Critical Role of Myeloperoxidase and Nicotinamide Adenine Dinucleotide Phosphate–Oxidase in High-Burden Systemic Infection of Mice with Candida albicans

Yasuaki Aratani,1 Fumiaki Kura,2 Haruo Watanabe,2 Hisayoshi Akagawa,3 Yukie Takano,3 Kazuo Suzuki,3 Mary C. Dinauer,4 Nobuyo Maeda,5 and Hideki Koyama1

Oxygen metabolites generated by myeloperoxidase (MPO) and nicotinamide adenine dinucleotide phosphate (NADPH)–oxidase contribute to microbial killing by phagocytes. To compare the importance of the 2 enzymes for host defense, MPO-deficient (MPO−/−) mice and NADPH-oxidase–deficient mice with chronic granulomatous disease (CGD mice) were intraperitoneally infected with 3 different doses of Candida albicans, and their infection severity was analyzed. CGD mice had increased mortality and exhibited increased tissue fungal burden in a dose-dependent manner, whereas normal mice showed no symptoms. Of interest, at the highest dose, the mortality of MPO−/− mice was comparable to that of CGD mice, but at the lowest dose, it was the same as that of normal mice. At the middle dose, the number of fungi disseminated into various organs of the MPO−/− mice was comparable to that of the CGD mice at day 6 of infection, but it was significantly lower at day 14. These results suggest that MPO and NADPH-oxidase are equally important for early host defense against a large inoculum of Candida.

Several lines of evidence suggest that neutrophils are the main cellular component in the immune system responsible for the defense against Candida albicans [1, 2] and that oxidative products generated by neutrophils are one of the important host defense mechanisms [3]. The most efficient microbicidal system employed by neutrophils depends on the reactive oxygen variants generated by NADPH-oxidase and myeloperoxidase (MPO). In the phagocytosis-associated respiratory burst, phagocytic cells generate the superoxide anion (O2−) via NADPH-oxidase, a multicomponent enzyme localized in the plasma membrane of the cells. Patients with chronic granulomatous disease (CGD), in which granulocytes are unable to produce O2− because of deficiency in NADPH-oxidase, typically present clinical symptoms early in life, with recurrent infections, and die during childhood [3, 4]. In a mouse model of X-linked CGD, intratracheal challenge with Aspergillus fumigatus resulted in high rates of mortality [5, 6]. MPO is found mainly in neutrophils; it produces a strong oxidant, hypochlorous acid (HOCl), from hydrogen peroxide (H2O2) and the chloride ion (Cl−) [7, 8]. Phagocytes deficient in MPO express a marked defect in fungal killing in vitro [9, 10]. We recently reported that MPO-deficient (MPO−/−) mice generated by targeted disruption of the gene exhibit an increased susceptibility to pulmonary [11, 12] and systemic [11] infections with C. albicans and to pulmonary infection with A. fumigatus [12], demonstrating that MPO is an important enzyme for host defense against those fungi in mice. However, the significance of MPO versus NADPH-oxidase is still unclear, because individuals with MPO deficiency are usually healthy, although an increased susceptibility to infections, particularly to C. albicans infection, has been reported in some MPO-deficient patients [10, 13, 14]. To better understand the contributions of MPO and NADPH-oxidase to antifungal defense mechanisms in vivo, we compared the susceptibility of MPO−/− mice [11] and mice with X-linked CGD (X-CGD mice) [6] to systemic infections with C. albicans.

Materials and Methods

Animals. All mice used were 8–10-week-old female C57BL/6 mice purchased from the Japan SLC. MPO−/− mice [11] and X-
CGD mice (gp91phox knockout) [6] were backcrossed at least 10 times with C57BL/6 mice to ensure similar genetic backgrounds. Before infection, all animals were housed under specific pathogen-free conditions.

Experimental infection with C. albicans. Stock cultures of C. albicans (ATCC 18804) were prepared on agar slant medium, as described elsewhere [11, 12]. Wild-type, MPO<sup>-/-</sup>, and X-CGD mice were injected intraperitoneally with 1 mL of fungal suspensions. At 6 and 14 days after the challenge, 5 mice in each group were killed, and lungs, brain, heart, liver, kidneys, and spleen were removed aseptically and were homogenized in sterile saline. Appropriate dilutions of the homogenates were plated in duplicate onto the Candida GS plates (Eiken Chemical) and incubated for 24 h at 37°C. The number of viable fungi was calculated from the number of colonies grown on the plate and was expressed in colony-forming units (cfu) per organ. Data were recorded as the mean log colony-forming units and were expressed in colony-forming units on days after the challenge.

Numbers of fungi are shown in figure 1. When infected with 2 × 10<sup>6</sup> fungi/mouse, the disseminated fungus was undetectable in any organ of wild-type and MPO<sup>-/-</sup> mice at day 6 after the challenge, except for small amounts of fungi recovered from lung and liver tissues of MPO<sup>-/-</sup> mice. Compared with those recovered from the MPO<sup>-/-</sup> mice, significantly higher numbers of fungi (P < .01) were recovered from every organ except the brain in the X-CGD mice, although relatively fewer were observed in the heart (figure 2). Of interest, a dramatically enhanced fungus load, equal to that seen in the X-CGD mice at 9 days after the challenge, whereas the MPO<sup>-/-</sup> mice died during this same time period (figure 1A). When infected with a 20-fold higher dose of fungi (4.6 × 10<sup>7</sup> fungi/mouse), deaths were first observed in MPO<sup>-/-</sup> and X-CGD mice at day 9 and day 6 after the challenge, respectively. All X-CGD mice died before day 35. In contrast, the MPO<sup>-/-</sup> mice surviving for > 24 days all survived (figure 1B). Of interest, at the highest dose of fungi administered (6.9 × 10<sup>7</sup> fungi/mouse), MPO<sup>-/-</sup> mice died at a rate almost equivalent to that of the X-CGD mice, showing 100% mortality before day 13 after the challenge, whereas all wild-type mice remained alive during the 40-day observation period (figure 1C). These results indicate that the mortality of MPO<sup>-/-</sup> mice approached that of X-CGD mice with increasing numbers of injected fungi.

Dissemination of C. albicans into organs. To investigate the dissemination of fungi into tissues, wild-type, MPO<sup>-/-</sup>, and X-CGD mice (n = 5 mice/group) were killed at 6 and 14 days after the challenge; the lungs, brain, heart, liver, kidneys, and spleen were removed; and the numbers of fungi recovered were determined (figure 2). When C. albicans was administered at a dose of 2.3 × 10<sup>6</sup> fungi/mouse, the disseminated fungus was almost undetectable in any organ of wild-type and MPO<sup>-/-</sup> mice at day 6 after the challenge, except for small amounts of fungi recovered from lung and liver tissues of MPO<sup>-/-</sup> mice. Compared with those recovered from the MPO<sup>-/-</sup> mice, significantly higher numbers of fungi (P < .01) were recovered from every organ except the brain in the X-CGD mice, although relatively fewer were observed in the heart (figure 2). Of interest, a dramatically enhanced fungus load, equal to that seen in the X-CGD mice at 9 days after the challenge, whereas the MPO<sup>-/-</sup> mice died during this same time period (figure 1A). When infected with a 20-fold higher dose of fungi (4.6 × 10<sup>7</sup> fungi/mouse), deaths were first observed in MPO<sup>-/-</sup> and X-CGD mice at day 9 and day 6 after the challenge, respectively. All X-CGD mice died before day 35. In contrast, the MPO<sup>-/-</sup> mice surviving for > 24 days all survived (figure 1B). Of interest, at the highest dose of fungi administered (6.9 × 10<sup>7</sup> fungi/mouse), MPO<sup>-/-</sup> mice died at a rate almost equivalent to that of the X-CGD mice, showing 100% mortality before day 13 after the challenge, whereas all wild-type mice remained alive during the 40-day observation period (figure 1C). These results indicate that the mortality of MPO<sup>-/-</sup> mice approached that of X-CGD mice with increasing numbers of injected fungi.

Conclusion. Survival of mice after Candida albicans infection. Wild-type mice (black circles), myeloperoxidase-deficient (MPO<sup>-/-</sup>) mice (open circles), and mice with X-linked chronic granulomatous disease (X-CGD) mice (black triangles) were intraperitoneally infected with 2.3 × 10<sup>6</sup> cfu (A), 4.6 × 10<sup>6</sup> cfu (B), or 6.9 × 10<sup>7</sup> cfu (C) of Candida per mouse. The mice were observed daily, and the percentage of surviving mice was plotted vs. time after the infection. The nos. of wild-type, MPO<sup>-/-</sup>, and X-CGD mice used were, respectively, 8, 10, and 13 in panel A, 7, 17, and 10 in panel B, and 9, 9, and 9 in panel C. In panel B, there were significant differences between wild-type and MPO<sup>-/-</sup> mice (P < .001, log-rank test) and between MPO<sup>-/-</sup> and X-CGD mice (P < .01, log-rank test).
day 6 after the challenge (figure 2), was observed in all organs of the MPO−/− mice that had been infected with a 20-fold higher amount of fungi (4.6 × 10⁶ fungi/mouse). These results indicate that MPO−/− mice are less susceptible than X-CGD mice at lower doses of fungi and that the 2 genotypes are comparably susceptible to high doses of fungi.

The numbers of C. albicans fungi recovered from the lung (P < .01), heart (P < .05), and spleen (P < .05) of X-CGD mice were lower than those in wild-type mice. In contrast, no significant differences were observed in the brain, liver, and kidney.
mice increased with time, being significantly greater in those organs at day 14 after the challenge than at day 6. Similarly, the number of fungi tended to have increased in the brain, liver, and kidney by day 14, although these differences were not significant ($P > .05$). In contrast, in MPO$^{-/-}$ mice, the number of fungi detected at day 14 tended to have decreased from that detected at day 6, although no significant difference was found for all the organs ($P > .05$). As a result, at day 14, the numbers of fungi detected in the lung ($P < .01$), heart ($P < .01$), liver ($P < .01$), kidney ($P < .05$), and spleen ($P < .01$) of the MPO$^{-/-}$ mice were significantly lower than those of the X-CGD mice (figure 2).

Taken together, these results strongly suggest that the importance of MPO is comparable to that of NADPH-oxidase at the early stage of host defense against a high-challenge dose of C. albicans.

### Discussion

In this study, we compared the susceptibility of MPO$^{-/-}$ and X-CGD mice to systemic infection with C. albicans, to define the importance of MPO, and found that MPO and NADPH-oxidase appear to be equally important for host defense against a large inoculum of Candida.

At the lowest dose of C. albicans (2.3 × 10$^7$ cfu/mouse), X-CGD mice were much more susceptible to the fungi than normal and MPO$^{-/-}$ mice. The X-CGD mice exhibited 100% mortality, and much larger numbers of fungi were recovered from almost all of their organs at day 6 of infection. These results suggest that, in killing of a low burden of the fungi in vivo, O$_2^-$ and/or H$_2$O$_2$ produced by NADPH-oxidase play a more important role than HOCl derived from the MPO/H$_2$O$_2$/Cl$^-$ system. However, the difference in mortality between MPO$^{-/-}$ and X-CGD mice became smaller as the challenge dose of fungi increased, and the survival curve of the MPO$^{-/-}$ mice infected with the highest dose (6.9 × 10$^7$ fungi/mouse) was virtually identical to that of the X-CGD mice. These results strongly suggest that both HOCl and O$_2^-$ produced from neutrophils can kill invading C. albicans but that HOCl is less important than O$_2^-$ against a lower dose of infection.

At the middle challenge dose (4.6 × 10$^6$ fungi/mouse), no difference was observed between MPO$^{-/-}$ and X-CGD mice in the number of fungi disseminated into all organs at day 6 of infection. These results are consistent with the observation that the times of the onset of deaths in MPO$^{-/-}$ and X-CGD mice were similar (figure 1B). The numbers of fungi recovered from the lung, heart, and spleen in the X-CGD mice at day 14 were significantly higher than those recovered at day 6. In contrast, the number of fungi recovered from MPO$^{-/-}$ mice at day 14 was slightly, not significantly, lower than that recovered at day 6. However, the number of fungi at day 14 was determined in the surviving mice. These mice probably had slightly lower organ burdens than those that had died between days 6 and 14. Therefore, we should consider that the outgrowth at day 14 might be slightly underestimated, when compared with that at day 6. Although we did not follow up the experiment thereafter, we speculate that the number of the fungi in MPO$^{-/-}$ mice continued to decrease over time, because all MPO$^{-/-}$ mice alive at 24 days after the challenge survived, as observed in figure 1B. In contrast, fungus dissemination in X-CGD mice seemed to be unlikely to decrease after day 14, because all had died by day 35.

In this study, mice were challenged with C. albicans by intraperitoneal route. In the peritoneal lining, there are host defense systems, such as peritoneal macrophages and neutrophils, that could contribute to preventing or delaying systemic invasion. Therefore, it remains possible that MPO deficiency and NADPH-oxidase deficiency may differently affect the elimination of C. albicans from the peritoneal cavity or the penetration of the fungi into the bloodstream. Further experiments with different challenge routes (intratracheal or intravenous) are required to define the contribution of MPO and NADPH-oxidase to disseminated candidiasis.

Hereditary MPO deficiency is a common neutrophil defect, with an estimated incidence of 1 in 2000 persons in the United States [10], and most individuals with this deficiency are healthy. However, an increased susceptibility to C. albicans infection has been reported for some MPO-deficient patients [10, 13, 14], which has also been observed in MPO$^{-/-}$ mice [11, 12]. Furthermore, our present results suggest that MPO-deficient individuals could exhibit problems similar to those of CGD patients if exposed to a large amount of C. albicans.

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### References

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