

Cellular and Extracellular Myeloperoxidase in Pyogenic Inflammation

By Peter P. Bradley, Robert D. Christensen, and Gerald Rothstein

We explored the effect of *in vitro* phagocytosis and *in vivo* inflammation on the MPO content of functioning neutrophils and on the ability of these cells to export active MPO into the extracellular environment. After ingestion of staphylococci, neutrophils retained 52% of their MPO and released 8% into the medium in active form; the remaining 40% of their MPO could no longer be detected. During bacterial infection induced by intradermally injecting staphylococci, neutrophils harvested from minced infected lesions contained 52% of the MPO of circulating neutrophils that had not reached the lesions. Extracellular fluid

from the lesions contained active MPO secreted by the neutrophils, and concentrations of 10–45 U/ml were detected. These data demonstrate that functioning neutrophils can lose approximately half of their MPO. *In vitro*, 4%–8% of neutrophilic MPO appears in the extracellular space and 40% is inactivated. *In vivo*, the MPO content of inflammatory neutrophils also decreases, and MPO appears in the extracellular fluid in active form where it is available to participate in a variety of physiologic processes.

IT HAS BEEN PROPOSED that when neutrophils migrate to sites of inflammation, they release cellular contents and enzymes, which then act on cells or microorganisms in the surrounding tissue.^{1,2} One such enzyme is myeloperoxidase (MPO), which has been shown to have both antimicrobial and cytotoxic properties,^{3,4} as well as the ability to inactivate chemotactic factors,⁵ thereby limiting the migration of neutrophils. Thus, the fate of neutrophilic MPO and its secretion during phagocytosis, and more broadly, the inflammatory process *in vivo*, is of interest. In the present study, we employed methods to extract total cellular MPO in soluble form, assuring a complete assay of the enzyme. In this way, we investigated the content of MPO in neutrophils during the phagocytosis of bacteria *in vitro* and the liberation of MPO into the surrounding medium during this process. We also measured the MPO in both circulating neutrophils and neutrophils that had migrated to sites of bacterial infection *in vivo*. It was found that the process of phagocytosis induces a loss of total extractable neutrophilic MPO and that a fraction of the lost enzyme can be found extracellularly in active form. A similar phenomenon was also detected *in vivo*: neutrophils in infected lesions exhibited reduced MPO content, and MPO could be

detected in soluble and active form in the extracellular fluid. These data establish that the loss of neutrophilic MPO during cellular function *in vivo* is associated with accumulation of the enzyme in the extracellular fluid where it could exert a variety of biologic effects.

MATERIALS AND METHODS

Preparation of Neutrophils

Peripheral venous blood from normal human subjects or caval blood from adult male or female Sprague-Dawley rats was collected in syringes containing 10–15 U of heparin (Panheparin, Abbott Labs., N. Chicago, Ill) per milliliter of blood. The leukocytes were separated in discontinuous gradients of Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.) and sodium diatrizoate (Winthrop Lab., New York, N.Y.).⁶ Neutrophils thus collected were washed three times and suspended in McCoy's 5A medium (MSA) (Flow Labs., Inglewood, Calif.). Contaminating erythrocytes were subjected to hypotonic lysis in 3 vol of distilled water for 30 sec, followed by the addition of 1 vol of 3.5% NaCl. Final cell concentrations were determined by electronic counting (Coulter Electronics, Hialeah, Fla.). Two-hundred cell differential counts were performed using Wright-stained smears. The cell suspensions thus prepared from human blood contained 98.8% ± 0.3% (mean ± SEM) neutrophils, 0.3% ± 0.3% lymphocytes, and 1.0% ± 0.3% eosinophils. Specimens prepared from rat blood were 75% ± 5% neutrophils, 21% ± 5% lymphocytes, 1.8% ± 0.5% monocytes, and 2.4% ± 0.5% eosinophils.

Bacteria

Staphylococcus aureus ATCC no. 25923 was grown overnight in tryptic soy broth (Difco Lab., Detroit, Mich.). For *in vitro* studies, bacterial suspensions were autoclaved and frozen at –70°C until used. The bacteria were washed and suspended in medium, and the concentration of bacteria determined by means of McFarland standards.⁷ Opsonized bacteria were prepared by incubating 2 × 10¹⁰ organisms in medium with 80% human serum at 37°C for 1 hr.

Assay of Myeloperoxidase (MPO)

MPO was extracted from cells by suspending the material in 2.0 ml 0.5% hexadecyltrimethylammonium bromide (HTAB) (Sigma Chemical Co., St. Louis, Mo.) in 50 mM potassium phosphate buffer, pH 6.0, to solubilize MPO.⁸ For MPO measurement in cell-free medium or plasma, aliquots were mixed with an equal

From the Department of Medicine, University of Utah College of Medicine, Salt Lake City, Utah.

Supported in part by Research Training Grant AM-7715 from the National Institutes of Health.

This work was presented in part at the Annual Meeting of the Western Section of the American Federation for Clinical Research, Carmel, Calif. February 6, 1981. An abstract has been published in *Clinical Research* 29:40, 1981.

Submitted October 5, 1981; accepted April 20, 1982.

Address reprint requests to Peter P. Bradley, M.D., Division of Hematology-Oncology, University of Utah Medical Center, 50 North Medical Drive, Salt Lake City, Utah 84132.

© 1982 by Grune & Stratton, Inc.

0006-4971/82/6003-0010\$01.00/0

volume of 1.0% HTAB in 100 mM buffer, pH 6.0. Specimens were subjected to sonication in an ice bath for 10 sec, 40 W (Heat Systems Ultrasonics, Plainview, N.Y.), after which they were freeze-thawed 3 times. Sonication was repeated, and the specimens were centrifuged at 40,000 g for 15 min; the resulting supernate or pellet was assayed.

MPO was assayed spectrophotometrically: 0.1 ml of the material to be measured was mixed with 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/ml *o*-dianisidine dihydrochloride (Sigma Chemical Co.) and 0.0005% hydrogen peroxide (Mallinckrodt, Paris, Ky.) in a final volume of 3 ml. Then the absorbance change at 460 nm was measured with a Beckman DU spectrophotometer (Beckman Instruments Lab., Fullerton, Calif.) with a recording attachment (Gilford Instrument Labs., Oberlin, Ohio). A Cary spectrophotometer Model 118 (Varian, Palo Alto, Calif.) was employed for turbid suspensions. One unit of activity was defined as that degrading 1 μ mole of peroxide/min at 25°C.⁹ Staining of smears for MPO activity was performed by the method of Kaplow.¹⁰

In Vitro Experiments

Suspensions for phagocytosis were prepared by mixing human neutrophils with opsonized bacteria in 1.0 ml medium in sterile polypropylene tubes (Falcon Plastics, Oxnard, Calif.), and were incubated at 37°C in a Dubnoff incubator (Lab-Line Instruments, Melrose Park, Ill.) at 100 excursions/min. Aliquots of the same number of neutrophils were incubated without bacteria as controls. After 1 hr, the suspensions were chilled in an ice bath and centrifuged at 800 g for 5 min. MPO activity was determined in the supernates mixed with 1.0% HTAB or in the pellets suspended in 0.5% HTAB.

Disruption experiments were performed by suspending neutrophils in 3 ml medium (M5A) in a Potter-Elvehjem type homogenizer and homogenizing them for 10 min in an ice bath by means of a Teflon plunger powered by a variable-speed drill (Black & Decker Mfg. Co., Towson, Md.). Care was taken to avoid any foaming. The homogenizer was rinsed twice with medium and the pooled suspension centrifuged at 40,000 g for 15 min to yield a cell-free supernate and pellet for MPO assay.

Statistical Analysis

Data were analyzed by means of Student's *t* test.

RESULTS

In Vitro Release of Neutrophilic MPO

Within resting neutrophils, MPO is tightly associated with the primary granules.¹¹ Indeed, quantitative recovery of MPO from neutrophils requires treatment of the cells with detergent to render the enzyme activity soluble in aqueous medium.^{3,12-14} Because MPO may function extracellularly as well as within the cell, we examined the retention of MPO activity within the neutrophil or its release in active form into the surrounding medium when neutrophils were stimulated to perform phagocytosis. Neutrophils (5×10^6) were mixed with 5×10^9 *S. aureus* and incubated at 37°C so that phagocytosis could occur. After 1 hr, greater than 90% of the neutrophils contained 30 or more bacteria and exhibited a qualitative decrease in MPO staining. Then the suspensions were centrifuged,

and the MPO activity recovered from the supernates or pellets was compared with that of an aliquot of resting neutrophils incubated without bacteria. As shown in Fig. 1, after neutrophils were allowed to ingest bacteria, a reduction in cellular MPO was observed, and some MPO appeared in the extracellular medium. The MPO of neutrophils that had ingested bacteria was $52.2\% \pm 2.1\%$ of the value prior to phagocytosis, $8.2\% \pm 0.5\%$ appeared in the medium, and $39.6\% \pm 2.5\%$ could not be detected, having apparently become inactive. In order to determine the distribution of MPO found in the medium, the material was subjected to centrifugation at 40,000 g for 15 min, yielding a pellet and supernatant fraction. It was found that $41.5\% \pm 5.1\%$ of the MPO in the medium appeared in the supernatant and $58.5\% \pm 5.1\%$ in the pellet. Thus, it can be concluded that some of the active MPO exported by neutrophils during phagocytosis appears to be associated with suspended subcellular particles, while the remainder is soluble.

In smears of neutrophils that had been allowed to ingest bacteria in vitro, neutrophils were frequently observed in varying stages of disintegration. Therefore, we examined the possibility that disruption itself might also contribute to the release of MPO extracellularly as well as loss in intracellular MPO. Human neutrophils (10.5×10^6) were suspended and homogenized in medium for 10 min in an ice bath with a Potter-Elvehjem homogenizer. The homogenizer was rinsed twice with medium and the pooled suspension centrifuged at 40,000 g for 15 min. The MPO activity recovered from the resulting supernate or pellet was

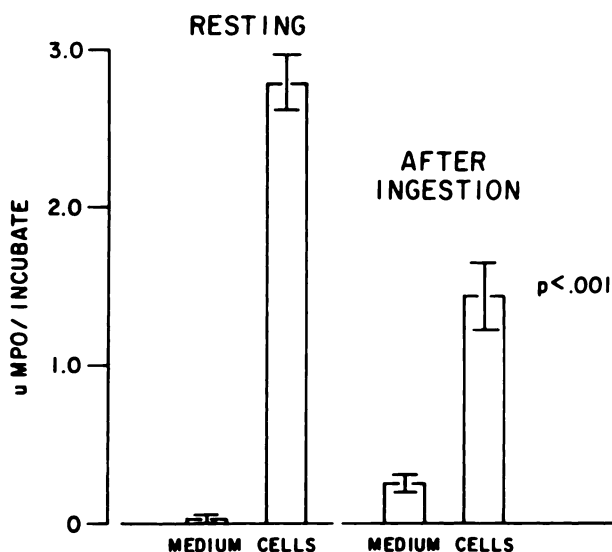


Fig. 1. Effect of ingestion of bacteria upon the MPO of neutrophils. The means \pm SE for groups of seven values are shown for MPO recovered from suspensions of resting or phagocytosing neutrophils.

compared to that of an aliquot of the same number of resting neutrophils. As shown in Fig. 2, disruption was associated with a loss of $30.5\% \pm 2.7\%$ of the MPO of intact neutrophils, and 0.290 ± 0.590 U was recoverable from the medium. Cell-free extracts of MPO from neutrophils were also subjected to homogenization and did not display loss of enzyme activity, indicating that homogenization did not inactivate soluble MPO, which had been released during disruption of neutrophils.

Cellular and Extracellular MPO During *In Vivo* Pyogenic Infection

Changes in neutrophil and extracellular MPO were also examined in an *in vivo* animal model of pyogenic infection. Adult rats were given $1.3\text{--}2.5 \times 10^9$ staphylococci (5×10^{10} organisms/ml) intradermally in the skin of the back with a tuberculin syringe fitted with a no. 25 needle. A well localized, 5-mm, indurated, red lesion developed. Twenty-four hours after bacterial injection, the animals were sacrificed. MPO activity was examined in circulating neutrophils and in neutrophils and extracellular fluid obtained from the infected lesions. In order to measure the MPO of circulating neutrophils, animals were phlebotomized from the inferior vena cava with a heparinized syringe. Suspensions of neutrophils were then prepared by centrifugation on discontinuous gradients and subjected to extraction of MPO activity. Neutrophils from the blood of infected rats contained $4.07 \pm 0.20 \times 10^{-7}$ U MPO/neutrophil, as compared with the value of $5.04 \pm 0.21 \times 10^{-7}$ U MPO/neutrophil for control uninfected animals ($p < 0.05$). In order to explore the possibility that differences in the content of monocytes

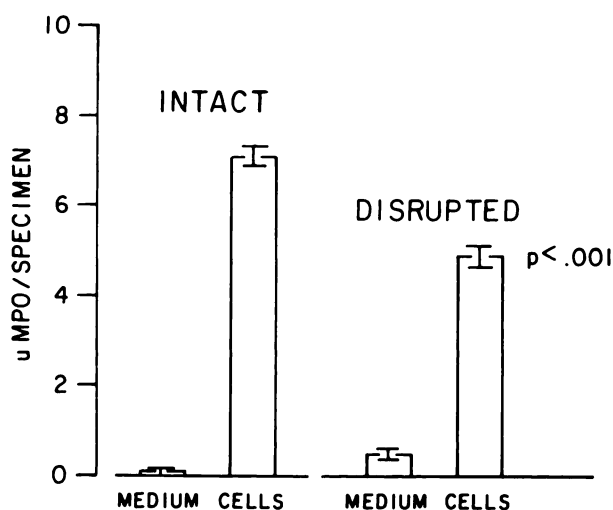


Fig. 2. Effect of disruption upon the MPO of neutrophils. The means \pm SE for groups of seven values are shown for MPO recovered from suspensions of resting or homogenized neutrophils.

or eosinophils might account for the lower value in the infected animals, differential counts were performed on suspensions of the purified cells. In specimens from infected rats, the concentration of monocytes was $2.6\% \pm 0.8\%$ and of neutrophils was $84.4\% \pm 2.8\%$, as compared to a value of $1.8\% \pm 0.5\%$ monocytes and $74.6\% \pm 4.7\%$ neutrophils for the controls. Further, since we found that rat monocytes contain only $9.36 \pm 2.20 \times 10^{-9}$ U MPO/cell,¹⁵ monocytes could have been expected to account for only $0.049\% \pm 0.001\%$ of the sample MPO in any case. In contrast, eosinophils contain considerable peroxidase activity,¹⁶ and reduction in the content of eosinophils present in the specimens from infected animals could contribute to the observed decrease in MPO per neutrophil. However, the concentration of eosinophils in specimens from infected animals was $4.4\% \pm 1.0\%$, as compared to $2.4\% \pm 0.5\%$ for controls. Thus, the decrease in MPO could not be ascribed to changes in the concentrations of other granulocytes present in the suspensions of neutrophils.

The content of MPO of neutrophils within the inflammatory lesions was examined by studying neutrophils shed from the lesions during gentle mincing of the lesions with scissors in medium. In this manner, suspensions of $1.32\text{--}4.50 \times 10^3$ cells/cu mm consisting of $90.8\% \pm 0.9\%$ neutrophils were prepared. Microscopic examination indicated that $36\% \pm 4\%$ of the neutrophils had ingested 10 or more staphylococci. Free bacteria did not stain for MPO. MPO assay of neutrophils from the lesions disclosed a value of $2.76 \pm 0.24 \times 10^{-7}$ U MPO/neutrophil ($p < 0.01$).

The reduction in neutrophilic MPO after the cells had reached the lesions suggested that MPO might have been released into the extracellular fluid and accumulated there. In order to test this hypothesis, we examined the extracellular fluid from inflammatory lesions for MPO activity. First, the lesions were placed in 2 ml of ice-cold M5A and minced into 16 pieces with a pair of scissors. The M5A, now containing tissue fluid from the mincing, was collected by centrifugation at $40,000$ g for 15 min at 4°C and was assayed for MPO activity. It was found that the extracellular fluid thus collected from infected lesions contained 2.738 ± 0.509 U MPO/lesion. Since this maneuver undoubtedly does not liberate all of the extracellular fluid from the lesions, this may be considered a minimum estimate of the extracellular MPO present in the lesions. Total lesional MPO, including both HTAB-extracted intracellular plus soluble extracellular enzyme, was evaluated by subjecting minced lesions to Potter-Elvehjem homogenization, sonication, and freeze-thawing. In 8 animals, the recovered MPO was found to be 18.226 ± 1.287 U/lesion. Thus, at least $15.8\% \pm$

3.0% of total lesional MPO was present extracellularly and in soluble active form. In order to measure the concentration of MPO in extracellular fluid, other lesions were harvested 24 hr after inoculation, placed on a watch glass, and the indurated area was gently incised once with a scalpel blade. Then, the fluid within the incision (approximately 0.01 ml) was collected in a capillary tube and centrifuged at 14,000 g for 5 min to yield a cell-free supernate. The supernate volume was measured and subjected to analysis for MPO. In 5 such experiments, it was found that the MPO concentration was 23.8 ± 6.3 U/ml.

Because of the decrease in the MPO of circulating neutrophils during infection and the accumulation of active MPO in the extracellular fluid of the lesions, we postulated that MPO might also accumulate in plasma during infection. However, when cell-free plasma (40,000 g for 15 min) was prepared from animals with 24-hr inflammatory lesions, its peroxidase activity was found to be 0.0049 ± 0.0012 U/ml, as compared to that of uninfected controls 0.0050 ± 0.0008 U/ml ($p > 0.4$).

DISCUSSION

Although MPO is a major constituent of neutrophils,¹⁶ its role in the physiology of the neutrophil has not been completely defined. Previous studies have indicated that MPO may function both intracellularly and extracellularly.^{17,18} From the *in vitro* studies of Baehner et al.¹⁹ and Henson,²⁰ it has been demonstrated that during phagocytosis neutrophilic MPO appears in the extracellular fluid. The present experiments are in keeping with those observations, and we have attempted to extend our understanding of the fate of MPO by employing methods to assay total cellular MPO and to study it *in vitro* and *in vivo*. *In vitro* studies were carried out to explore the fate of the active MPO of neutrophils performing phagocytosis. After neutrophils were allowed to ingest bacteria, 52% of initial MPO could be found in the cells, 8% in the medium, and 40% of their MPO activity was lost. Since neutrophils can become disrupted during phagocytosis, we also homogenized neutrophils to determine

if disruption alone released MPO. Mechanically disrupted neutrophils lost 31% of their initial MPO, and 4% of the predisruption cellular MPO appeared in the medium in soluble active form. Thus, during these *in vitro* studies, neutrophils retained much of their MPO while releasing a portion into the extracellular environment in active form.

Further studies were performed to determine if these events occur *in vivo*. Experimental pyogenic infection was induced by the intradermal injection of bacteria, and MPO activity was evaluated either in neutrophils obtained from blood or those harvested directly from inflammatory lesions. Circulating neutrophils from infected animals contained significantly less MPO than neutrophils from control uninfected animals, suggesting that during infection neutrophils undergo activation and lose MPO during their transit in the blood. Neutrophils from the inflamed sites had sustained a 48% decrease in MPO activity, as in the *in vitro* studies. Extracellular MPO was present in concentrations of 20–45 U/ml of extracellular fluid. Interestingly, it has been shown that MPO can participate in bacterial iodination,²¹ mediate toxicity to mammalian cells,¹⁸ or inactivate chemoattractants⁶ at concentrations of 0.024–0.064 U/ml, values well below those we have demonstrated *in vivo* in inflammatory fluid. Thus, the present data indicate that sufficient concentrations of active MPO appear in inflammatory fluid to cause similar effects in the intact animal.

In conclusion, we assessed the effect of *in vitro* phagocytosis or disruption of neutrophils and *in vivo* pyogenic infection on the cellular content of MPO and on the appearance of the enzyme in soluble form in the extracellular environment. It was found that in all of these experimental situations, there was a decrease in the MPO within neutrophils. *In vitro*, 4%–8% of the original MPO activity was found in the extracellular fluid. *In vivo*, MPO was also released, and concentrations exceeding those previously shown to be active *in vitro* were demonstrated in extracellular fluid. These experiments support the concept that neutrophils can export MPO into the extracellular environment where it may participate in a variety of biologic effects.

REFERENCES

1. Weissman G, Uhr JW: Studies on lysosomes. IX. Localization of bacteriophages and thorotrast and their inflammatory properties. *Biochem Pharmacol* 17(Suppl):5, 1968
2. Weissman G, Spilberg I, Krakauer K: Arthritis induced in rabbits by lysates of granulocyte lysosomes. *Arthritis Rheum* 12:103, 1969
3. Klebanoff SJ: Myeloperoxidase-halide-hydrogen peroxide antibacterial system. *J Bacteriol* 95:2131, 1968
4. Edelson PJ, Cohn ZA: Peroxidase-mediated mammalian cell cytotoxicity. *J Exp Med* 138:318, 1973
5. Clark RA, Klebanoff SJ: Chemotactic factor inactivation by the myeloperoxidase-hydrogen peroxide-halide system. *J Clin Invest* 64:913, 1979
6. English D, Anderson BR: Single-step separation of red blood cells, granulocytes and mononuclear leukocyte on discontinuous density gradients of Ficoll-Hypaque. *J Immunol Meth* 5:249, 1974
7. Lennette EH, Spaulding ED, Truant SP: *Manual of Clinical Microbiology*. Washington DC, American Society of Microbiology, 1974, p 934
8. Renlund DG, Macfarlane JL, Christensen RD, Lynch RE,

Rothstein G: A quantitative and sensitive method for measurement of myeloperoxidase. *Clin Res* 28:75A, 1980 (abstr)

9. Worthington Enzyme Manual. Freehold, NJ, Worthington Biochemical Corp, 1972, p 43
10. Kaplow LS: Simplified myeloperoxidase stain using benzidine dihydrochloride. *Blood* 26:215, 1965
11. Patriarca P, Cramer R, Marussi M, Rossi F, Romeo D: Mode of activation of granule-bound NADPH oxidase in leukocytes during phagocytosis. *Biochim Biophys Acta* 237:335, 1971
12. Zeya HI, Spitznagel JK: Cationic proteins of polymorphonuclear leukocyte lysosomes. I. Resolution of antibacterial and enzymatic activities. *J Bacteriol* 91:750, 1966
13. Salmon SE, Cline MJ, Schultz J, Lehrer RI: Myeloperoxidase deficiency. Immunologic study of a genetic leukocyte defect. *N Engl J Med* 282:250, 1970
14. Evans WH, Wilson S, Mage MG, Grieshaber CK, Himmelhock SR: Biochemical characterization of isolated immature granulocytes from guinea pig bone marrow. *J Reticuloendothel Soc* 9:209, 1971
15. Bradley PP, Priebe DA, Christensen RD, Rothstein G: Measurement of cutaneous inflammation: Estimation of neutrophil content with an enzyme marker. *J Invest Dermatol* 78:206, 1982
16. Mage MG, Evans WH, Himmelhock SR, McHugh L: Immunological identification and quantification of guinea pig heterophil and eosinophil peroxidases. *J Reticuloendothel Soc* 9:201, 1971
17. Schultz J, Kaminker K: Myeloperoxidase of the leukocyte of normal human blood. I. Content and localization. *Arch Biochem Biophys* 96:465, 1962
18. Klebanoff SJ, Hamon CB: Role of myeloperoxidase-mediated antimicrobial systems in intact leukocytes. *J Reticuloendothel Soc* 12:170, 1972
19. Clark RA, Klebanoff SJ: Neutrophil-mediated tumor cell cytotoxicity: Role of the peroxidase system. *J Exp Med* 141:1442, 1975
20. Baehner RL, Karnovsky MJ, Karnovsky ML: Degranulation of leukocytes in chronic granulomatous disease. *J Clin Invest* 48:187, 1969
21. Henson PM: The immunologic release of constituents from neutrophil leukocytes. I. The role of antibody and complement on nonphagocytosable surfaces or phagocytosable particles. *J Immunol* 107:1535, 1971
22. Klebanoff SJ: Iodination of bacteria: A bactericidal mechanism. *J Exp Med* 126:1063, 1967