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Severe Impairment in Early Host Defense against *Candida albicans* in Mice Deficient in Myeloperoxidase

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Myeloperoxidase (MPO) catalyzes the reaction of hydrogen peroxide with chloride ion to produce hypochlorous acid (HOCl), which is used for microbial killing by phagocytic cells. Despite the important role of MPO in host defense, however, MPO deficiency is relatively common in humans, and most of these individuals are in good health. To define the in vivo role of MPO, we have generated by gene targeting mice having no MPO activity in their neutrophils and monocytes. The mice without MPO developed normally, were fertile, and showed normal clearance of intraperitoneal *Staphylococcus aureus*. However, they showed increased susceptibility to pneumonia and death following intratracheal infection with *Candida albicans*. Furthermore, the lack of MPO significantly enhanced the dissemination of intraperitoneally injected *C. albicans* into various organs during the first 7 days. Thus, MPO is important for early host defense against fungal infection, and the inability to generate HOCl cannot be compensated for by other oxygen-dependent systems in vivo in mice. The mutant mice serve as a model for studying pulmonary and systemic candidiasis.

Neutrophil granulocytes are the first line of defense against invading microorganisms such as bacteria, viruses, and fungi. Apart from other defense mechanisms (4), the generation of reactive oxygen compounds plays an important role in defense. Myeloperoxidase (MPO; EC 1.11.1.7) is a cationic heme-containing enzyme found in primary azurophilic granules of neutrophils and primary lysosomes of monocytes (3, 23). In chemoattractant-activated neutrophils, MPO transforms hydrogen peroxide (H_2O_2) generated during the oxidative burst to highly cytotoxic hypochlorous acid (HOCl) in the presence of chloride ion (Cl^-) (2). This MPO-H_2O_2-Cl^- system appears to be important in microbial killing by neutrophils (6, 22, 24, 35, 37, 52, 53). It may also be involved in their cytotoxicity against tumor cells (7, 31) and in tissue damage at sites of inflammation, where neutrophils can release both MPO and H_2O_2 (5, 10–12, 18).

Both human MPO and murine MPO are encoded by a single gene (*Mpo* in mice) (39, 54), and the respective genes have been cloned and sequenced (19, 46). MPO is synthesized in bone marrow during the late myeloblastic and promyelocytic stages of myeloid maturation (55). MPO isolated from mature human neutrophils has a molecular mass of 150 kDa and is composed of two heavy chains (59 kDa) and two light chains (14 kDa) (1, 36, 51).

Hereditary MPO deficiency appears to be the most common biochemical defect of neutrophils and is not geographically restricted (6, 22, 31, 34, 37, 40, 41, 43). An estimated prevalence of 1 of 2,000 to 4,000 individuals has been reported in the United States (37) and Italy (8). Considering the important role of HOCl for effective killing of microorganisms, it is surprising that most individuals with MPO deficiency are healthy. This fact stands in marked contrast to the situation for patients with chronic granulomatous disease (CGD), in whom granulocytes are deficient in NADPH oxidase and consequently do not produce any reactive oxygen compound. Patients with CGD typically have clinical symptoms early in life and recurrent infections that can lead to death during childhood. However, an increased susceptibility to infections, particularly those caused by *Candida albicans*, has been reported for some MPO-deficient patients (6, 29, 35, 37). In these patients, who were also affected with diabetes, it was not determined whether the infections were due to MPO deficiency or whether other disorders were also relevant. An interplay of other mechanisms seems to compensate for the inability of MPO-deficient cells to generate HOCl from H_2O_2.

Here, we report the generation of mice with a nonfunctional allele for MPO by targeted homologous recombination with mouse ES cells. The enzyme activity of MPO is absent in neutrophils and monocytes from homozygous mutant mice. These mice also exhibit increased susceptibility to infection with *C. albicans*.

MATERIALS AND METHODS

Cloning of the mouse *Mpo* gene and construction of a targeting vector. A 396-bp DNA fragment containing exons 5 and 6 of the mouse *Mpo* gene was amplified from ES cell genomic DNA with primers designed from the published sequence (46). This fragment was used as a probe to screen a λ phage library made from strain 129 mouse genomic DNA. A clone containing a part of the *Mpo* gene was isolated, and its restriction map and a partial nucleotide sequence were determined. For constructing the targeting vector (Fig. 1A), a 2.2-kb fragment containing exons 1 to 5 and a 5.9-kb fragment containing exons 8 to 11 were used as the two homologous arms flanking the *neo* gene. The herpes simplex virus (HSV) thymidine kinase (TK) gene was positioned downstream of the longer arm (32).

Gene targeting and production of mice by use of modified ES cells. BK4 cells, a subclone of E14TG2a derived from strain 129/Ola mice, were cultured on feeder cells as described previously (44). The targeting vector mentioned above was linearized with *Not*I and introduced into ES cells by electroporation. Colonies doubly resistant to G418 (200 μg/ml) and ganciclovir (2 μM) were screened for homologous recombination by Southern blot analysis following digestion of genomic DNA with *Bgl*II by use of the 722-bp PCR-amplified fragment containing exon 5 of the *Mpo* gene as a probe. The resulting cells with a disrupted *Mpo* gene were injected into blastocysts to obtain chimeras as described previously

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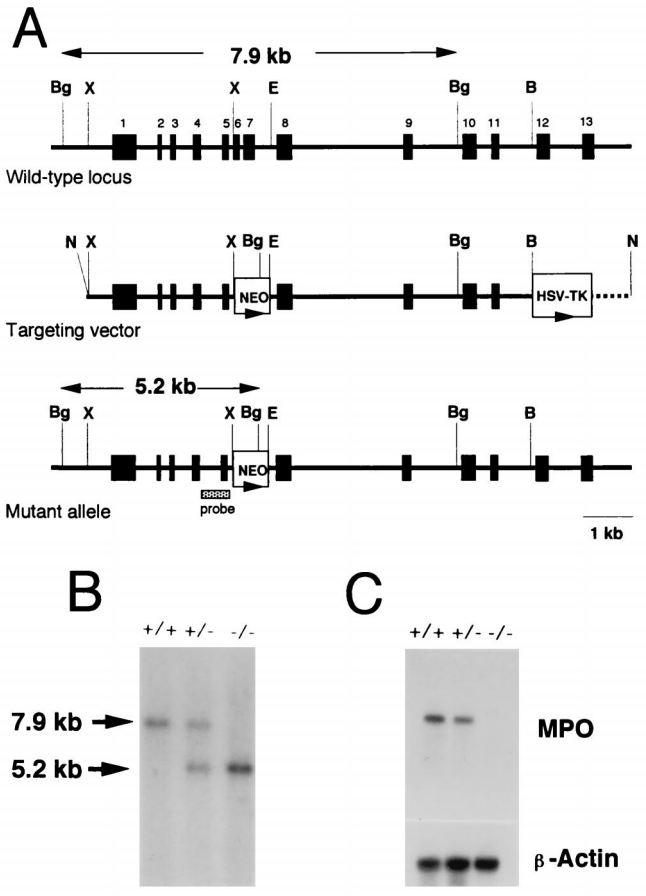

FIG. 1. Targeted disruption of the mouse *Mpo* gene and germ line transmission of the disrupted allele. (A) Structures of the wild-type *Mpo* locus, targeting vector, and mutant allele generated by homologous recombination. Exons are shown as black boxes and numbered. The targeting vector contains the *neo* gene (NEO) in place of the *Xba*I-*Eco*RI region containing exons 6 and 7. The HSV TK gene is attached to the end of the region of homology. The broken line indicates the vector sequence. The lengths of diagnostic *Bgl*II restriction fragments and the location of a probe used for Southern blot analysis are shown. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; N, *Not*I; X, *Xba*I. (B) Southern blot analysis. Genomic DNA was isolated from tail snips of the offspring of a heterozygous cross, digested with *Bgl*II, and analyzed by Southern hybridization with the DNA probe indicated in panel A. Genotypes are indicated as wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mice. (C) Northern blot analysis of bone marrow mRNA. Total RNA (10 μg) isolated from bone marrow of wild-type (+/+), heterozygous mutant (+/−), and homozygous mutant (−/−) mice was electrophoresed, blotted, and hybridized to a human *MPO* cDNA probe. The amounts of RNA loaded are indicated by hybridization to a cDNA probe for chicken β-actin.

(25, 47). Animals classified as chimeric by coat color were mated with strain C57BL/6 mice, and F_1 animals heterozygous for the disrupted *Mpo* gene were obtained. Interbreeding of heterozygous offspring was used to produce mice homozygous for the modified MPO allele.

Northern blotting of bone marrow cells. Total RNA was isolated from bone marrow cells with TRIzol (Gibco BRL) as recommended by the manufacturer. Each sample (10 μg) was electrophoresed in a 2.2 M formaldehyde–1% agarose gel, transferred to a Hybond nylon membrane (Amersham Corp.), and probed with a 2.2-kb *Pst*I fragment of a human *MPO* cDNA vector (pH17) (17) containing exons 2 through 12 of the *MPO* gene. This fragment is 85% identical to that in mouse cDNA. A cDNA clone for chicken β-actin was used as a probe to evaluate the amount of mRNA loaded. mRNA levels were estimated by densitometric analysis of autoradiograms after serial exposures.

Cell counting and measurement of MPO activity. Blood was drawn from the retro-orbital plexus of mice into EDTA-containing tubes. Blood cell analysis was carried out by flow cytometry with Technicon H-1 by the method recommended by the manufacturer. Briefly, separation of leukocytes was performed by peroxidase staining and simultaneous measurement of light scattering (Perox-Channel). For peroxidase staining, erythrocytes were lsyed in the Technicon H-1-integrated Perox-Chamber, and leukocytes were fixed with formalin and stained for peroxidase with H_2O_2 and 4-chloro-1-naphthol as a chromogen. The cell distribution pattern was plotted by a so-called leukogram with peroxidase activity on the *x* axis and light scattering on the *y* axis and was analyzed by a multispecies software program developed for animal blood samples. If intracellular MPO activity decreases, neutrophils and monocytes shift to the left.

Thioglycolate-induced peritonitis. Mice were injected intraperitoneally with 1 ml of 3% fluid thioglycolate medium (Difco). After 4 h, peritoneal exudate cells were harvested by peritoneal lavage with 20 ml of phosphate-buffered saline (PBS). Total cell numbers were determined with a hemocytometer. The percentage of neutrophils was determined by microscopic examination of Wright-Giemsa-stained samples.

Cytochemical and biochemical determination of MPO activity. Isolated neutrophil-rich peritoneal exudate cells were stained for MPO activity with the 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate system from Sigma (St. Louis, Mo.) following formalin-acetone fixation of the cells. Briefly, oxidation of the substrate TMB in MPO-positive cells yields a blue insoluble reaction product which is visualized by light microscopy. MPO activity was quantitatively measured by the method of Suzuki et al. (45) with some modifications. Peritoneal exudate neutrophils were adjusted to 5×10^6 cells/ml and incubated with *N*-formyl-Met-Leu-Phe (1 μM) and cytochalasin B (5 μg/ml) for 10 min at 37°C. Triton X-100 (final concentration, 0.1%) was added to the cell suspensions for total MPO release. Aliquots of the cell extracts were incubated with the TMB liquid substrate system, and the oxidized product was detected spectrophotometrically. The activity was expressed as the initial rate of increase in the absorbance at 655 nm.

Generation of HOCl and O_2^- from neutrophils. HOCl generation by peritoneal exudate neutrophils was measured by the chlorination of monochlorodimedon (MCD) (21). Peritoneal exudate cells (2×10^6/ml) were incubated in PBS containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose, 100 ng of phorbol myrisate acetate (PMA) per ml, and 20 μM MCD at 37°C for 20 min. At the end of this period, samples were ice chilled and centrifuged at 12,000 × *g* for 5 min. The activity in supernatants of reaction mixtures with or without cells was measured at 290 nm. The amount of HOCl generated was calculated by use of a molar linear absorption coefficient of 19,000 M^{-1}/cm (16).

O_2^- generation was determined as the superoxide dismutase (SOD)-inhibitable reduction of cytochrome *c* (14). Cytochrome *c* (40 μM) with or without 20 μg of SOD per ml was added to peritoneal exudate cells (2×10^6/ml) that had been stimulated with 100 ng of PMA per ml at 37°C for 5 min, and O_2^- generation in the samples was continuously measured for another 5 min at 550 nm with a spectrophotometer. O_2^- generated from the cells was calculated as the difference between levels in SOD-containing samples and those in samples not containing SOD by use of an absorption coefficient of 21,000 M^{-1}/cm (14).

Experimental infections with *C. albicans* and *Staphylococcus aureus*. Stock cultures of *C. albicans* (ATCC 18804) were cultured on 2% agar slant medium (pH 6.4) containing 83 mM glucose, 2 mM $MgSO_4 \cdot 7H_2O$, 7.4 mM KH_2PO_4, 0.5% Polypeptone, and 0.2% yeast extract for 10 days at 37°C. Blastoconidia of *C. albicans* grown on the slant were transferred to 1.2% agar plates containing 28 mM glucose and 0.2% Polypeptone. After cultivation for 2 days at 37°C, the blastoconidia were harvested in sterile saline. The number of fungi was counted with a hemocytometer and adjusted to 2×10^8 cells/ml; the viable number was also determined by plating the diluted samples on agar plates. Wild-type, heterozygous, and homozygous mutant mice were injected by the intratracheal route with 0.02 ml of the fungal suspension. At 0.5, 20, 80, and 120 h after the challenge, the organs were removed aseptically and homogenized in sterile saline. At least five mice were used per group. Aliquots of the homogenates were plated in duplicate on agar plates with Guanofuracin-Sabouraud medium (Eiken Chemical Co., Tokyo, Japan) and incubated for 48 h at 37°C. The number of viable *C. albicans* was calculated from the number of colonies grown on the plates and was expressed in CFU. Data were recorded as the mean $\log_{10}$ CFU per organ.

In studies of systemic infections, wild-type and homozygous mutant mice were injected intraperitoneally with 4×10^6 cells of *C. albicans*. Seven days later, the organs were removed, homogenized, and plated on agar plates as described above. Five mice were used per group.

For intraperitoneal infection with *S. aureus*, mice were injected intraperitoneally with 0.1 ml of a suspension (7×10^8/ml) of *S. aureus* (FDA209P) that had been grown for 24 h on a plate with Trypticase soy agar at 37°C. Twenty-four and 48 h later, peritoneal exudate fluid was harvested with PBS, diluted, and cultured for viable *S. aureus* on Trypticase soy agar at 37°C. Three to five mice of different genotypes were studied at each time point.

Preparation of sections and slides. Lungs were fixed in a buffered 4% paraformaldehyde solution, dehydrated in ethanol, and embedded in paraffin for sectioning. Sections were prepared, and hematoxylin and eosin (H&E) staining and Grocott staining were carried out by standard protocols.

RESULTS

Targeted disruption of the mouse *Mpo* gene. The targeting strategy used to disrupt the *Mpo* coding sequence is illustrated

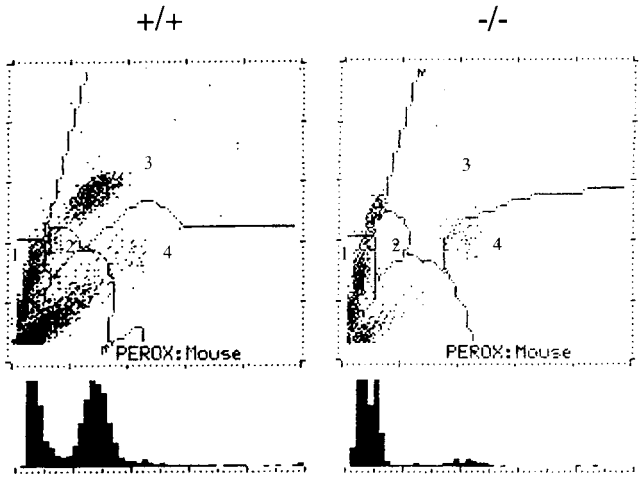

FIG. 2. Leukograms of Technicon H-1 analysis of wild-type (+/+) and homozygous mutant (−/−) mice. Light scattering is plotted on the *y* axis, and peroxidase (PEROX) activity is plotted on the *x* axis. The numbers indicate the following cells: 1, lymphocytes; 2, monocytes; 3, neutrophils; 4, eosinophils. The lower the peroxidase activity of a single cell, the more the cell is located toward the left on the *x* axis. Histograms of peroxidase activity are shown below the graphs.

in Fig. 1A. The targeting vector was constructed by removing a 0.7-kb *Xba*I-*Eco*RI fragment containing exon 6 through a part of intron 7 and replacing it with the *neo* gene. In addition, a copy of the HSV TK gene was placed at the 3′ end of the construct. Both of the selectable marker genes were inserted in the same transcriptional orientation as the *Mpo* gene. Male chimeric mice generated by use of correctly targeted ES cells transmitted the mutation of the *Mpo* gene through the germ line when mated with C57BL/6 female mice (Fig. 1B). Heterozygote ($Mpo^{+/-}$) mating then produced homozygous mutant mice ($Mpo^{-/-}$) at the expected Mendelian frequency. The mutant mice were viable, exhibited normal growth and development, and were fertile. As shown in Fig. 1C, Northern blot analysis of mRNA isolated from the bone marrow of homozygous mutant mice showed no significant hybridization to the human *MPO* cDNA probe, whereas the bone marrow of wild-type mice ($Mpo^{+/+}$) contained mRNA for *Mpo*, indicating the absence of any detectable transcript for the *Mpo* gene in the mutants. The heterozygous mice showed the expected 50% reduction in mRNA levels.

Identification of MPO-deficient neutrophils and monocytes. Figure 2 shows the leukograms for the peroxidase activity of peripheral blood cells from wild-type and homozygous mutant mice determined with Technicon H-1. When stained vesicles are lacking, the light scattering changes characteristically, leading to a cluster location upward on the *y* axis and a shift to the left on the *x* axis. The neutrophil clusters from homozygous mutant mice clearly shifted to the left, unstained cell area, and no stained cells were observed in the normal neutrophil area. The mutant mice also showed no clusters in the monocyte area. These results strongly suggested that the neutrophils and the monocytes of the homozygous mice that we generated are completely deficient in MPO. However, the clusters of their eosinophils did not shift to the left, demonstrating that eosinophil peroxidase is genetically distinct from MPO.

MPO activity in peritoneal exudate neutrophils. In order to explore MPO deficiency in further detail, neutrophils were isolated from the thioglycolate-injected peritoneal cavity of wild-type, heterozygous, and homozygous mice. Judging from the microscopic examination of Wright-Giemsa-stained samples, more than 95% of the exudate cells recovered 4 h after injection were neutrophils, regardless of their genotypes. Cytochemical staining with TMB revealed that more than 95% of the exudate cells in wild-type and heterozygous mice were peroxidase positive (Fig. 3A). In contrast, the majority of homozygous mutant mice were peroxidase negative. Quantitative analysis by spectrophotometry demonstrated that the average MPO activity in wild-type mice was 1.7 nmol/min/10^6 cells (Fig. 3B). Heterozygous animals showed the expected 50% reduction in MPO activity. Homozygous mutant mice showed only a low level of MPO activity. This low level of activity likely represents peroxidase activity supplied by eosinophils, because eosinophil peroxidase is normally active in homozygous mutant mice (Fig. 2) and is able to oxidize TMB. From these results, we conclude that homozygous mutant mice have no detectable MPO activity, while heterozygous mice have half the level of MPO activity present in wild-type littermates.

HOCl and O_2^- generation from normal and MPO-deficient neutrophils. During the respiratory burst, neutrophils generate

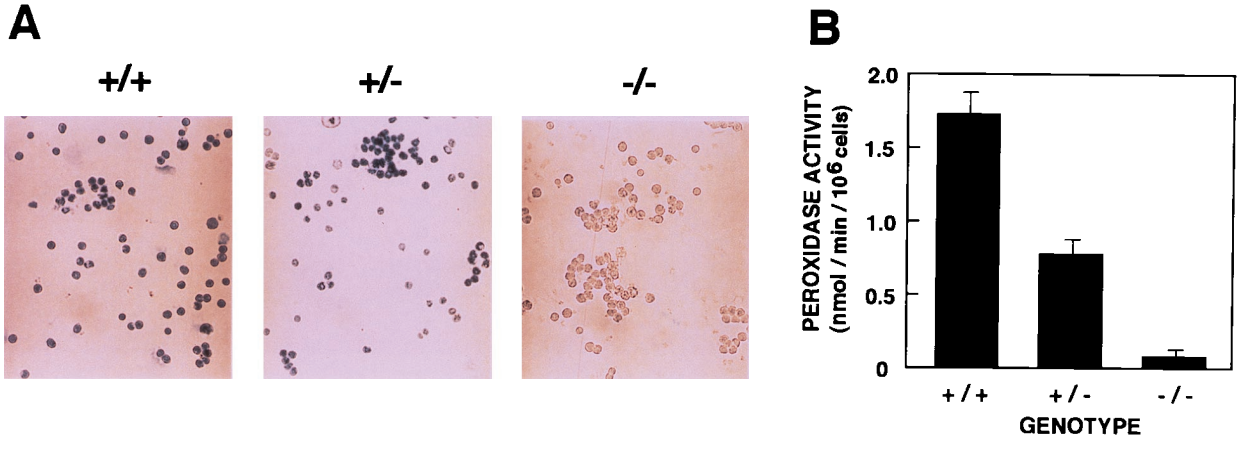

FIG. 3. MPO activity of neutrophils. Wild-type (+/+), heterozygous mutant (+/−), and homozygous mutant (−/−) mice were injected with thioglycolate in the peritoneal cavity, and peritoneal exudate cells were collected 4 h later. (A) The cells were fixed and stained with TMB for peroxidase activity. (B) Peroxidase activities in total peritoneal exudate cells were measured with TMB as a substrate. Three animals of different genotypes, each tested in duplicate, were used. Results represent means ± standard deviations.

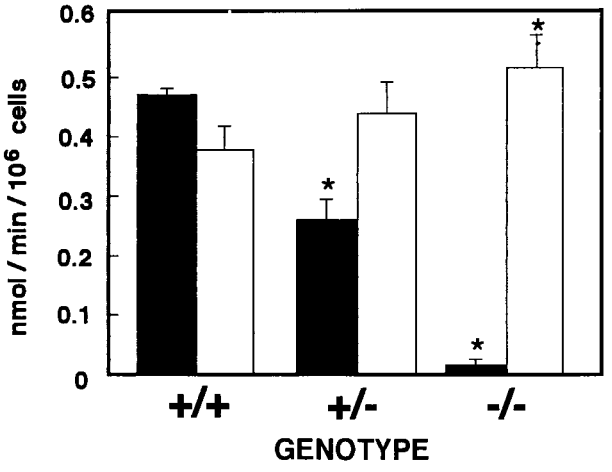

FIG. 4. HOCl and O_2^- generation from neutrophils of wild-type (+/+), heterozygous mutant (+/−), and homozygous mutant (−/−) mice. The levels of HOCl (closed bars) and O_2^- (open bars) generated from PMA-stimulated peritoneal exudate neutrophils (2×10^5 cells) were determined by the chlorination of MCD and the SOD-inhibitable reduction of cytochrome *c*, respectively. Three animals of each genotype were used in each experiment, and assays were performed in triplicate. Results represent means ± standard deviations. The asterisk indicates a *P* value of <0.05 for mutant versus wild-type mice, as determined by Student's *t* test.

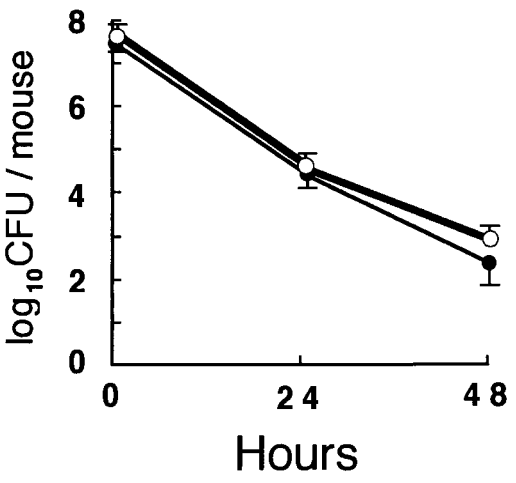

FIG. 5. Clearance of viable *S. aureus* from the peritoneal cavity. Peritoneal exudate fluid was cultured 24 and 48 h after intraperitoneal injection of 7×10^7 CFU of *S. aureus*. Results represent mean $\log_{10}$ CFU ± standard deviations obtained from three to five wild-type mice (closed circles) and homozygous mutant mice (open circles).

O_2^- via the NADPH oxidase system. O_2^- is then reduced to H_2O_2, from which HOCl is generated by the MPO reaction.

The rapid reaction of MCD with HOCl to form dichlorodimedon (21) was used to determine the level of HOCl generation from the peritoneal exudate neutrophils (Fig. 4). The average level of HOCl generation from wild-type neutrophils was 0.47 nmol/min/10^6 cells. On the other hand, as anticipated from the absence of peroxidase activity (Fig. 2 and 3), the neutrophils of homozygous mutant mice produced HOCl at an almost undetectable level. Because EPO also produces HOCl from H_2O_2, a low level of HOCl detected in homozygous mutant mice could be supplied from eosinophils. The neutrophils of heterozygous mutant mice generated from half the normal level of HOCl. These results demonstrate that the levels of HOCl generated from the neutrophils in each genotype correlate well with the MPO activities measured with TMB as a substrate (Fig. 3).

O_2^- generation by peritoneal exudate neutrophils was measured as SOD-inhibitable cytochrome *c* reduction. The average level of O_2^- generation from neutrophils of wild-type mice was 0.39 nmol/min/10^6 cells (Fig. 4) and was completely inhibited in the presence of SOD (data not shown). The neutrophils of homozygous mutant mice generated a slightly higher level of O_2^- (0.50 nmol/min/10^6 cells) than did those of wild-type mice (*P*, <0.05) (Fig. 4). A slight enhancement of O_2^- generation was also seen in heterozygous mutant mice, although it did not reach statistical significance (*P*, 0.075).

Clearance of *S. aureus* in vivo. To assess whether MPO-deficient mice have enhanced susceptibility to *S. aureus* infection in vivo, mice were intraperitoneally challenged with 7×10^7 cells of *S. aureus*. Most of the cells injected disappeared by 48 h both in wild-type and in homozygous mutant mice, and there was no difference in the rates of clearance between those mice (Fig. 5). Gross anatomical experiments revealed no obvious inflammation of the lungs, liver, heart, kidneys, and intestines.

Pulmonary infection with *C. albicans*. To investigate the susceptibility of MPO-deficient mice to *C. albicans* compared to that of wild-type littermates, 26 wild-type, 18 heterozygous mutant, and 27 homozygous mutant mice were challenged with 4×10^6 fungi intratracheally. This is a relatively low dose, since the 50% lethal dose for mice challenged by the intratracheal route was estimated to be in excess of 10^8 yeast cells by Sawyer (42). On the following day, signs of severe infection, including ruffled fur and hunched posture, were observed only in the homozygous mutant mice. The wild-type mice were able to eliminate the fungi effectively, and approximately 2,000 fungi were recovered from their lungs 120 h after infection (Fig. 6A). In contrast, in the mutant mice, the clearance of viable *C. albicans* was significantly delayed, and nearly 500 times as many viable fungi were cultured from their lungs (Fig. 6A). In addition, the survival of the mutant mice was dramatically decreased, and 18 of the 27 mice died by 5 days after the challenge. We also determined the numbers of viable *C. albicans* cultured from other tissues. In the kidneys (Fig. 6B), the number of *C. albicans* in the wild-type mice was almost below the detectable level, whereas that in the homozygous mutant mice was time dependently increased, and about 6,000 fungi were detected at 120 h. In the brain, heart, and liver of both wild-type and homozygous mutant mice, the numbers of *C. albicans* were all below 100 fungi per each organ from 20 to 120 h. *C. albicans* was not detectable in the spleen. There was no difference in the numbers of viable fungi obtained from the mutant mice that had died or that had been weakened. Although the clearance of fungi from the lungs in the heterozygous mutant mice was slightly delayed at 80 h compared to that in the wild-type mice, almost the same number of fungi disappeared by 120 h (Fig. 6A).

Only one wild-type mouse died 3 days after the challenge as a consequence of lung bleeding, but no gross or histological abnormalities were observed in the lungs of this mouse or those of the wild-type mice still alive by 120 h (Fig. 7A and B). In contrast, the lungs of the mutant mice 120 h after the challenge showed diffuse enlargement and brownish discoloration (Fig. 7A). Additionally, patchy white, soft, and spherical masses 1 to 5 mm in diameter were observed on the surfaces of every lobe (Fig. 7A). H&E-stained sections of the lungs revealed massive infiltration of alveolar and peribronchiolar spaces with neutrophil polymorphonuclear leukocytes, and

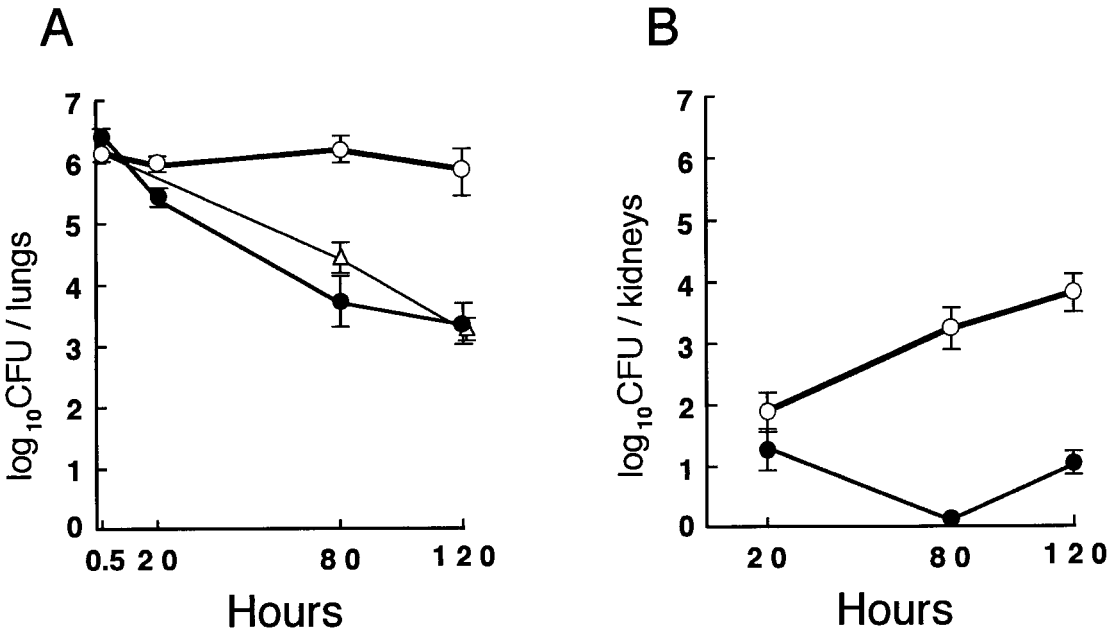

FIG. 6. Pulmonary infection with *C. albicans* in wild-type, heterozygous mutant, and homozygous mutant mice. Wild-type (closed circles), heterozygous mutant (open triangles), and homozygous mutant (open circles) mice were injected intratracheally with 4×10^6 CFU of *C. albicans*. At the indicated times after the challenge, whole lungs (A) and kidneys (B) were homogenized, and aliquots of the homogenates were plated on Guanofuracin-Sabouraud agar plates. Five mice or more were used in each group. Results represent mean $\log_{10}$ CFU per organ ± standard deviations.

fairly extensive edema filled many other alveolar spaces (Fig. 7C). In peribronchiolar spaces around neutrophil infiltrates, mucus infiltration was also observed. Furthermore, some of the peribronchial epithelial cells were observed to be peeling off (Fig. 7D). By Grocott staining, hyphae of *C. albicans* were detected in several alveolar spaces of the mutant mice (Fig. 7E) but not the wild-type mice (data not shown). These clinical features are characteristic of alveolar and peribronchiolar pneumonia, and the most likely explanation for the death of the mutant mice infected with *C. albicans* is severe impairment of lung functions caused by myeloid cell infiltration and edema.

Systemic infection with *C. albicans* via the intraperitoneal route. To evaluate the susceptibility of wild-type and MPO-deficient mice, *C. albicans* was administered intraperitoneally at a dose of 4×10^6 fungi/mouse. Seven days later, the lungs, brain, kidneys, spleen, and liver were removed from five animals in each group and pooled to determine the dissemination of the fungi into these organs. Gross anatomical evaluation revealed no obvious inflammation in any of those organs. In the wild-type mice, the highest and lowest numbers of fungi were found in the liver and the brain, respectively (Fig. 8), consistent with a previous report (20). In contrast, significantly higher numbers of fungi were disseminated into every organ in the homozygous mutant mice, and approximately 290, 11, 110, 17, and 10 times as many viable fungi were cultured from the lungs, brain, kidneys, spleen, and liver, respectively (Fig. 8). Unlike the results of the intratracheal infection, no obvious signs of severe infection were observed in either wild-type or homozygous mutant mice during a period of 7 days after the challenge. When mice were challenged intraperitoneally at a high dose (10^8 fungi/mouse), all homozygous mutant mice died by 2 days, while all wild-type mice survived for 7 days without many signs of distress. These results demonstrate that MPO is very important for the murine host defense against *C. albicans* and that the lack of MPO enhances *C. albicans* dissemination, especially into the lungs and kidneys.

DISCUSSION

MPO-deficient mice generated by disruption of the *Mpo* locus by homologous recombination represent the first genetically defined model for the inherited human MPO deficiency. The mutation disrupts the *Mpo* gene and completely eliminates expression of the gene, as demonstrated by the absence of the mRNA in the bone marrow and of enzyme activity in the neutrophils and monocytes of homozygous mutant mice. No HOCl generation by neutrophils in homozygous mutant mice was observed. O_2^- generation was slightly increased in our MPO-deficient mice, consistent with the deficiency in humans (22, 41). Heterozygous mutant mice had half the normal levels of mRNA, enzyme activity, and HOCl generation.

Homozygous mutant mice completely lacking MPO activity are born and develop normally but exhibited markedly enhanced susceptibility to infection with *C. albicans*. Mutant mice intratracheally exposed to even a low dose of *C. albicans* developed severe alveolar and peribronchiolar pneumonia (Fig. 7) resulting in the death of two-thirds of the mice. Although this challenge was time dependently disseminated into the kidneys (Fig. 6B), this process was unlikely to be the main cause of death, because mice intraperitoneally infected with *C. albicans* were in good health for at least 7 days even when a slightly higher number of fungi was present in the kidneys (Fig. 8). Rather, the severe pneumonia was more likely to be the main cause of death of the mutant mice intratracheally challenged with *C. albicans*. It is well known that MPO plus H_2O_2 plus Cl^- forms a cytotoxic triad in vitro which is toxic for fungi (9, 49) and that neutrophils genetically deficient in MPO fail to kill them (37). In addition, neutrophils are often the first cells to be recruited to a site of infection, where they show a higher level of phagocytic activity against *C. albicans* than do alveolar macrophages (26). Taken together, the results indicate that it is most plausible that neutrophils with MPO represent the early stage of defense in the lungs against *C. albicans* and that

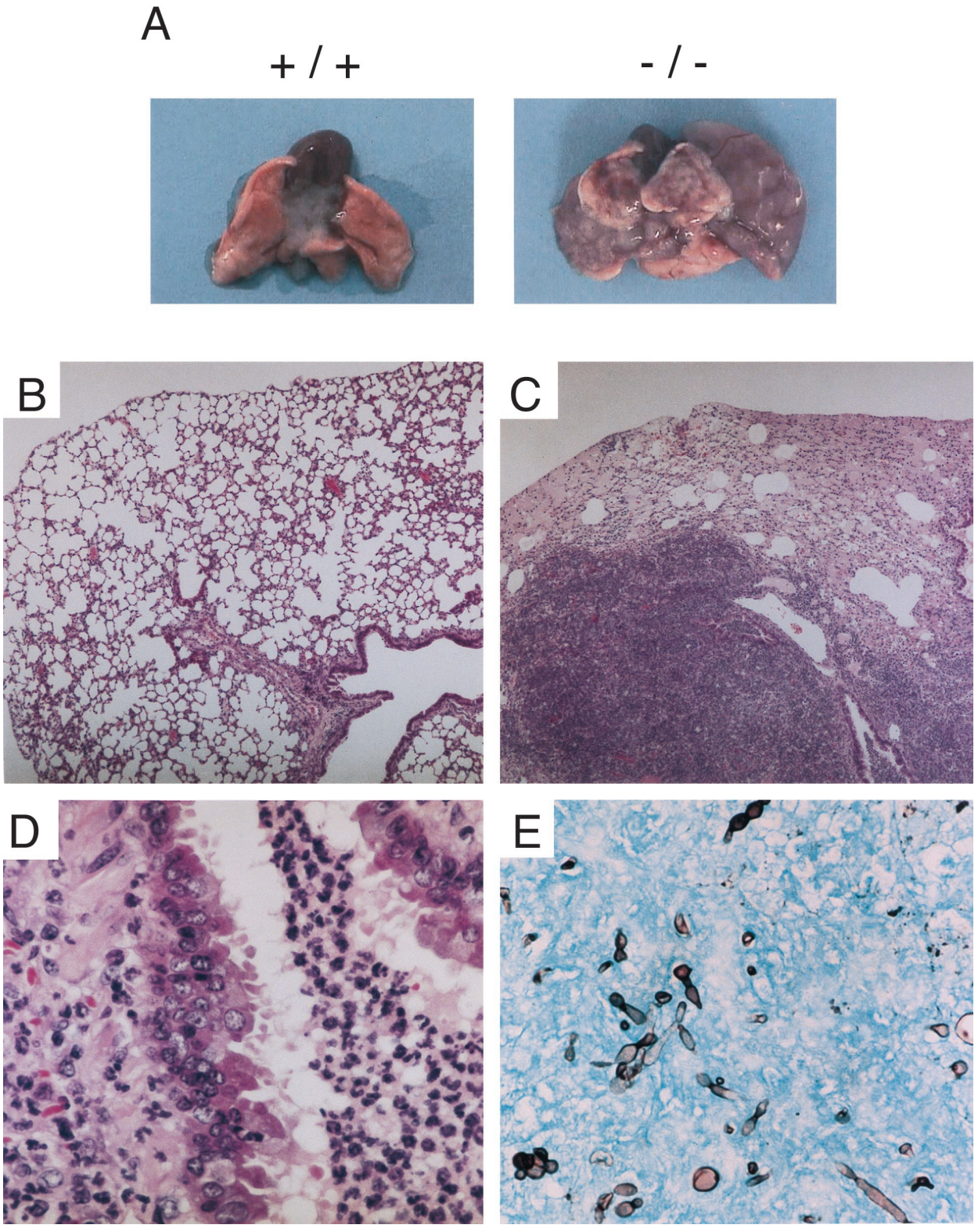

FIG. 7. Lung pathology observed in wild-type and homozygous mutant mice 120 h after intratracheal challenge with *C. albicans*. (A) Representative gross appearance of lungs from wild-type (+/+) and homozygous mutant (−/−) mice. (B) H&E-stained section from a wild-type mouse. Magnification, ×40. (C) H&E-stained section from a representative homozygous mutant mouse. Magnification, ×40. (D) Same sample as C but at a magnification of ×400. (E) Grocott-stained section from a homozygous mutant mouse. Magnification, ×400.

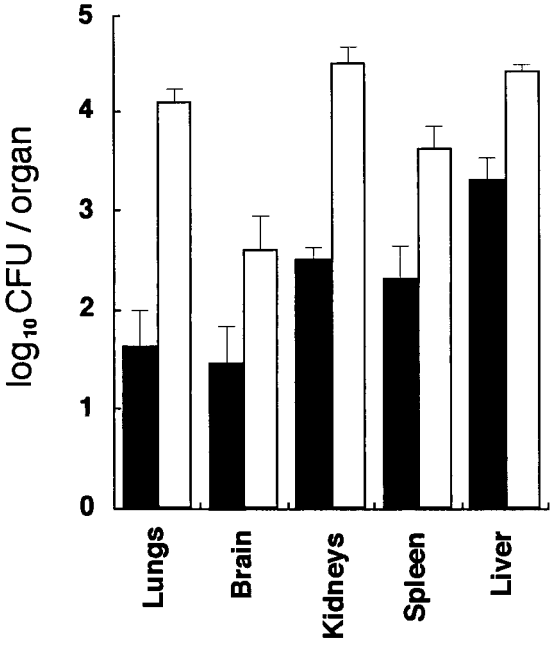

FIG. 8. Numbers of *C. albicans* cultured from various organs of mice after intraperitoneal challenge. Wild-type mice (closed bars) and homozygous mutant mice (open bars) were injected intraperitoneally with 4×10^6 cells of *C. albicans*. Seven days after the challenge, the organs were removed and homogenized, and aliquots of the homogenates were plated on Guanofuracin-Sabouraud agar plates. Five mice were used in each group. Results represent mean $\log_{10}$ CFU per organ ± standard deviations.

inefficient killing due to the lack of MPO causes a continuous recruitment of neutrophils into the lungs, leading to congestion of the lungs.

Heterozygous mice, having about half the normal level of MPO activity (Fig. 3 and 4), showed only a slight delay in the killing of *C. albicans* in the lungs compared to that in wild-type mice at the tested dose (Fig. 6). These results strongly suggest that HOCl produced from neutrophils at half the normal rate could be sufficient in vivo for exhibiting complete fungal killing. Alternatively, neutrophils with normal and suboptimal MPO activities may equally enhance secondary antifungal effector activities, such as that of macrophages. Although alveolar macrophages are less able to kill *C. albicans* than neutrophils (26), those exposed to MPO are known to exhibit an enhanced respiratory burst (30), resulting in augmentation of the macrophage-mediated cytotoxicity for *C. albicans* (28, 33). Furthermore, MPO stimulates macrophages to secrete cytokines, such as tumor necrosis factor alpha (27), which causes neutrophils to degranulate and to release more MPO into the microenvironment and which potentiates *Candida* killing by neutrophils in vitro (13). Hence, a feedback loop is established until the pathogen is removed. Further studies on the susceptibility of heterozygous mice with strain backgrounds different from 129/C57BL to various doses of fungi are important to clarify the risk of patients with partial MPO deficiency for fungal infection.

The increase in fungal load found in the lungs, brain, kidneys, spleen, and liver of systemically infected MPO-deficient mice may imply a possible unique role for MPO against *Candida* infection in each organ. Among the organs, the lungs and the kidneys were most affected by the lack of MPO, while the other organs were less affected. It has been reported that depletion of neutrophils in mice increases the susceptibility to systemic infection with *C. albicans* and that the fungal burden in the kidneys increases 100-fold on day 4, while the brain is little affected (15). Our results obtained with MPO-deficient mice are similar to those obtained with neutrophil-depleted mice (15). Furthermore, intraperitoneal administration of purified MPO has been reported to increase the resistance of mice to *Candida* infection (50), suggesting that peritoneal neutrophils without MPO are impaired in their ability to protect against invasion from the intraperitoneal route. Collectively, these results indicate that neutrophils with MPO play a critical role against systemic candidiasis, especially in the lungs and kidneys, while other defense systems, such as the phagocytic function of macrophages, appear to be more effective in the brain, spleen, and liver.

When homozygous mutant mice were challenged with the same dose of *C. albicans* (4×10^6 fungi/mouse) by different routes (intratracheal and intraperitoneal), the mice showed a higher level of resistance to challenge by the intraperitoneal route, and no death occurred within 7 days. As peritoneal macrophages have higher levels of phagocytic activity than do alveolar macrophages (48), the mechanism of resistance may be due to the presence of an environment rich in macrophage activity in the area where the systemic invasion is prevented or delayed by phagocytosis.

Homozygous mutant mice did not exhibit a remarkable difference from wild-type mice in the rate of clearance of *S. aureus* from the peritoneal cavity (Fig. 5). This finding is consistent with the fact that most humans deficient in MPO show no symptoms and with the observations that although the in vitro killing of *S. aureus* in MPO-deficient neutrophils is significantly less effective than normal at early time periods, it reaches the normal rate by approximately 1 h (37) or 2 h (22) after the organism is ingested. The lack of impairment in the clearance of *S. aureus* in our homozygous mutant mice in vivo is also in clear contrast to the results obtained with a mouse model of CGD; because of a mutation in the NADPH oxidase gene, these mice are unable to generate O_2^-, and the clearance of *S. aureus* from the peritoneal cavity is retarded (38). It is thus likely that reactive oxygen species other than HOCl, such as O_2^-, are sufficient for neutrophils to kill *S. aureus*. Further studies with MPO-deficient mice are warranted to define the role of MPO in *S. aureus* infection.

Oxidants generated by neutrophils and monocytes have been implicated in the tissue damage that occurs when these phagocytic cells penetrate tissue in various chronic inflammatory conditions (10–12). However, to date there is a lack of convincing evidence that the MPO-H_2O_2-Cl^- system causes tissue injury in chronic inflammation in vivo. MPO-deficient mice should therefore be invaluable for evaluating the role of phagocyte-derived oxidants not only in host defense against various pathogens but also in tissue injury and resolution of the inflammatory response and for refining treatment strategies.

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