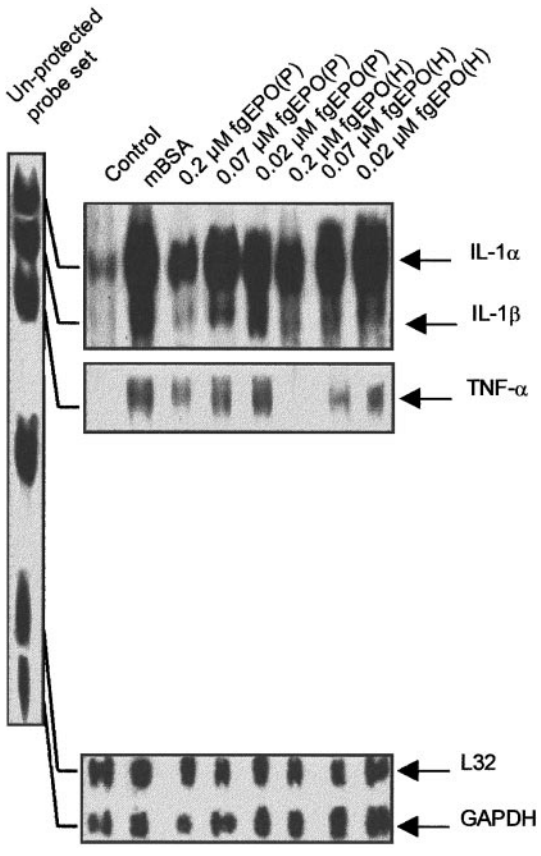

FIG. 10. Effects of fgEPO on "proinflammatory" cytokine mRNA levels in mBSA-induced Mϕ. One hundred microliters of TG-Mϕ, at 1.2×10^6 cells/ml, was added to the wells of a microtiter plate. After a 2-h attachment, monolayers were washed and treated in one of the following ways: (1) media alone, (2) media containing 12.5 μg/ml mBSA, or (3) media containing 12.5 μg/ml mBSA with various concentrations of fgEPO. After 2 h of incubation with treatments, media were removed and cells lysed in TRIzol. Total RNA was extracted and assayed for proinflammatory cytokine mRNA (TNF-α, IL-1α, and IL-1β) via RPA. The internal standards utilized were the constitutive mRNA for L32 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

teine protease that can be isolated from the liver, is available in insufficient quantity for use in an extensive study. Therefore papain, a similar and more readily available cysteine protease, was employed in this research. Papain covalently bound to an agarose matrix allows for the efficient treatment of a target protein sample while ensuring quick and complete removal of the protease following digestion. Enzymatically active pEPO treated *in vitro* with the above protease resulted in partial digestion products, fgEPO(P), similar to those isolated *in vivo*, fgEPO(H), as determined by PAGE analysis (Fig. 1). Both fgEPO preparations contained partial degradation products with similar molecular weight ranges. This suggests that cysteine protease digestion of pEPO is a plausible mechanism for the production of fgEPO observed *in vivo*. PBS incubated with beads for the same length of time as pEPO did not exhibit protein contamination. Likewise, BSA exposed to papain-coated beads retained the same protein concentration as that not exposed to beads. Therefore, no detectable leaching of the beads was observed.

Increases in inflammation attributed to the Mϕ are predominantly mediated through the release of proinflammatory cytokines including TNF-α, IL-1, and IL-6. It is also well established that a temporal relationship exists in the secretion of these cytokines. Vassalli demonstrated that LPS-challenged Mϕ secrete increased levels of TNF-α first (30). A cytokine cascade progresses with IL-1 being secreted second and IL-6 being secreted last. Confirmation of the above was provided by studies in which antibody to TNF-α inhibited LPS-mediated plasma elevations of IL-1 and IL-6 (31). The present study directly measured secretion of

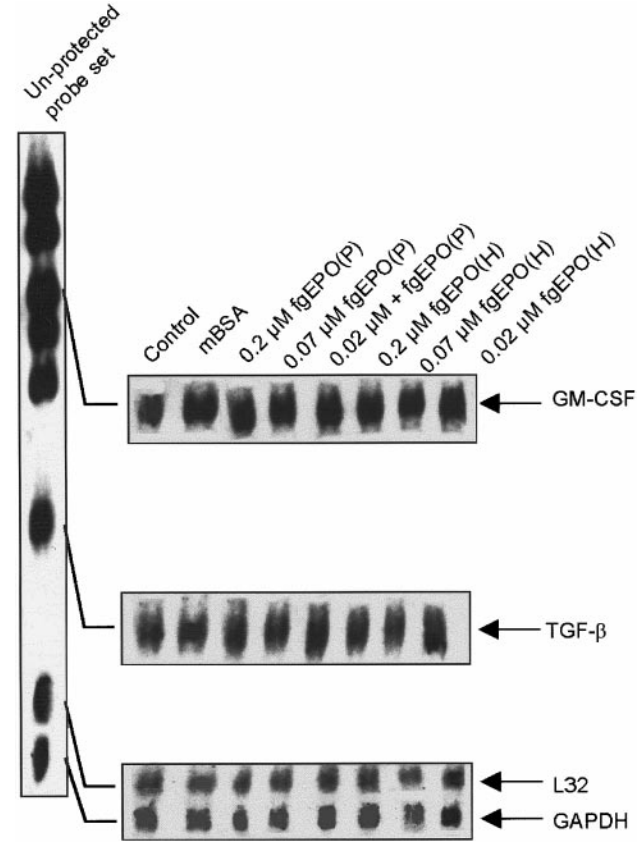

FIG. 11. Effects of fgEPO on "regulatory" cytokine mRNA levels in mBSA-induced Mϕ. One hundred microliters of TG-Mϕ, at 1.2×10^6 cells/ml, was added to the wells of a microtiter plate. After a 2-h attachment, monolayers were washed and treated in one of the following ways: (1) media alone, (2) media containing 12.5 μg/ml mBSA, or (3) media containing 12.5 μg/ml mBSA with various concentrations of fgEPO. After 4 h incubation with treatments, medium was removed and cells were lysed in TRIzol. Total RNA was extracted and assayed for "regulatory" cytokine mRNA (GM-CSF and TGF-β) via RPA. The internal standards utilized were the constitutive mRNA for L32 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

TNF-α. Mannosylated BSA-stimulated TG-Mϕ, which were exposed to fgEPO, demonstrated a dose- and time-dependent decrease in the secretion of TNF-α (Figs. 3 and 4). Since fgEPO was not toxic to Mϕ, as demonstrated by trypan blue exclusion dye, decreases in cytokine secretion were not the result of direct cytotoxicity as observed when pEPO was employed. The decrease in TNF-α secretion could possibly be explained as follows: Since both mBSA and fgEPO are mannosylated proteins, there could be a competition for the same receptor. The Mϕ mannose receptor is one of the scavenger receptors that bind mBSA. This receptor requires that a certain configuration of subunits be bound for either stimulation or inhibition. It is possible that either fgEPO's configuration or exposure of more mannose groups during fragmentation results in inhibitory signaling rather than the stimulatory ligand–receptor interaction observed with mBSA.

The above, however, could only partially account for the inhibition in cytokine secretion observed. When these investigators employed nonmannosylated stimulants of Mϕ cytokine secretion such as (1) LPS, a classical Mϕ activator, (2) IFN-γ, and (3) Poly I:C, similar results were obtained. In other experiments, when LPS or mBSA were added to Mϕ for 1 h, washed off, and fgEPO was subsequently added, similar decreases in TNF-α secretion were observed (data not shown). Because the same effect was seen irrespective of the sequence in which treatments were added or the type of stimulant employed, competition for receptor binding seems unlikely as the only viable explanation for decreased cytokine secretion. A possible explanation not involving one of the scavenger receptors (i.e., the MMR) is the effect of protein charge on its binding potential (32). The high cationicity of EPO could enable this enzyme to bind readily to cells (32, 33). Electrostatic forces could enable nonspecific binding to alternate receptors. Binding to other receptors could result in altered cytokine secretion similar to that reported above. Therefore, the data indicate that multiple receptors could be involved in the decreased cytokine levels.

Since various stimulants demonstrated similar effects in the presence of fgEPO, a series of experiments was performed to determine the effect of fgEPO on transcription of various cytokine genes. These investigators relatively quantified cytokine mRNA levels via an RNase protection assay. Thioglycollate-induced Mϕ were treated with either mBSA alone or mBSA and various concentrations of fgEPO and mRNA extracted as described previously. As seen in Fig. 10, there was a dose-dependent decrease in the amount of cytokine mRNA for IL-1α, IL-1β, and TNF-α. It is noteworthy to mention that IL-1α is the soluble form and IL-1β is the membrane-bound form of this cytokine in the murine system. Therefore, the data suggest that exposure of Mϕ to fgEPO results in a decrease in both soluble IL-1α and, to a greater extent, membrane-bound IL-1β. Although the level of secreted IL-1α was not directly determined, it is well established that this cytokine is secreted following TNF-α. With reference to TNFα, IL-1α, and IL-1β, these data demonstrate that observed decreases in cytokine secretion were a direct result of decreased cytokine mRNA transcription. There were no differences observed between all treatments in the constitutively expressed mRNA for the GAPDH and L32 maintenance proteins (Figs. 10 and 11). Finally, there were no differences observed in the transcription of mRNA for either TGF-β or GM-CSF (Fig. 11). Therefore, the observed decrease in cytokine mRNA was not the result of a global decrease in cellular mRNA transcription but was specific for only the proinflammatory cytokines cited in this study.

There are a number of clinical situations where these data could be relevant. Since fgEPO inhibited TNF-α secretion, there are several consequences of this activity. First, there would be diminished release of other proinflammatory mediators, i.e., IL-1 and IL-6. The absence of TNF-α also could affect cellular infiltration to an area of inflammation through the fact that expression of cellular adhesion molecules would not be up-regulated and this would inhibit neutrophil extravasation. Since neutrophils would not extravasate, an extension of this concept would be the lack of Mϕ stimulation via MPO released from neutrophils. Second, since TNF-α induces IL-1, diminished IL-1 induction would result in a loss of a chemoattractant for cells at a site of inflammation. Third, as observed with TNF-α, the levels of IL-6 secreted by resident Mϕ exposed to a stimulant and fgEPO decreased in a time-dependent manner (Fig. 4). Since IL-6 is also considered to be a proinflammatory cytokine, diminution of this cytokine would also help to inhibit inflammation by not inducing the acute phase response which can initiate the complement cascade. Therefore, all of the above would serve to reduce inflammation associated with various disease states.

It is also interesting to note that fgEPO not only inhibited proinflammatory cytokines but also caused a diminution in the secretion of IFN-α/β. At least two possible consequences of this could occur. One is the fact that IFN-α/β is antiviral in action and therefore inhibition of its expression would render a host more susceptible to a virus infection. Another consequence is the fact that IFN-α/β induces secretion of IFN-γ by NK cells. Since IFN-γ is a potent Mϕ activating factor, fgEPO, through inhibition of IFN-α/β, could indirectly prevent Mϕ activation.

During the course of asthma, EPO concentration is reduced (34). Perhaps this is due to the fact that only the active enzyme was measured. The measurement of both EPO and fgEPO might reveal a different conclusion. Also, it is known that EPO enhances epithelial release of GM-CSF, which would cause neutrophil de-

granulation (35). Since neutrophils are one of the first cells at a site of inflammation (36), during the initial phases of inflammation more MPO and iMPO than EPO could be present. As a result of this, there would be an up-regulation of Mϕ functions (17–20). However, as the inflammatory process associated with asthma progresses, fewer neutrophils and more eosinophils are present. Therefore, EPO as well as fgEPO would be more abundant during this phase. The presence of the later could result in less cytokine secretion which would suggest that the eosinophil, through release of fgEPO, would tend to return the immune system to homeostasis.

In summary, this study utilized fgEPO that was isolated from the granule of hypereosinophilic patients fgEPO(H) as well as those obtained from the *in vitro* exposure of papain-coated agarose beads to intact fgEPO(P). We have postulated that fgEPO may arise as a result of the proteolytic digestion of EPO within the eosinophil granule or in the microenvironment. Furthermore, these results indicate that fgEPO is capable of modulating the Mϕ-mediated transcription and secretion of various cytokines, such as TNF-α, IL-1α/β, IL-6, and IFN-α/β, but not TGF-β and GM-CSF. These altered cytokine profiles indicate a new role for an inactive form of EPO and indicate a dichotomy of function between active EPO and inactive EPO. While EPO functions to destroy invading pathogens by producing various cytotoxic mediators, fgEPO functions to modulate Mϕ responses to down-regulate the ongoing inflammatory process thereby returning the body to homeostasis.

There could also be a temporal role in the differing functions of EPO and fgEPO. Neutrophils are among the first cells recruited to an infection site and readily release MPO into the microenvironment (37, 38). Macrophages bind MPO via the MMR (39) and release various cytokines (such as IL-1 and IL-8) that activate Th2 cells and others (i.e., the eosinophil) (submitted for publication). Together, these cells collectively function to destroy the invading pathogen. Eosinophils aid in this cycle to destroy the pathogen by releasing various cytokines of their own and by releasing enzymatically active EPO that can also bind to and activate Mϕ. Therefore, at a site of inflammation or infection, the Mϕ would initially be activated by MPO. As the process continued, EPO would replace MPO as the activator. Both EPO and fgEPO should simultaneously be present in the microenvironment. Fragments of EPO *may* function to inhibit the formation of a Mϕ-mediated Th1 response and prolong the Th2-type reactions being driven by eosinophil-derived cytokines.

In addition to the above explanation, there is an alternate hypothesis for the possible function of fgEPO at an inflammation site. Data from our laboratory have shown that fgEPO can decrease Mϕ cytokine responses without effecting Mϕ phagocytosis and killing of a pathogen (submitted for publication). Collectively, these functions of fgEPO could serve to clear the invading pathogen and return the body to homeostasis. Taken together, these results further support the concept that there is an interaction between the eosinophil and Mϕ at the site of inflammation that could affect certain diseases including asthma, allergy, or helminth infections.

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