0022-202X/82/7803-0206$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 78:206–209, 1982

Vol. 78, No. 3
Printed in U.S.A.

# Measurement of Cutaneous Inflammation: Estimation of Neutrophil Content with an Enzyme Marker

PETER P. BRADLEY, M.D., DENNIS A. PRIEBAT, M.D., ROBERT D. CHRISTENSEN, M.D., AND GERALD ROTHSTEIN, M.D.

*The Department of Medicine, University of Utah College of Medicine, Salt Lake City, Utah*

**We examined the hypothesis that myeloperoxidase (MPO), a plentiful constituent of neutrophils, might seve as a marker for tissue neutrophil content. To completely extract MPO from either neutrophils or skin, hexadecyltrimethylammonium bromide (HTAB) was used to solubilize the enzyme. With this detergent treatment, 97.8 ± 0.2% of total recoverable MPO was extracted from neutrophils with a single HTAB treatment; 93.1 ± 1.0% was solubilized with a single treatment of skin. Neutrophil MPO was directly related to neutrophil number; with the dianisidine-$H_2O_2$ assay as few as $10^4$ neutrophils could be detected. The background level of MPO within uninflamed tissue was 0.385 ± 0.018 units per gram of tissue, equivalent to only 7.64 ± 0.36 × $10^5$ neutrophils. In experimental staphylococcal infection, skin specimens contained 34.8 ± 3.8 units MPO per gram, equivalent to 8.55 ± 0.93 × $10^7$ neutrophils. These studies demonstrate that MPO can be used as a marker for skin neutrophil content: it is recoverable from skin in soluble form, and is directly related to neutrophil number. Further, normal skin possesses a low background of MPO compared to that of inflamed skin.**

The accumulation of neutrophils is a characteristic feature of such cutaneous inflammatory diseases as pyogenic infection and dermatitis herpetiformis [1]. Indeed, the presence of neutrophils in the lesions may bear a relationship to the pathophysiology of the process. During infection, neutrophils may ingest and kill organisms to reverse the process; in dermatitis herpetiformis, neutrophils may accentuate the inflammatory process as they secrete their toxic contents [2]. In either case, the quantity of neutrophils in skin can be taken as one measure of the intensity of the disease process, and therefore, a means of quantifying skin neutrophil content is of considerable interest. However, the study of skin neutrophil accumulation during inflammation has been hampered by a lack of the means to quantify the total number of neutrophils present in the entire tissue lesion. The present study derives from our hypothesis that a naturally occurring constituent of neutrophils, myeloperoxidase (MPO), might be utilized as a marker for tissue neutrophil content. Thus, in this report, we describe a method for extracting MPO in soluble form from neutrophils, uninflamed skin, and skin subjected to infection with staphylococci. Total MPO was related to neutrophil number, uninflamed skin possessed a low background of MPO and bacterially infected skin exhibited an accumulation of MPO. Therefore, we propose that the measurement of skin MPO provides a quantitative index of tissue neutrophil content.

Manuscript received May 5, 1981; accepted for publication July 29, 1981.

This work was supported in part by Research Training Grant #AM-07715 from the National Institute of Health.

This work was presented in part at the Annual Meeting of the American Federation for Clinical Research, San Francisco, CA, April 27, 1981. An abstract has been published in *Clinical Research* 29:280A, 1981.

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

Abbreviations:
HTAB: hexadecyltrimethylammonium bromide
MPO: myeloperoxidase

## MATERIALS AND METHODS

### *Preparation of Neutrophils*

Vena cava blood was collected from adult male or female Sprague-Dawley rats in syringes containing 10–15 units of heparin (Panheparin, Abbott Labs., N. Chicago, IL) per ml of blood. The leukocytes were separated in discontinuous gradients of Ficoll (Pharmacia Fine Chemicals, Piscataway, NJ) and sodium diatrizoate (Winthrop Lab., NY, NY) [3]. Neutrophils thus collected were washed 3 times and suspended in McCoy's 5A medium (M5A) (Flow Labs., Inglewood, CA). Contaminating erythrocytes were subjected to hypotonic lysis in 3 vol of distilled water for 30 seconds, followed by the addition of 1 vol 3.5% NaCl. Final cell concentrations were determined by electronic counting (Coulter Electronics, Hialeah, FL). Two hundred cell differential counts were performed using Wright's stained smears. The cell suspension contained 75 ± 5% (mean ± SEM) neutrophils, 21 ± 5% lymphocytes, 1.8 ± 0.5% monocytes and 2.4 ± 0.5% eosinophils.

### *Preparation of Mononuclear Cells*

Monocyte-lymphocyte suspensions were obtained from heparinized rat blood by ficoll-sodium diatrizoate (Ficoll-Paque, Pharmacia) separation [4]. The resulting monocyte-lymphocyte suspensions contained 17 ± 1% monocytes and 83 ± 1% lymphocytes and were free from contaminating neutrophils.

### *Bacteria*

*Staphylococcus aureus* ATCC#25923 were grown overnight in tryptic soy broth (Difco Lab., Detroit, MI) and were washed and suspended in M5A prior to use. The concentration of bacteria was determined by McFarland standards [5].

### *Myeloperoxidase Assay*

Myeloperoxidase was extracted from cells or homogenized tissue by suspending the material in 0.5% hexadecyltrimethylammonium bromide (HTAB) (Sigma Chemical Co., St. Louis, MO) in 50 mM potassium phosphate buffer, pH 6.0, before sonication in an ice bath for 10 seconds (Heat Systems-Ultrasonics, Plainview, NY). The specimens were freeze-thawed 3 times, after which sonication was repeated. Suspensions were then centrifuged at 40,000 ×*g* for 15 min and the resulting supernate or pellet assayed.

MPO was assayed spectrophotometrically: 0.1 ml of the material to be measured was mixed with 2.9 ml of 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). These conditions were employed because they confer increased sensitivity to the assay [6]. The change in absorbance at 460 nm was measured with a Beckman DU spectrophotometer (Beckman Instruments, Fullerton, CA) with a recording attachment (Gilford Instrument Labs., Oberlin, OH). Turbid suspensions were assayed in the photomultiplier compartment of a Cary Spectrophotometer Model 118 (Varian, Palo Alto, CA). One unit of MPO activity was defined as that degrading one micromole of peroxide per minute at 25°C [7].

### *Statistical Analysis*

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

## RESULTS

### *Extraction of Neutrophil Myeloperoxidase (MPO) Activity*

Since MPO is located within the primary granules of neutrophils [8], extraction of MPO depends upon procedures to disrupt the granules, as well as ones which render MPO soluble in

aqueous solution. To evaluate the recovery of neutrophil MPO in soluble form, 0.57 to 4.10 × $10^6$ neutrophils were subjected to sonication and freeze-thawing in 2 ml 50 mM phosphate buffer pH 6.0 or 2 ml 50 mM phosphate buffer containing 0.5% HTAB. The suspensions were centrifuged at 40,000 ×*g* for 15 min and the MPO activity in the supernate or in the pellet assayed. As shown in Fig 1, when the cells were disrupted in buffer alone, most of the activity remained associated with the particles. In contrast, when neutrophils were disrupted in HTAB, recovery of activity was enhanced and virtually all the activity appeared in the supernate. The pellets from the HTAB-treated cells were extracted with 2 ml HTAB, and the extracts and the pellets assayed. The initial extract contained 97.8 ± 0.2% of the total recoverable activity. After 3 extractions, no additional MPO was obtained in soluble form and 1.4 ± 0.4% of the activity remained in the pellet.

*Determination of MPO Per Neutrophil*

To determine neutrophil MPO content, neutrophils were obtained from caval blood from adult rats, and the cellular MPO was extracted with 0.5% HTAB. In 12 experiments, the MPO recovered from 0.75–4.02 × $10^6$ neutrophils was 5.04 ± 0.21 × $10^{-7}$ U per neutrophil and was proportional to the number of neutrophils subjected to extraction. The sensitivity of the method permitted MPO extraction from as few as 0.035 × $10^6$ neutrophils (Fig 2).

*MPO Activity of Uninflamed Skin*

To determine the background levels of peroxidase in control uninflamed skin, circular full thickness specimens of skin 15 mm diameter were excised from the shaved dorsum of rats. The skin was very finely minced with scissors in a beaker containing

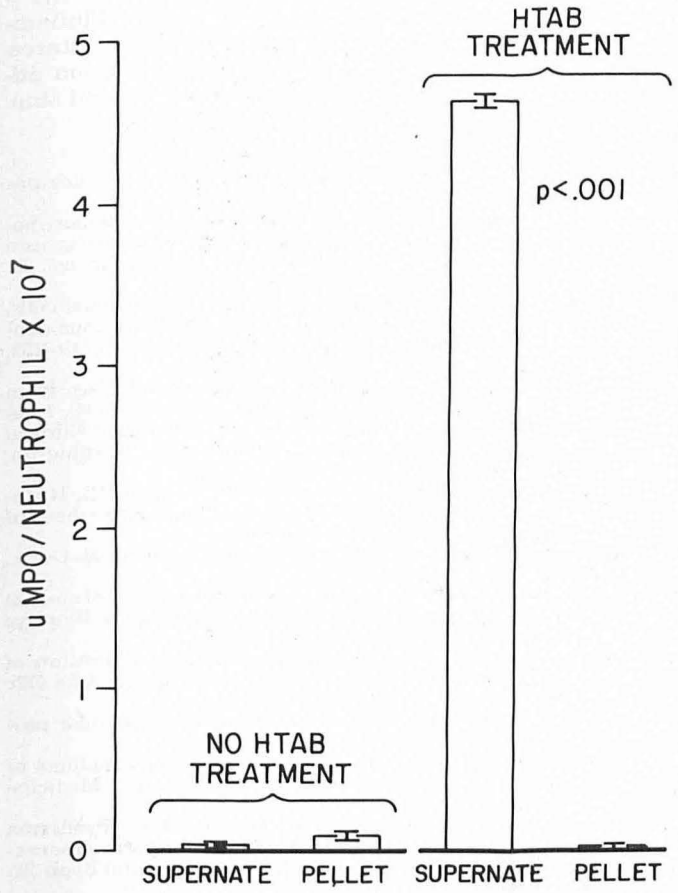


FIG 1. Effect of hexadecyltrimethylammonium bromide (HTAB) upon MPO recovery. MPO recovered from either the supernate or pellet fraction of disrupted neutrophils is shown. The *bars* represent the mean values and the *brackets* indicate standard error for groups of 6 or more values.

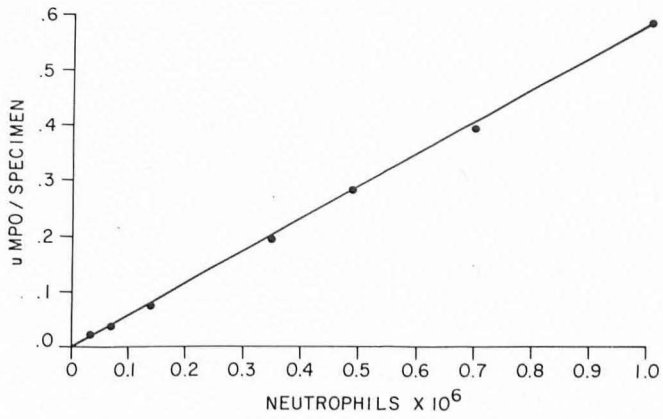


FIG 2. Relationship of recovered MPO activity to neutrophil number. Each *point* represents a single value for MPO recovered from specimens containing various numbers of blood neutrophils.

1 ml 0.5% HTAB in 50 mM phosphate buffer pH 6.0. The suspension was transferred to a Potter-Elvejhem type homogenizer, and the beaker was rinsed twice with 1 ml HTAB, the rinses being added to the homogenizer. The suspension was homogenized for 10 min in ice bath by means of a Teflon plunger powered by a variable speed drill (Black & Decker Mfg. Co., Towson, MD). The homogenizer was rinsed twice with 1 ml HTAB and the pooled homogenate (5 ml) was subjected to sonication and freeze-thawing. The suspension was centrifuged at 40,000 ×*g* for 15 min and the supernate reserved. The homogenized tissue pellet was extracted 4 times with 5 ml HTAB, and the extracts and pellet assayed. In six experiments, the first plus second extracts contained 81.7 ± 0.9% of the total recoverable activity. After 4 extractions of the pellet, no additional MPO activity could be extracted and the residual pellet exhibited 9.8 ± 0.9% of the total recoverable activity (soluble plus particle associated). The skin specimens, which weighed 0.2900 ± 0.0045 gm, contained 0.114 ± 0.005 U peroxidase per specimen, or 0.385 ± 0.018 U per gram wet tissue. Based upon 5.04 × $10^{-7}$ U MPO per neutrophil, the peroxidase activity recoverable from normal skin was equivalent to 7.64 ± 0.36 × $10^5$ neutrophils per gram of tissue, or 2.26 ± 0.10 × $10^5$ neutrophils per skin specimen.

To determine whether skin tissue might interfere with the recovery of neutrophil MPO in inflammatory lesions, neutrophils were combined with specimens of uninflamed skin, and the MPO content of the neutrophils plus skin was compared to that of an aliquot containing the same number of neutrophils alone. In 10 experiments, skin plus 0.61 to 3.68 × $10^6$ neutrophils was homonogenized in 0.5% HTAB after which the specimens were subjected to sonication and freeze-thawing. The MPO activity recovered in the initial extract from skin plus neutrophils ranged from 0.233 to 2.007 U MPO and was 97 ± 3% of that obtained from neutrophils alone, indicating that skin did not itself interfere with MPO recovery.

*MPO Assay in Cutaneous Inflammatory Lesions*

In order to induce experimental cutaneous inflammation, rats received 1.3–2.5 × $10^9$ staphylococci (5 × $10^{10}$ organisms per ml) by intradermal injection into the skin of the dorsum. Two such injections were given to each animal and resulted in erythema and swelling at the injection sites. Twenty-four hours after inoculation, the animals were phlebotomized and sacrificed, and the inflammatory lesions were excised. The lesions were minced in medium and the inflammatory suspension collected to determine its composition. The inflammatory cells shed during mincing were 90.8 ± 0.9% neutrophils, 3.1 ± 0.5% monocytes, 0.2 ± 0.1% lymphocytes, and 5.8 ± 0.7% damaged nucleated cells. The minced tissue was suspended in 3.0 ml 0.5% HTAB and subjected to MPO extraction by homogenization in a Potter-Elvejhem homogenizer as described above. The sus-

*Recovery of myeloperoxidase activity from inflamed skin: Mean values (±SE) are given for units of myeloperoxidase and percent of total activity in 5 experiments*

| | uMPO | % Total Activity |
|---|---|---|
| First extract | 16.00 ± 2.98 | 93.1 ± 1.0 |
| Extracts 2–6 | 0.88 ± 0.29 | 4.9 ± 0.8 |
| Extracts 7–11 | 0.09 ± 0.06 | 0.4 ± 0.2 |
| Residual pellet | 0.24 ± 0.02 | 1.6 ± 0.3 |
| Total activity | 17.21 ± 3.27 | 100.0 ± 19.0 |

pension was removed and the homogenizer washed twice with 1 ml HTAB, the washes being added to the suspension. Then the suspension was centrifuged at 40,000 ×*g* for 15 min and the supernate reserved. The pellet was extracted 10 additional times with 5 ml HTAB, and the extracts and pellet assayed. The result of 5 such experiments, shown in the Table, demonstrates that direct extraction of inflamed tissue yielded 98.0 ± 0.3% of the activity in soluble form, the remainder being associated with the tissue pellet. Of the total recoverable activity, 17.21 ± 3.27 U per specimen, 93.1 ± 1.0% was contained in the initial extract. By means of the blood neutrophil content, 4.07 ± 0.20 × $10^{-7}$ U per neutrophil in the infected animals, the MPO from inflamed skin was found to be equivalent to 4.23 ± 0.80 × $10^{7}$ neutrophils per specimen.

*Nonneutrophil Contribution to Lesion Peroxidase Content*

The possibility that monocyte peroxidase activity might contribute to the inflammatory lesion MPO content was also evaluated. Since monocytes are granulocytes which also possess MPO activity [9], their MPO content was evaluated in blood monocyte-lymphocyte suspensions. In 4 such specimens, 0.67–1.40 × $10^{6}$ monocytes contained 9.36 ± 2.20 × $10^{-9}$ U MPO per monocyte. Thus, the level of MPO activity in monocytes (which comprised only 3.1 ± 0.5% of the inflammatory cells) could account for only 0.06 ± 0.01% of the MPO recovered from 24 hr inflammatory lesions. Although eosinophils contain peroxidase, no eosinophils appeared in the lesions and thus did not contribute activity. *S. aureus* (5 × $10^{10}$) had no detectable peroxidase activity.

## DISCUSSION

Accumulation of neutrophils is a prominent feature of a number of inflammatory diseases of the skin [1, 10–12]. Indeed, an ability to estimate the quantity of neutrophils in inflamed tissue might prove useful to judge the intensity of inflammation or the effect of experimental or therapeutic maneuvers to alter the inflammatory response. Furthermore, a means to quantify tissue neutrophil content might be useful in exploring factors which influence neutrophil migration to skin *in vivo*. We reasoned that MPO, a constituent of neutrophils, would be a marker for tissue neutrophils, since the enzyme is abundant in neutrophils comprising as much as 5% of their mass [8]. However, MPO is tightly associated with the cell granules as noted by Patriarca, Cramer et al [13] and as demostrated by the present cell disruption studies. When cells were disrupted in aqueous buffer alone, 71% of the MPO activity remained associated with the particulate fraction of the cells, and only trace amounts were found in the supernate. In contrast, treatment with HTAB, an ionic detergent previously shown to solubilize MPO [13, 14, 15, 16] resulted in extraction of 99% of the MPO to the soluble fraction of disrupted cells, with enhanced recovery of activity as well. HTAB extraction of neutrophils in the presence of skin was effective in recovering 97% of the added activity, indicating that soluble extraction of MPO from neutrophils which had accumulated in inflamed skin would be possible. Subsequent experiments to study neutrophil accumulation in bacterially infected skin revealed that HTAB treatment permitted 93% of the recoverable activity to appear in the first extract. Inflammatory cells shed from the lesion during mincing were primarily neutrophils (91%) with relatively small numbers of monocytes, which contributed only 0.06% of the total lesion MPO. Thus, in these lesions, MPO content was taken as a reflection of the quantity of neutrophils which had migrated there during the infection. By comparing the total lesion MPO with the average blood neutrophil MPO from the same animal, it was possible to calculate a minimum number of neutrophils which must have migrated to the lesion to contribute the MPO therein.

Although the number of neutrophils in inflamed skin may be estimated by the MPO method, the calculation of the neutrophil number warrants further discussion. First, quantification of tissue neutrophil content is necessarily a measure of the net accumulation of the cells or their markers. Thus, it might be expected that due to neutrophil or MPO loss, any measurement would be a minimum estimate of neutrophil accumulation in the lesion, and might underestimate the total number of neutrophils which had migrated there prior to assay. Also, neutrophils stimulated by the ingestion of staphylococci may contain less MPO than do resting neutrophils [17]. For this reason, interepretation must be tempered by the concept that tissue MPO levels reflect the minimum number of blood neutrophils which must have reached the lesion to contribute their MPO, and that the measurement may represent an underestimate of the total migration of neutrophils to the lesion before sampling.

From the data, we conclude that MPO is a marker for tissue neutrophil content: MPO was extractable in soluble form by HTAB, whether from neutrophils alone or from neutrophils in the presence of skin. Further, MPO bore a direct relationship to the number of cells from which it was extracted. Control uninflamed skin possessed a low background of MPO, but when neutrophils accumulated in bacterially infected skin, the skin MPO increased one hundred fold and could be recovered in soluble form by HTAB treatment. We propose that skin MPO content may be useful as a measure of the neutrophil inflammatory response in a variety of clinical and experimental states. Of course, use of this approach in humans depends upon adaptation of the technique specifically for human cells and skin.

## REFERENCES

1. Katz SI, Strober W: The pathogenesis of dermatitis herpetiformis. J Invest Dermatol 70:63–75, 1978
2. Stendahl O, Molin L, Dahlgren C: The inhibition of polymorphonuclear leukocyte cytotoxicity by dapsone. A possible mechanism in the treatment of dermatitis herpetiformis. J Clin Invest 62: 214–220, 1978
3. English D, Anderson BR: Single-step separation of red blood cells, granulocytes and mononuclear leukocytes on discontinuous density gradients of Ficoll-Hypaque. J Immunol Methods 5:249–252, 1974
4. Boyum A: Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest 21 (suppl 97):77–89, 1968
5. Lennete EH, Spaulding ED, Truant SP (eds.): Manual of Clinical Microbiology. American Society of Microbiology, Washington, D.C., 1974, p 934
6. Renlund DG, Macfarlane JL, Christensen RD, Lynch RE, Rothstein G: A quantitative and sensitive method for measurement of myeloperoxidase. Clin Res 28:75A, 1980
7. Worthington Enzyme Manual. Worthington Biochemical Corp., Freehold, NJ, 1972, pp 43–45
8. Schultz J, Kaminker K: Myeloperoxidase of the leukocyte of normal human blood. I. Content and localization. Arch Biochem Biophys 96:465–467, 1962
9. Bos A, Wever R, Roos D: Characterization and quantification of the peroxidase in human monocytes. Biochim Biophys Acta 525: 37–44, 1978
10. Kingery FAJ, Chinn HD, Saunders TS: Generalized pustular psoriasis. Arch Dermatol 84:74–81, 1961
11. Winkelman RK, Ditto WB: Cutaneous and visceral symptoms of necrotizing or "allergic" angiitis: A study of 38 cases. Medicine 43:59–89, 1964
12. Brunsting LA, Geockerman WH, O'Leary PA: Pyoderma (echthyma) gangrenosum. Clinical and experimental observations in five cases occurring in adults. Arch Dermatol Syph 22: 655–680, 1930
13. 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 237:335–338, 1971

14. Zeya HI, Spitznagel JK: Cationic proteins of polymorphonuclear leukocyte lysosomes. I. Resolution of antibacterial and enzymatic activities. J Bacteriol 91:750–754, 1966
15. Salmon SE, Cline MJ, Schultz J, Lehrer RI: Myeloperoxidase deficiency. Immunologic study of a genetic leukocyte defect. N Engl J Med 282:250–253, 1970
16. Evans WH, Wilson S, Mage MG, Grieshaber CK, Himmelhock SR: Biochemical characterization of isolated immature granulocytes from guinea pig bone marrow. J Reticuloendothel Soc 9:209–224, 1971
17. Bradley P, Rothstein G: Release of myeloperoxidase during inflammation. Clin Res 29:40A, 1981

## Announcement

The International Society for Bioengineering and the Skin wish to announce the availability of a discussion document on a standard for *in vivo* measurement of dermal physical properties. Price: £10.00 (UK). $US 25.00 (Overseas). incl. p.p. Professor R. Marks, Professor of Dermatology, Department of Medicine, Welsh National School of Medicine, Heath Park, Cardiff. CF4 4XN., U.K.