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PEROXIDASE-MEDIATED OXYGENATION AND MICROBICIDAL ACTIVITY

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Abstract—It is known that a peroxidase, H_2O_2, and a halide form a "cytotoxic triad." As a result of the interactions of the components of the triad, reactive oxygen intermediates (ROI) are formed that help to destroy various invading pathogens including *Candida.* The present study was undertaken to determine if equivalent units of peroxidase induced equivalent levels of macrophage-mediated killing of *Candida.* Murine peritoneal macrophages were exposed to various concentrations of eosinophil peroxidase (EPO), myeloperoxidase (MPO), and horseradish peroxidase (HRP). Luminol-dependent chemiluminescence studies showed that equivalent units of peroxidase, as determined by oxidation of guaiacol, demonstrated a hierarchical pattern of ROI production. Macrophage phagocytosis and candidicidal activity, as measured by a fluorescence acridine orange assay, also demonstrated the same hierarchical pattern of EPO > MPO > HRP. Therefore, enzymatically equivalent peroxidases do not demonstrate equivalent candidicidal activity. These data indicate a distinct order of peroxidases relative to their ability to stimulate chemiluminescence and macrophage-mediated killing.

INTRODUCTION

Immunosuppression as a result of the widespread advance of AIDS and drug therapy required for transplant patients has lead to increased prevalence of *Can-*

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dida (1, 2). Phagocytosis and subsequent killing of this pathogen is paramount to the efficient clearance and resolution of this type of infection. Phagocytosis of pathogens at the site of infection or injury is among the macrophages (Mϕ) earliest functions (3). It has also been documented that phagocytosis is strongly correlated with increased production of reactive oxygen intermediates (ROI). Increased ROI production is usually observed after perturbation of the cell membrane and is associated with the respiratory burst (RB) (3).

Peroxidases are heme-containing enzymes that function to catalyze the conversion of hydrogen peroxide (H_2O_2) to more toxic ROI such as superoxide anion (O_2^-), and hydroxyl radicals (OH). These enzymes can also react with H_2O_2 to form hypohalous acids, which may participate in the cytotoxic triad. This triad functions in the destruction of various invading pathogens, including bacteria, fungi and mammalian cells (4–6).

Previous studies in this laboratory have shown that Mϕ exposed to peroxidases have enhanced functions including: (a) killing potential (7), (b) microbicidal activity (7–9), and (c) increased cytokine gene transcription and secretion (unpublished data). Recently, experiments performed in this laboratory have suggested that eosinophil peroxidase (EPO) may be more efficient than myeloperoxidase (MPO) in enhancing ROI, phagocytosis and killing of *Candida* by Mϕ. In vivo, these enzymes are utilized to kill pathogens of differing sizes, i.e., EPO functions in the elimination of large extracellular pathogens (4, 10) and MPO is utilized in the destruction of smaller intracellular pathogens (7, 11, 12), both peroxidases are capable of enhancing Mϕ-mediated candidicidal activity.

With regard to dye dehydrogenation, such as that seen with luminol-dependent chemiluminescence (CL), this technique provides a measure of enzymatic (i.e., peroxidase) capacity to oxidize dye. This activity does not, however, necessarily equate to halide oxidizing capacity or microbicidal action of a particular substrate. In addition to differences in halide dependency and different pH optima, the microbicidal activities of these peroxidases is dependent on their cationicity. Each of the peroxidases utilized in this study are cationic proteins with EPO being the most basic followed by MPO and horseradish peroxidase (HRP) (13).

At a site of infection the neutrophil is often the first cell to interact with the pathogen (3). During phagocytosis of the pathogen, the neutrophil will release MPO into the extracellular environment where the enzyme can be either bound to the Mϕ via the MMR (14) or phagocytosed by this cell (15). Other investigators have reported that Mϕ engulf MPO and utilize the enzyme for cytocidal activities (15, 16). With respect to *Candida* both the neutrophil and the Mϕ are thought to be associated with the clearance of the organism from the body (17). Although evidence exists to suggest that cell wall proteins and mannoproteins from *Candida* are responsible for eliciting a host immune response including that of the Mϕ (18), the exact mechanism of Mϕ clearance of *Candida* has yet to be fully elucidated.

Very little work has been presented that examines the differences in microbial activity of MPO, EPO and HRP. The present study compares the candidicidal activity or killing potential of MPO, EPO and HRP and elucidates what appears to be a hierarchy of Mϕ-mediated killing potential with respect to stimulation by different peroxidases.

MATERIALS AND METHODS

Animals. Age matched, C57BL/6J mice, 18–22 g male animals were purchased from Jackson Laboratories (Bar Harbor, Maine). All animals were used in accordance with federal guidelines and were maintained in facilities that abide by federal guidelines for animal care.

Materials. Candida albicans, strain 3153A, was generously provided by Dr. L. Chaffin, Texas Tech University Health Sciences Center, Lubbock, Texas. Purified EPO was provided by ExOxEmis Corporation, Little Rock, AR, and purified MPO was generously provided by Dr. Robert C. Allen, San Antonio, TX. Dulbecco's modified Eagle media (DMEM), and guinea pig complement (serum), GIBCO Laboratories (Long Island, New York), yeast extract peptone-dextrose (YPD), Difco Laboratories (Detroit, Michigan). Phosphate buffered saline pH. 7.2 (PBS) was prepared as needed. The following reagents were purchased from Sigma Scientific (St. Louis, Missouri): guaiacol, HRP, gentamycin sulfate, HEPES, hydrogen peroxide, potassium phosphate, bovine serum albumin fraction V (≥99% globulin free) (BSA), acridine orange (color index #46005, dye content 90%), and dimethyl sulfoxide (DMSO). Additional reagents utilized that are commercially available were: Crystal violet (color index #42555, dye content 95%), sodium bicarbonate, Fisher (Pittsburgh, Pennsylvania), fetal bovine serum (FBS), Intergen (Purchase, New York), Auto-POW, ICN Labs (Aurora, Ohio), 16-well tissue culture treated chamber slides, NUNC, Inc. (Napierville, Illinois), clear bottom 96-well microtiter plates, Packard Instruments (Meriden, Connecticut). A 20 mM stock solution of luminol, Eastman Kodak (Rochester, New York) dissolved in DMSO was prepared and stored at 4°C until used. All reagents were tested for endotoxin contamination using a Limulus amebocyte lysate test (LAL), Associates of Cape Cod (Woods Hole, Maine). Working concentrations of peroxidases utilized contained ≥0.03 ng/ml of endotoxin.

Macrophage Collection. Murine resident peritoneal Mϕ were collected as described previously (19). Mice were sacrificed by cervical dislocation and the Mϕ collected by gentle peritoneal lavage using PBS at 4°C. Cells were centrifuged at 300 g for 10 min. Cells were washed and resuspended in either Auto-Pow MEM or DMEM without gentamycin or FBS. Mϕ were counted using a hemacytometer and adjusted to 1×10^6 Mϕ/ml. 100 μl of the cell suspension were added to each well of a 96-well Packard tissue culture plate or a Nunc 16-well slide. After a 2 h incubation period at 37°C under 5% CO_2, the non-adherent cells were removed by washing with PBS and the appropriate assay was performed. The Mϕ population was approximately 99% as determined by microscopy.

Candida albicans. C. albicans were cultured in 10 ml of YPD broth at 30°C with light agitation. After 14.5 h, the stationary growth phase was reached and the density was determined to be approximately 5×10^8 cfu/ml as measured by a spread plate technique. The cell suspension was then centrifuged at 15,900 g for 15 min at 4°C and washed once in PBS. Yeast cells were opsonized twice for 30 min at 30°C with 400 μl of guinea pig complement using gentle agitation. After opsonization, cells were washed twice with PBS and diluted to 5×10^6 yeast/ml in DMEM supplemented with 10% FBS.

Enzymatic Activity. EPO, MPO, and HRP were evaluated for enzymatic activity using guaiacol as a substrate. To prepare the reagent solutions: Reagent A, contained potassium phosphate, 1.12 ml of 8.9 M guaiacol and 98.9 ml of H_2O. Reagent B, contained 28.9 μl of H_2O_2 and 9.9 ml of H_2O. Using a quartz cuvette, the absorbance of the H_2O_2 solution was determined by spectropho-

tometer at 240 nm. Approximately 2.9 ml of reagent A and 20 μl of reagent B were added to 35 μl of peroxidase sample, inverted several times to mix thoroughly and the absorbance measured at 470 nm after 1 min. Units of activity per ml of enzyme were calculated using the following formula (sigma):

$$\frac{(\text{abs @ 1 min})(\text{dilution factor})}{(1.0)(\text{sample volume})} = \text{Units/ml}$$

Chemiluminescence Assay. A modified method for chemiluminescence (CL) was employed similar to that described previously (20). Briefly, resident peritoneal Mϕ were obtained as described and suspended at 1×10^6 cells/ml in Auto-POW media supplemented with 0.6 g/dl HEPES, 0.2 g/dl sodium bicarbonate, and 1.0 g/dl BSA. This mixture was termed CI media. Each well of a 96-well "Packard" microtiter plate was seeded with 1.2×10^5 cells. Following a 60 min incubation at 37°C under 5% CO_2, the monolayer was washed with CL media three times to remove non-adherent cells. After washing, 50 μl of CL media was added to each well and the cultures were allowed to equilibrate for an additional 10 min at 37°C. After equilibration, the following were added to each well: 50 μl of luminol (160 μM working concentration), 50 μl of zymozan opsonized with guinea pig complement (10 zymozan particles: Mϕ), and either 50 μl of media alone for the control wells or 50 μl of media containing equivalent units of one of the following: EPO, MPO, or HRP. The plate was placed in a Dynatech ML 3000 plate luminometer and luminescence was read every 2 s and recorded for 2 min intervals. Results were plotted as time vs. relative light units (RLU). The mean of triplicate treatments ± S.E.M. was determined. Each experiment was repeated at least two times.

Phagocytosis Assay. The procedure employed was similar to that described by Lian et al. (21). Peritoneal Mϕ were obtained, as described earlier, in DMEM and 100 μl of 1×10^6 Mϕ/ml of the cell suspension were seeded onto a 16-well tissue culture chamber slide. Following a 2 hr incubation at 37°C under 5% CO_2, the cells were washed gently three times with DMEM without FBS. *C. albicans* was prepared in the manner described earlier. Mϕ monolayers were treated according to the following sequence: (1) Mϕ were exposed for 10 min to EPO, MPO or HRP. (2) After exposure the monolayers were washed and *C. albicans*, suspended in DMEM supplemented with 10% FBS was added at a ratio of 5 : 1 *Candida* per Mϕ (60 min at 37°C). (3) After incubation, monolayers were washed to remove any undigested *Candida.* (4) The cells were stained with acridine orange (0.1 mg/ml) for 90 s, and counterstained with crystal violet (1 mg/ml) for 60 s. Crystal violet was employed to quench the extracellular *Candida.* Mϕ-mediated phagocytosis and intracellular killing of *Candida* was microscopically determined on a BX-4 Olympus fluorescence microscope. *Candida* that fluoresced green were considered live and those that fluoresced orange/red were considered dead. Values represented the mean ± S.E.M. of three 100-cell counts.

In an attempt to ensure the probity of the assay procedure, the following was performed: (1) The viability of the *Candida* was tested using a microbiological plate count and trypan blue staining, (2) *Candida* which were boiled for 60 min and were used as a positive control to ensure the staining of dead cells was appropriate, and (3) slides were periodically read "blind" by a second party to ensure accuracy of count. Each experiment was repeated at least two times.

Statistical Analyses. All data represent the mean ± SEM of replicate counts. A one-way ANOVA and Student-Newmann-Keuls tests were used to determine significance levels among groups.

RESULTS

Experiments were done to determine if equivalent units of three different peroxidases equally enhanced certain Mϕ functions. Highly purified porcine EPO

and MPO were used along with commercially available HRP. Resident murine Mϕ were exposed to the following U/ml of enzymes: 10, 30, 90, 100, 200, and 270. Of these concentrations, only 10 and 30 U/ml were not toxic to Mϕ. Concentrations exceeding 90 U/ml resulted in erratic results, possibly as a consequence of damage to the Mϕ.

Concentrations as low as 3.3 U/ml of peroxidase were sufficient to cause an increase in CL above that of the control (data not shown). When 10 U/ml of peroxidases were utilized, maximum CL (measured at 30 min) was approximately 1200 RLU for EPO, 1100 RLU for MPO and 600 RLU for HRP (Figure 1A). Although similar results were obtained when 30 U/ml peroxidases were employed, the differences between the three peroxidases (measured at 60 min) were more pronounced (Fig. 1B) i.e. 1200 RLU for EPO, 800 RLU for MPO, and 600 RLU for HRP. In addition to the differences noted between peak RLU production for the 10 and 30 U/ml peroxidase doses, there were also differences noted in the kinetics between these doses. While the 10 U/ml dose of peroxidases peaked at 30 min, the 30 U/ml dose demonstrated increasing CL beyond 60 min.

Since the RB is highly correlated with phagocytosis and microbicidal activity, initial experiments were done to determine the effect of 10 and 30 U/ml of each peroxidase on Mϕ-mediated phagocytosis. Typically, EPO induced more phagocytosis than either MPO or HRP. However, there was no significant difference between MPO and HRP in Mϕ-mediated phagocytosis of *Candida.* Ten units of EPO induced 38% phagocytosis, whereas 10 U/ml of MPO and HRP both induced 29% phagocytosis, compared to control values of 22% (Figure 2). Again, if 30 U/ml of each peroxidase were employed, the percent of phagocytosis was higher but the pattern of activation remained the same. Also, the number of yeast per cell was not greater in the treated cultures compared to controls. In treated cultures, however, there were more Mϕ with ingested organisms than in the controls (data not shown).

Figure 3 depicts the results of studies involving the kinetics of phagocytosis of *Candida.* When 10 U/ml of each peroxidase were employed, EPO was superior to either MPO or HRP in inducing uptake of *Candida* by Mϕ. However, there were no marked reproducible differences between phagocytosis induced by either MPO or HRP for all time periods observed.

In order to determine if equivalent units of each peroxidase induced equivalent Mϕ candidicidal activity, a direct comparison of killing potential was done using either 10 or 30 U/ml of each peroxidase (Figure 4). When 10 U/ml of each peroxidase were utilized, EPO exhibited approximately 43% killing, MPO approximately 35% killing, and HRP approximately 31% killing. If 30 U/ml were employed, EPO induced approximately 73% killing, MPO approximately 52% killing, and HRP approximately 44% killing. In all experiments, EPO induced greater killing of *Candida* compared to MPO which was greater than

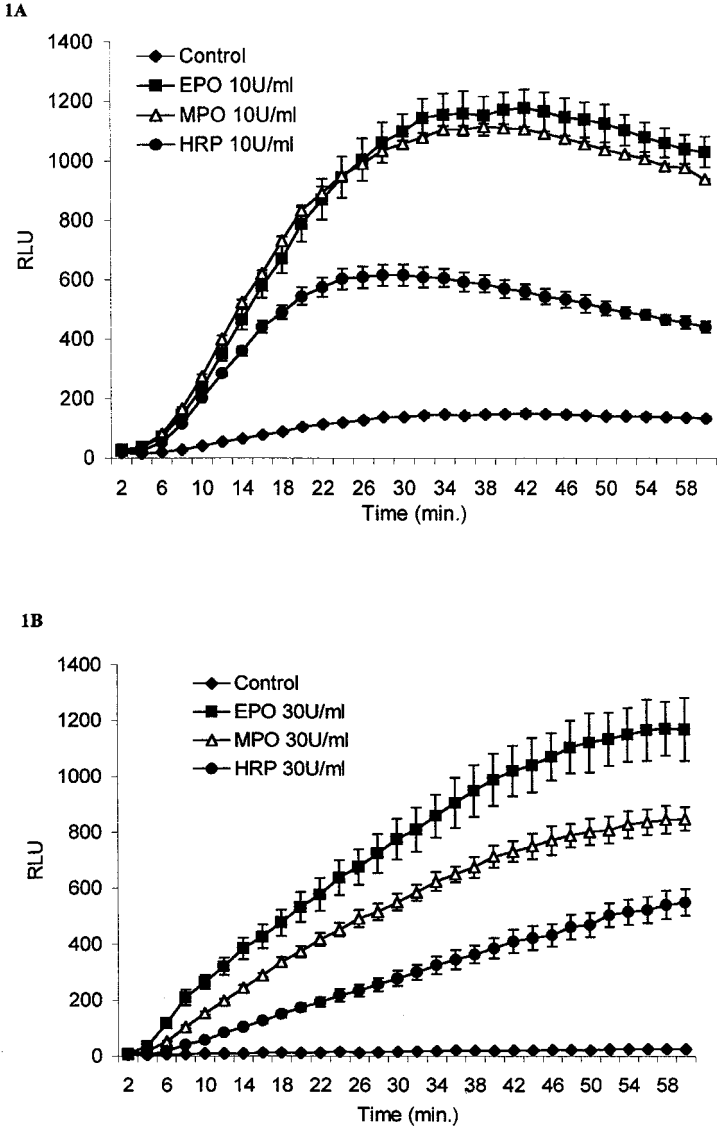

Fig. 1. Peroxidases enhance Mϕ Chemiluminescence. Resident macrophages were cultured on a 96-well tissue culture plate for 2 h. Macrophage monolayers were simultaneously treated with either media alone or media containing either 10 U/ml (1A) or 30 U/ml (1B) of various peroxidases. Luminol and zymozan were added to each well and then incubated at 37°C. The amount of light released for a 60 min period was measured using a Dynatech plate luminometer. Values represent the mean ± *SEM* of triplicate counts.

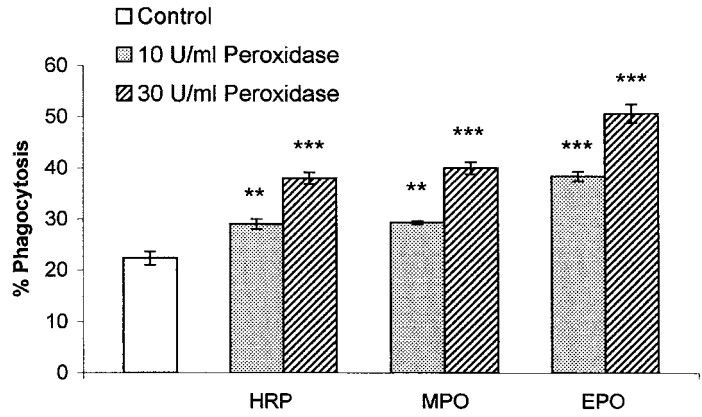

Fig. 2. Perioxidases enhance Mϕ Phagocytosis of *C. Albicans* in dose dependent manner. Resident Mϕ were cultured on a 16-well tissue culture plate for 2 h. Macrophage monolayers were simultaneously exposed for 10 min to either media alone or media containing either 10 U/ml or 30 U/ml of peroxidases. The Mϕ monolayers were washed and yeast was added to each well at a ratio of 5 : 1 *Candida*/Mϕ and incubated at 37°C for 60 min. Following incubation, the cells were stained with acridine orange and the number of cells with ingested bacteria were counted. Each value represents the mean $\pm SEM$ of three 100-cell counts. $^{**}P \leq 0.01$ $^{***}P \leq 0.001$

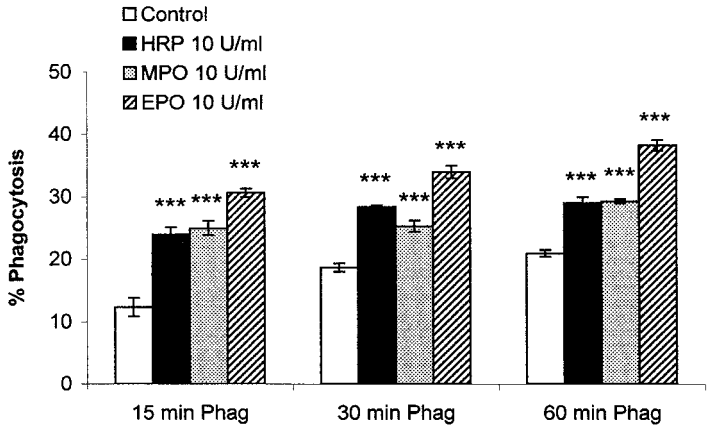

Fig. 3. Peroxidases enhance Mϕ Phagocytosis of *C. albicans* in Time-Dependent Manner. Resident Mϕ were cultured on a 16-well tissue culture plate for 2 h. Macrophage monolayers were simultaneously exposed for 10 min to either media alone or media containing 10 U/ml of peroxidases. The Mϕ monolayers were washed and yeast added to each well at a ratio of 5 : 1 *Candida*/Mϕ and incubated at 37°C for 15, 30 or 60 min. Following incubation, the cells were stained with acridine orange and the number of cells with ingested bacteria were counted. Each value represents the mean $\pm SEM$ of three 100-cell counts. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$

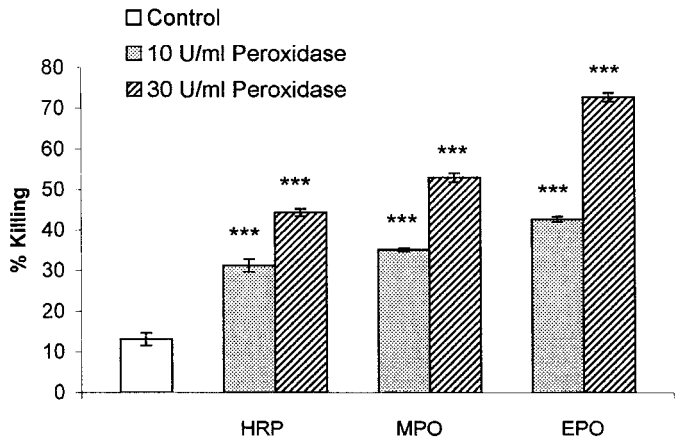

Fig. 4. Peroxidases enhance Mϕ killing of *C. albicans* in Dose-Dependent Manner. Resident Mϕ were cultured on a 16-well tissue culture plate for 2 h. Macrophage monolayers were simultaneously exposed for 10 min to either media alone or media containing either 10 U/ml or 30 U/ml of peroxidases. The Mϕ monolayers were washed and yeast added to each well at a ratio of 5 : 1 *Candida*/Mϕ and incubated at 37°C for 60 min. Following incubation, the cells were stained with acridine orange and the number of cells with ingested bacteria were counted. Each value represents the mean $\pm SEM$ of three 100-cell counts. $^{***}P \leq 0.001$

HRP. At the lower concentrations, there were no marked differences between MPO and HRP. However, with the higher concentrations, the differences in the killing potentials of the Mϕ were obvious. Both EPO and MPO induced markedly more killing than HRP. Also, regardless of the amount employed, each peroxidase significantly enhanced killing above control cultures ($P \geq 0.001$).

Once it had been established that each of the peroxidases induced a dose-dependent increase in candidicidal activity, kinetics studies were undertaken. Figure 5 depicts these results. At all time periods, EPO induced the most killing and HRP the least. Also, there was a time-related increase in candidicidal activity observed.

DISCUSSION

Previously a study has not been done to investigate whether equivalent units of enzymatic activity implied equal killing potential in biological systems. The present study was undertaken to determine if equivalent units of enzymatic activity of three different peroxidases would induce similar Mϕ-mediated killing of *Candida.* Differences in killing could help explain the varying roles of peroxidases in biological situations such as that found at a site of inflammation.

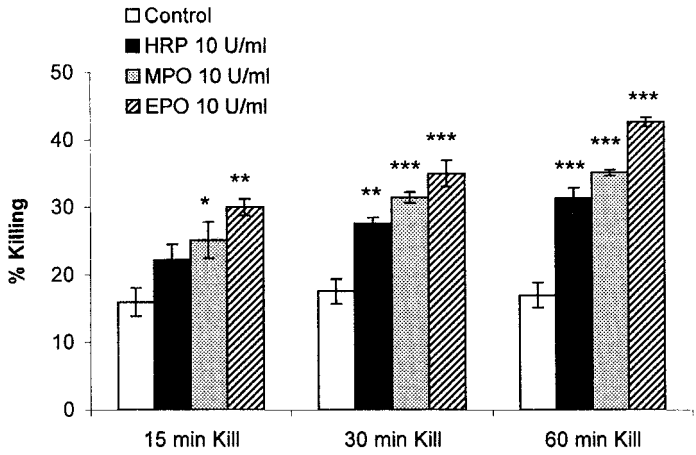

Fig. 5. Peroxidases enhance Mϕ killing in a time-dependent manner. Resident Mϕ were cultured on a 16-well tissue culture plate for 2 h. Macrophage monolayers were simultaneously exposed for 10 min to either media alone or media containing 10 U/ml of peroxidases. The Mϕ monolayers were washed and yeast added to each well at a ratio of 5 : 1 *Candida*/Mϕ and incubated at 37°C for 15, 30 or 60 min. Following incubation, the cells were stained with acridine orange and the number of cells with ingested bacteria were counted. Each value represents the mean $\pm SEM$ of three 100-cell counts. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$

Other investigators have reported that in vivo approximately 15 μg/ml of enzymatically active MPO are present at a site of inflammation (13). Since the present study employed approximately 3.4 μg/ml of MPO, this concentration was within "physiological range." With respect to EPO, it is known that EPO rapidly binds to and is internalized by neutrophils at a concentration 10 fold *less* than MPO. Also, EPO binds to a much wider variety of cells than MPO (13, 22). Therefore, since EPO is extremely cytophilic, there does not seem to be a consensus as to the amount of EPO one would find at a site of inflammation or helminth infection. The physiological range of EPO has not been accurately determined.

Throughout the present study, resident peritoneal Mϕ were employed. These cells were utilized because resident Mϕ express higher levels of the Mϕ mannose receptor (MMR) than inflammatory or activated Mϕ (23, 24). Of the three peroxidases used in this study, two (MPO and HRP) can enter the cell via the MMR (14, 25). In addition, it is known that EPO is a highly mannosylated compound like MPO and HRP. Since EPO has a mannose content of 4.5% (MPO is 4.6%) (22) and competition studies using a known MMR ligand blocked EPO binding (8), the MMR could be one of the possible receptors involved in the uptake of EPO. However, other scavenger receptors present on Mϕ which bind mannosylated substances cannot be ruled out (26).

The rationale for a 10 min peroxidase exposure time for Mϕ is as follows: Other studies have indicated that MMR expressed on the surface of a Mϕ is recycled every 6–8 min (23). Ten minutes should allow enough time for the MMR to recycle. Based on the above, both phagocytosis and candidicidal activity were measured after 10 min exposure to peroxidase and usually 60 min exposure to *Candida.*

Chemiluminescence is associated with the RB and the production of various ROI. The RB is highly correlated with phagocytosis and microbicidal activity and, as such, oxygen intermediates produced during the RB can be used as a measure of the killing potential of the cell. Luminol-dependent CL can be driven by active peroxidases to enhance the production of light. The amount of light detected (RLU) in the presence of luminol is a direct measure of Mϕ ROI production when challenged with zymosan is an indirect measure of Mϕ killing potential.

Previous studies by the present investigators and others, have shown that either HRP or MPO enhances the production of ROI secreted by Mϕ (7, 8, 14). In the present study, EPO was the most potent inducer of ROI as measured by CL (Figures 1a and 1b). This peroxidase was followed closely by MPO, which was superior to HRP (Figures 1a and 1b). If 30 U/ml of the peroxidase were used, the observed differences were more obvious than when 10 U/ml was employed (Figure 1b). This pattern was obtained only if at least 160 μM of luminol were utilized. The shape of the curve obtained with lower concentrations of luminol showed a rapid rise in RLU followed by a precipitous decline. However, when 160 μM of luminol were used, there was a rapid rise of RLU followed by a slow decline indicating that the luminol was not limiting. When luminol was not limiting, the above pattern occurred repeatedly.

Once it had been established that there appeared to be a hierarchy of potency with respect to ROI production, phagocytosis and candidicidal activity were measured to determine if a similar pattern would evolve (Figures 2 and 4). Since the RB with its resultant ROI production is highly correlated with phagocytosis and candidicidal activity (1), results obtained were not unexpected. Macrophages that were exposed to various concentrations of the three peroxidases demonstrated a pattern of enhanced function similar to that seen with CL studies. With respect to phagocytosis, EPO enhanced Mϕ-mediated ingestion of yeast to a greater extent than that observed with either MPO or HRP. Although the overall percentages were increased, a similar pattern was observed with 30 U/ml (Figure 2). It should be noted that the number of *Candida* per Mϕ was not markedly increased, rather the number of Mϕ ingesting yeast were greater in the presence of a peroxidase compared to controls.

Peroxidase induced, Mϕ-mediated candidicidal activity has been shown to be ROI dependent (7, 8, 20). Chemiluminescence studies indicated that at the 60 min time interval, when phagocytosis and candidicidal activity were measured,

Mϕ killing potential decreased with 10 U/ml peroxidases while ROI production continued to increase with 30 U/ml peroxidases (Figures 1A, 1B). Other studies were performed to determine the potential for each of the peroxidases to enhance Mϕ-mediated killing. When 10 U/ml peroxidases were employed, only moderate differences in Mϕ-candidicidal activity were observed (43% for EPO, 35% for MPO & 31% for HRP) (Figure 4). However, pronounced differences, most notably between EPO and MPO, were seen in Mϕ candidicidal activity with 30 U/ml peroxidases (73% for EPO, 53% for MPO & 44% for HRP (Figure 4). This hierarchy of candidicidal activity was confirmed by the CL data. Taken together, the data in this study indicate a definite correlation between peroxidase-induced Mϕ killing potential, as measured by CL, and Mϕ candidicidal activity, as measured by fluorescence microscopy.

There are several parameters that must be considered with regard to the data presented. First, each of the peroxidases is cationic. Cationicity of a protein affects its binding potential (21, 22). The IEP of a protein should be above 8.5 in order to obtain strong charge-mediated retention of the protein in a microenvironment (21, 22). The IEP of MPO is > 10 and that of EPO is > 11 (13). Thus, cationicity of the peroxidases would enhance their binding to cells by electrostatic means (21–23). The positive charge of EPO and MPO could also enable these enzymes to be bound more readily by different cells. Other investigators have reported that EPO is definitely more cytophilic than MPO (13). Once either EPO or MPO is bound to a cell, the enzyme can alter that cell's functions (14). In particular, the positive charge would cause the peroxidase to remain in a particular microenvironment longer resulting in activation of Mϕ (7, 8, 14). Charge, therefore, via enhancing binding and subsequent activation of certain cells could contribute to the ability of either MPO or EPO to perpetuate an inflammatory response (21, 22, 24). The above could explain why EPO always induced greater Mϕ-mediated responses than MPO.

Another factor that must be considered is the pH of the microenvironment. After a neutrophil ingests a pathogen, within 5 s, MPO is observed on the cell surface (25, 26). Likewise, when an eosinophil binds to an organism which cannot be phagocytized, the cell secretes EPO into the microenvironment (10). The optimal pH of MPO is 5 and of EPO is 4.6. However, even though both peroxidases have different optimal pH's, these molecules are exposed to the physiological pH of 7.2 in the milieu of the tissues. In addition, within the body, the most abundant halide is chloride. Although this is not the halide of choice for EPO, both physiological pH and chloride was chosen for this study in order to mimic in vivo conditions.

In addition to the pH requirements of the peroxidase, one must consider the optimal pH of MMR-ligand interaction (pH 7.0) (27). The present investigators have found that, if the pH of the media was below 6.8, there was no candidicidal activity above control values. Therefore, it seems that the pH requirement of

the MMR supercedes that of the peroxidases. The same could be true for other scavenger receptors which bind mannosylated compounds.

The present study demonstrates that in the microenvironment at physiologic pH, equivalent units of MPO and EPO have different killing potentials. It is known that an internalized peroxidase, such as MPO, remains active for hours within a Mϕ (14). Therefore, these enzymes would be exposed to different pH environments within cytoplasmic vesicles and the microenvironment. Also, at a site of inflammation, the first cell to arrive is the neutrophil (28). After degranulation of the neutrophil, MPO can bind to resident Mϕ and stimulate these cells to secrete ROI as well as various cytokines (14, 24, 29). Eosinophils arrive later in the process and release EPO into the milieu of inflammation. This peroxidase also activates Mϕ to secrete ROI which enhances the cells killing potential. Therefore, the concentration of either MPO or EPO and their different requirements for optimal enzymatic functions could affect the host's ability to clear a pathogen such as *Candida.*

REFERENCES

1. HIBBERD, P. L., and R. H. RUBIN. 1994. Clinical aspects of fungal infection in organ transplant recipients. *Clin. Infect. Dis.* **19(1):**S33–S40.
2. AMPEL, N. 1996. Emerging disease issues and fungal pathogens associated with HIV infection. *Emerging Infect. Dis.* **2(2):**109–116.
3. GREENBERG, S., and S. C. SILVERSTEIN. 1993. Phagocytosis. *In:* Paul, W. E., (editors) *Fundamental Immunol.* Raven Press, New York: 941–964.
4. JONG, E. C., A. F. MAHMOUD, and S. J. KLEBANOFF. 1981. Peroxidase-mediated toxicity to Schistosomula of *Schistosoma Mansoni. J. Immunol.* **126:**468–471.
5. JONG, E. C., and S. J. KLEBANOFF. 1980. Eosinophil-mediated mammalian tumor cell cytotoxicity: Role of the peroxidase system. *J. Immunol.* **124:**1949–1953.
6. KLEBANOFF, S. J. 1975. Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. *Semin. Hematol.* **12:**117–121.
7. LEFKOWITZ, S. S., M. P. GELDERMAN, D. L. LEFKOWITZ, N. MOGUILEVSKY, and A. BOLLEN. 1996. Phagocytosis and intracellular killing of *Candida albicans* by macrophages exposed to myeloperoxidase. *J. Infectious Dis.* **173:**1202–1207.
8. LEFKOWITZ, D. L., J. L. LINCOLN, K. R. HOWARD, R. STUART, S. S. LEFKOWITZ, and R. C. ALLEN. 1997. Macrophage-mediated candidicidal activity is augmented by exposure to eosinophil peroxidase: A paradigm for eosinophil-macrophage interaction. *Inflammation.* **21:**159–171.
9. LEFKOWITZ, D. L., J. L. LINCOLN, S. S. LEFKOWITZ, A. BOLLEN, and N. MOGUILEVSKY. 1997. Enhancement of macrophage-mediated bactericidal activity by macrophage-mannose receptor-ligand interaction. *Immunol. Cell Biol.* **75:**136–141.
10. BOS, A. J., R. WEVER, M. N. HAMMERS, and D. ROOS. 1981. Some enzymatic characteristics of eosinophil peroxidase from patients with eosinophilia and from healthy donors. *Infect. Immun.* **32**(2):427–431.
11. SCHESINGER, L. S. 1993. Macrophage phagocytosis of virulent but not attenuated strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors. *J. Immunol.* **150:**2920–2930.

12. LINCOLN, J. A., D. L. LEFKOWITZ, T. CAIN, A. CASTRO, K. C. MILLS, S. S. LEFKOWITZ, N. MOGUILEVSKY, and A. BOLLEN. 1995. Exogenous myeloperoxidase enhances bacterial phagocytosis and intracellular killing by macrophages. *Infect. Immun.* **63:**3042–3047.
13. ZABUCCHI, G., M. R. SORANZO, R. MENEGAZI, P. BERTONCIN, E. NARDON, and P. PARTRIARCA. 1989. Uptake of human eosinophil peroxidase and myeloperoxidase by cells involved in the inflammatory process. *J. Histochem. Cytochem.* **37:**499–508.
14. SHEPHERD, V. L., HOIDAL, J. 1990. Clearance of neutrophil-derived myeloperoxidase by the macrophage mannose receptor. *Am. J. Respir. Cell. Mol. Biol.* **2:**335–340.
15. LEUNG, K. P., and M. B. GORDEN. 1989. Uptake and utilization of human polymorphonuclear leukocyte granule myeloperoxidase by mouse peritoneal macrophages. *Cell Tissue Res.* **257:**653–656.
16. LEHRER, R. I. 1975. The fungicidal mechanisms of human monocytes. I. Evidence for myeloperoxidase-linked and myeloperoxidase-independent candidicidal mechanisms. *J. Clin. Invest.* **55:**338–346.
17. CALDERONE, R., and STURTEVANT, J. 1994. Macrophage interactions with *Candida. In:* Zwilling, B. S., and T. K. Eisenstein. (Editors) Macrophage-Pathogen Interactions. Marcel Dekker, NY. 505–515.
18. MARTINEZ, J. P., M. L. GIL, J. L. LOPEZ-RIBOT, and W. L. CHAFFIN. 1998. Serologic response to cell wall mannoproteins and proteins of *Candida albicans. Clin. Microbiol. Rev.* **11:**121–141.
19. LEFKOWITZ, D. L., S. S. LEFKOWITZ, R. Q. WEI, and J. EVERSE. 1986. Activation of macrophages with oxidative enzymes. *In:* DiSabato, G., Everse, J. (Editors) Methods in Enzymology. Academic Press: Orlando. 537–548.
20. SASADA, M., A. KUBO, T. NISHIMURA, T. KAKITA, T. MORIGUCHI, K. TYAMAMOTO, and H. UCHINO. 1987. Candidicidal activity of monocyte-derived macrophages: Relationship between *Candida* killing and oxygen radical generation by human macrophages. *J. Leukocyte Biol.* **41:**289–294.
21. VAN LENT, P. L. E. M., C. DEKKER, J. MOSTRERD, L. VAN DEN BERSSELAAR, and W. B. VAN DEN BERG. 1989. Allergic arthritis induced by cationic proteins: Role of molecular weight. *Immunol.* **67:**447–452.
22. VAN LENT, P. L. E. M., L. VAN DEN BERSSELAAR, G. J. F. GRUTTERS, and W. B. VAN DEN BERG. 1989. Fate of antigen after intravenous and intraarticular injection into mice. Role of molecular weight and charge. *J. Rheumatol.* **16(10):**1295–1303.
23. ZABUCCHI, G., R. MENEGAZZI, M. R. SORANZO, and P. PATRIARCA. 1986. Uptake of human eosinophil peroxidase by human neutrophils. *Am. J. Pathol.* **124:**510–518.
24. GELDERMAN, M. P., R. STUART, D. VIGERUST, S. FUHRMANN, D. L. LEFKOWITZ, R. C. ALLEN, S. S. LEFKOWITZ, and S. GRAHAM. 1998. Perpetuation of inflammation associated with experimental arthritis: The role of the macrophage activation by neutrophilic myeloperoxidase. *Mediators of Inflamm.* **7:**381–389.
25. PRYZWANSKY, K. B., E. K. MACRAE, J. K. SPITZNAGEL, and M. H. COONEY. 1979. Early degranulation of human neutrophils: Immunocytochemical studies of surface intracellular phagocytic events. *Cell.* **18:**1025–1033.
26. SAVILL, J. 1997. Apoptosis in resolution of inflammation. *J. Leukocyte Biol.* **61:**375–380.
27. STAHL, P. D. 1990. The macrophage mannose receptor: Current status. *Am. J. Respir. Cell Mol. Biol.* **2:**317–318.
28. SAVILL, J. S., A. H. WYLLIE, J. E. HENSON, M. J. WALPORT, P. M. HENSON, and C. HASLETT. 1989. Macrophage phagocytosis of aging neutrophils in inflammation. *J. Clin. Invest.* **83:**865–875.
29. MILLS, K. C., D. L. LEFKOWITZ, C. D. MORGAN, and S. S. LEFKOWITZ. 1992. Induction of cytokines by peroxidases in vivo. *Immunol. Infect. Dis.* **2:**45–50.