Aberrant Restriction Endonuclease Digests of DNA From Subjects With Hereditary Myeloperoxidase Deficiency

By William M. Nauseef

Myeloperoxidase (MPO) is a critical component in the oxygen-dependent microbicidal activity of neutrophils. Hereditary deficiency of MPO occurs commonly, but its genetic basis has not been determined. Previously we have reported the presence of an 89-kilodalton protein, likely pro-MPO, in normal and MPO-deficient neutrophils and hypothesized that the absence of peroxidase activity in neutrophils from affected subjects was the result of defective posttranslational processing of pro-MPO. In this study we analyzed nucleic acids from three completely and two partially MPO-deficient individuals by using a cDNA probe for MPO. The affected individuals studied are unrelated to one another. Neutrophils from all affected subjects lacked mature MPO subunits; however, a monospecific antibody for MPO identified in these cells a high–molecular weight protein that is the same size as pro-MPO. Northern blots demonstrated that the amount and size of RNA (3.3 kilobases [kb]) in a completely deficient subject was normal. BglII digests of genomic DNA from control individuals (n – 14) contained three fragments that hybridized with cDNA for MPO under very stringent conditions. In contrast, BglII digests of genomic DNA from completely MPO-deficient individuals contained an extra fragment of 2.1 kb that was not present in DNA from controls. In addition, two different endonuclease digest patterns were found in MPO-deficient individuals who were biochemically and phenotypically identical. We conclude from these studies that (a) hereditary MPO deficiency is not associated with a major deletion or rearrangement of the MPO gene; (b) myeloid precursors in an MPO-deficient individual contain normal amounts of an mRNA that is the same size as that for MPO in normal individuals; and (c) the genetic basis for MPO deficiency may be heterogeneous, with at least two genotypes generating the same phenotype. These findings are consistent with the hypothesis that the genetic defect in MPO deficiency results in synthesis of a modified pro-MPO that undergoes defective posttranslational processing.

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METHODS

Materials. Restriction endonucleases and reaction buffers, proteinase K, HindIII λ phage, and Escherichia coli DNA polymerase I were obtained from the DNA Core Facility at the University of Iowa; dextran, Ficoll-Hypaque, and random oligonucleotides for priming (pdN6) were obtained from Pharmacia Fine Chemicals (Piscataway, NJ); agarose, guanidiinium isothiocyanate, cesium chloride, and phenol were of molecular biology grade and obtained from International Biotechnologies Inc (New Haven, CT); α-32P-deoxyxycytosine triphosphate (dCTP) (800 Ci/mmoll) and α-32P-deoxyguanosine triphosphate (dGTP) (800 Ci/mmoll) were obtained from New England Nuclear (Boston); other materials are listed in the text.

Cells. PMNs were isolated from heparinized venous blood by dextran sedimentation and Ficoll-Hypaque density centrifugation followed by hypotonic lysis of erythrocytes as previously described. Leukocytes were isolated from bone marrow aspirates by dextran sedimentation and hypotonic lysis of erythrocytes. All studies were conducted with the approval of the Human Investigation Committee at The University of Iowa.

Subjects. The MPO status of individuals was judged by three criteria: enzymatic activity, spectrophotometric evidence of MPO heme, and immunoechemical evidence of MPO-related peptides in Western blots (see later). Individuals (P.M., L.H., and J.Fu.) were classified as completely deficient because their PMNs had less than 10% of the peroxidase activity of normal PMNs, lacked spectroscopic evidence of MPO by oxidation-reduction difference spectroscopy, and lacked any mature MPO subunits by Western blot analysis.

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Submitted May 2, 1988; accepted August 15, 1988.

Supported by grants from the Veterans Administration (Merit Review) and the March of Dimes Birth Defects Foundation (Basil O’Connor Starter Research Grant 5-468).


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0006-4971/89/7301-0005$3.00/0

deficient subjects (C.W. and C.N.) had 10% to 50% of the peroxidase activity of normal PMNs and had 10% to 50% of normal MPO by spectroscopic and Western blot analysis. All subjects had the 89-Kd band in Western blots. The completely deficient subjects were not genetically related to one another; C.J.F. and C.H.F., two partially deficient subjects, are siblings. Subjects C.S.F. and J.Fu. are the parents of C.J.F. and C.H.F., but each have normal amounts of enzymatically active MPO. All of the deficient subjects (P.M., L.H., J.Fu., C.W., and C.N.) have been previously described. Control DNA was isolated from 14 unrelated individuals with normal MPO activity.

Assessment of MPO status. Peroxidase activity of solubilized PMNs was quantitated by spectrophotometric determination of o-dianisidine oxidation as previously described. Oxidation-reduction difference spectroscopy was performed by using a Perkin-Elmer dual-beam spectrophotometer equipped with a head-on photomultiplier tube. MPO was quantitated by using an extinction coefficient of 75 (mmol/L)/cm at 472 nm. In this assay the limit of detection of MPO is approximately 0.50 nmol/L.

Isolation and blotting of nucleic acids. Total cellular RNA was isolated from bone marrow leukocytes according to the method of Chirgwin et al and quantitated by absorbance at 260 nm. RNA was denatured, separated in 0.8% agarose formaldehyde gels, and blotted to nylon filters (Zetabind, Cuno, Inc, Meriden, CT). High-molecular weight (mol wt) DNA was isolated from PMNs by using a modification of the sodium dodecyl sulfate (SDS)–proteinase K digestion described by Pellicer et al. DNA was digested with a variety of restriction endonucleases, separated in 0.8% agarose gels, and blotted to nylon (Nytran, Schleicher & Schuell, Inc, Keene, NH). To ensure a complete digestion of DNA, a two-fold excess of restriction endonuclease was added and the reaction incubated for two or more hours at 37°C, followed by the addition of another twofold excess of enzyme, after which the incubation continued for an additional two or more hours. Prehybridization, hybridization of the o-labeled probe, and washing of filters were performed according to manufacturer’s specifications. The final washes were under stringent conditions using 0.1x SSC (1x SSC = 150 mmol/L NaCl, 15 mmol/L Na citrate) and 0.1% SDS at 65°C for one hour. Fragment sizes were determined on the basis of the migration of HindIII digestion fragments of λ phage in DNA gels or of a commercially provided RNA ladder (Bethesda Research Laboratories, Gaithersburg, MD) in RNA gels.

Probe. Blots were analyzed for MPO-related sequences by using a 2.2-kilobase (kb) insert cut with PstI from the previously described plasmid pMPO2. The insert was separated from pUC8 by electrophoresis in low-temperature-melting agarose, purifed by phenol-chloroform extraction of the melted gel slice, and labeled by random priming with α-32P-dCTP and α-32P-dGTP. Dot-blot analysis verified the absence of detectable pUC8 sequences in the isolated insert. The control probe for blots was a 770-base pair (bp) cDNA probe for chicken actin (Oncor, Gaithersburg, MD).

RESULTS

All subjects with complete or partial MPO deficiency had immunochemical evidence of a high–mol wt MPO–related peptide when solubilized PMNs were analyzed by electroblotting. PMNs from individuals with normal amounts of MPO, as determined by enzymatic and spectroscopic quantitation, contained the normal subunits of MPO described previously in addition to the broad band in the 80- to 90-Kd range (Fig 1). Individuals with a complete deficiency of MPO (P.M., J.Fu. and L.H.) lacked mature MPO subunits but contained the same high–mol wt band seen in PMNs from control subjects. PMNs from individuals with a partial deficiency of MPO contained decreased amounts of mature MPO subunits in addition to the high–mol wt species. These findings are identical to those of previous studies and are consistent with the hypothesis that MPO-deficient myeloid
cells synthesize a defective pro-MPO that is not processed normally.

To determine whether MPO-deficient cells contained an RNA species for MPO, RNA was isolated from the myeloid precursors obtained from bone marrow aspiration from one of the MPO-deficient subjects (P.M.) and compared with that from a control with normal MPO. Total cellular RNA (2.5 μg) was then analyzed by Northern blotting by using the labeled insert from pMPO2 and washing the blot under high stringency. As shown in Fig 2, myeloid precursors from the MPO-deficient individual contained RNA for MPO that was present in the same amount and was the same size as the 3.3-kb species seen in myeloid precursors from the control subject. The same blot was exposed to labeled actin probe, and equal amounts of 2.0-kb RNA were detected, indicating there was no difference in the amount of RNA in the samples that was subjected to electrophoresis (data not shown).

To determine whether MPO deficiency was the result of the deletion of all or a major part of the gene for MPO, Southern blot analysis of genomic DNA digested with a variety of restriction endonucleases and probed with the insert from pMPO2 was performed. After hybridization, the filters were washed under very stringent conditions to limit detection to sequences with very high homology with the probe. Digests of DNA from control and from MPO-deficient subjects with HincII, BamHII, SacI, Xhol, or PvuII resulted in identical bands when hybridized with the pMPO2 insert (data not shown).

However, Southern blots of BglII digests of DNA from control and from MPO-deficient individuals were different. On the basis of our sequence data of the full-length cDNA we knew there were two BglII sites at bases 2,250 and 2,463.9 In addition, sequence data from genomic cloning of MPO by Morishita et al19 and by Johnson et al (Keith Johnson, University of Toledo, personal communication, 1988) indicate there is a single BglII site in one of the introns. Thus we predicted there would be only three fragments after BglII digestion since the two sites in the cDNA sequence are only 200 bp apart and would produce a fragment too small to be visualized under the conditions of the electrophoresis.

The results of Southern analysis of the BglII digests are shown in Fig 3. As predicted, the BglII digestion of control DNA resulted in three fragments, a very large fragment and two smaller fragments of 3.3 and 2.6 kb. An identical pattern has been seen in all 14 unrelated control subjects studied to date. Digests of DNA from P.M. and J.Fu., two unrelated, completely MPO-deficient subjects, contained three fragments of the normal size and in addition had a 2.15-kb fragment. The digest of L.H., a completely deficient individual whose PMNs are biochemically and immunochemically indistinguishable from those of P.M. and J.Fu., contained the two larger BglII fragments but lacked the 2.6-kb fragment and had a more intense signal at 2.15 kb than did the other MPO-deficient subjects. The association of MPO deficiency and the presence of the 2.15-kb BglII fragment was statistically significant (P = .001, Fisher’s exact test considering only the completely deficient subjects).

The DNA of C.W., a partially deficient individual, had an apparently normal BglII digest. In contrast, C.N., a partially MPO-deficient individual indistinguishable biochemically and phenotypically from C.W., had a BglII digest identical (Fig 4) to that seen with DNA from completely deficient cells. Cells from C.S.F. and J.F. had normal MPO content and activity although they are the parents of two children with a partial MPO deficiency. The BglII digests of DNA from C.S.F. and J.F. contained normally-sized fragments, but the relative intensities of the 3.3- and 2.6-kb bands differed from that seen in the normal digest.

**DISCUSSION**

Until the late 1970s, only 15 patients from 12 families had been reported with a hereditary deficiency of MPO.12,20-26

![Fig 3](image3.png) **Fig 3.** Southern blot of BglII digests of genomic DNA. Control DNA digested with BglII had three fragments that hybridized with the MPO probe at 24, 3.3, and 2.6 kb. The same three bands were seen in digests of DNA from partially deficient subjects as well as from C.S.F. and J.Fu., two individuals with normal MPO content and activity but who have two children with a partial deficiency. However the digests of DNA from all three completely deficient subjects contained a 2.15-kb band not present in digests of DNA from control subjects. In addition L.H. lacked the normal 2.6-kb band, in contrast to the patterns of the other two completely deficient subjects.

![Fig 4](image4.png) **Fig 4.** Southern blot of BglII digests of genomic DNA. Three unrelated individuals with normal MPO activity in their cells had fragments of the same size as those found in control digests from different subjects shown in Fig 3. DNA from C.N., a partially MPO-deficient subject, contained a 2.15-kb band present in completely deficient subjects.
and the rarity of this disorder seemed consistent with the pivotal role of MPO in host defense. However, by using automated systems for leukocyte differential determination, several investigators have documented a prevalence as high as approximately 1 in 2,000 in population.27

In numerous studies the peroxidase activity of PMNs from family members of MPO-deficient individuals has been assessed, yet the pattern of inheritance is not completely clear.28 Some studies suggest that MPO deficiency is an autosomal recessive disorder,4,24-32 although in many pedigrees there is variable expression23 and often a suggestion of polygenic control of MPO expression with defects in structural as well as regulatory genes.27 Studies that use peroxidase activity as the sole criterion for MPO deficiency may be flawed by contamination of the preparations with low numbers of peroxidase-containing eosinophils, a caveat recently emphasized by Dri et al.31

On the basis of our immunocytochemical studies of MPO-related peptides in MPO-deficient subjects,8 we proposed that MPO deficiency results from a defect in posttranslational processing of an aberrant pro-MPO rather than a major deletion of the MPO gene. The studies presented here demonstrate that genetically unrelated individuals with PMNs that lack peroxidase activity, contain no spectroscopic evidence of the MPO chloride group, and lack immunocytochemical evidence of mature MPO subunits contained DNA that hybridized with a cDNA for MPO under very stringent conditions. We found no evidence for a major deletion of the gene for MPO since Southern blots of restriction endonuclease digests with a variety of enzymes were identical. The pattern seen with BglII digestion of genomic DNA from the completely MPO-deficient subjects demonstrated the existence of an extra BglII recognition site that resulted in the generation of a 2.15-kb fragment. This 2.15-kb fragment was absent from digests of DNA from 14 controls. Furthermore, the digest of DNA from one of the completely MPO-deficient subjects (L.H.) differed from that seen in DNA from P.M. or J.Fu., two other completely MPO-deficient individuals who phenotypically and biochemically were indistinguishable from L.H. The restriction pattern of DNA from one of the partially deficient individuals resembled the BglII digest of completely deficient individuals, whereas the others contained normal-sized fragments after BglII digestion. The significance of these findings in the partially deficient subjects and their relationship to the basis for complete deficiency await further study. In addition, the apparent cosegregation of the extra BglII fragment and the phenotype of MPO deficiency will require studies of larger pedigrees of affected families to be more convincingly demonstrated.

Myeloid precursors from an MPO-deficient individual contained an RNA species that hybridized with the MPO probe, as demonstrated by the single band seen on Northern blots (Fig 2). The RNA for MPO was present in normal amounts and was of normal size in the MPO-deficient precursors studied. Thus, the MPO-deficient subjects studied contain a gene that encodes for MPO since one subject had a normal-sized mRNA and all subjects had pro-MPO of a normal size.

MPO-deficient neutrophils contain normal amounts of other lysosomal enzymes that share the azurophilic granule,19,28,34,36 so it is very unlikely that a general defect in the processing of azurophilic granule lysosomal enzymes underlies MPO deficiency. Rather, these findings are consistent with the hypothesis that the genetic lesions in MPO deficiency result in the synthesis of a pro-MPO that is not processed normally. The defective posttranslational processing could reflect a loss or inaccessibility of the proteolytic cleavage site in the aberrant pro-MPO, proteolytic catabolism of pro-MPO before maturation into MPO subunits, or modification of glycosylation sites that results in defective intracellular trafficking to the intracellular site of processing. Similar defects have been reported as the underlying molecular lesions in other diseases. Martiniuk et al37 have characterized the defect in α-glucosidase deficiency in three affected patients. Of the two patients with an onset during infancy, one had no mRNA, and one had normal-sized mRNA for α-glucosidase. In neither case were Southern blots of endonuclease digestions of control DNA with a variety of enzymes different from those of glucosidase-deficient DNA. It is well recognized that conformation profoundly affects the processing and transport of nascent proteins during their synthesis. Recent studies of processing and transport of α-mannosidase38 and of β-glucosidase39 in Dictyostelium mutants illustrate this point. Single mutations result in changes in conformation of the precursor protein and accumulation within the lumen of the endoplasmic reticulum. Consequently, there is defective transport of the precursor to the lysosome, and as a direct result, there is less proteolytic maturation to enzymatically active native protein. Analogous examples of defective processing of proenzymes in human systems include primary sucrase-isomaltase deficiency40 and the Z variant of α1-antitrypsin deficiency.41

One can anticipate that MPO deficiency will resemble thalassemia in that other genotypes will be described that also produce the phenotype of MPO deficiency. For example, it is likely that individuals exist whose defect is the failure to process the RNA for MPO into messenger RNA, thereby resulting in the presence of an aberrant RNA species and an inability to synthesize any pro-MPO. Only sequencing of genomic DNA from individuals with a complete deficiency of MPO will identify the mutations responsible for this common genetic disorder of myeloid cells.

Although the data presented here do not distinguish among these possible mechanisms, it is possible to draw the following conclusions based on these studies. It is unlikely that the hereditary absence of functional MPO is the result of a major deletion of the MPO gene since most endonucleases used to digest genomic DNA resulted in patterns on Southern blots that were the same in MPO-deficient subjects as in controls. The RNA for MPO is of normal size in myeloid precursors from the individual with MPO deficiency, and there is evidence of pro-MPO in all peroxidase-deficient PMNs studied to date. In addition, if further studies confirm the apparent link between the extra BglII fragment and MPO deficiency, one must conclude that the functional deficiency of MPO is a biochemical phenotype with at least two different underlying genetic defects since distinctly different patterns in BglII digests were obtained in MPO-deficient subjects who phenotypically were indistin-
guishable from each other. Further characterization of the defect(s) in MPO deficiency should provide additional insight into events important for sorting and intracellular transport of myeloid proteins destined for the azurophilic granule.

ACKNOWLEDGMENT

The author acknowledges the helpful suggestions of Drs Jack T. Stapleton, Richard A. Anderson, Gordon D. Ginder, and Keith R. Johnson; the technical assistance of Nancy Filkins and Amy A. Wall; the secretarial assistance of Sherry Flanagan; and the constant support and encouragement of Dr Robert A. Clark.

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Aberrant restriction endonuclease digests of DNA from subjects with hereditary myeloperoxidase deficiency

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