Contribution of the myeloperoxidase-dependent oxidative system to host defence against *Cryptococcus neoformans*

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The in vivo contribution of reactive oxygen species produced by neutrophils against *Cryptococcus* infection is not widely recognized. Myeloperoxidase (MPO) is a neutrophil-specific enzyme that catalyses the production of hypohalous acids such as HOCl from H2O2. This study investigated the role of MPO in immunological defence against *Cryptococcus neoformans* in an MPO-deficient (MPO−/−) mouse model. The survival of MPO−/− mice infected either intranasally or intravenously with *C. neoformans* was lower than that of identically challenged wild-type mice. The MPO−/− mice that received intranasal injection of *C. neoformans* had significantly larger lung fungal burdens than wild-type mice. On day 7, MPO−/− mice had a significantly higher lung concentration of interleukin (IL)-4 and lower concentrations of IL-2, IL-12p70 and interferon (IFN)-c than wild-type mice, suggesting a weak Th1 response in the MPO−/− mice to *C. neoformans*. Pathologically, the MPO−/− mice with intranasal infection showed more severe pneumonia than wild-type mice, which was associated with an increase in the levels of IL-1α/β in the lungs. In addition, in MPO−/− mice, the pulmonary infection disseminated to the brain with occasional meningitis. The keratinocyte-derived cytokine (KC) level in the brain of infected MPO−/− mice was higher than that of control mice. Both intranasal and intravenous infections resulted in a higher number of fungi in the spleen of MPO−/− mice compared to wild-type, suggesting decreased resistance to *C. neoformans* not only in the lungs but also in the spleen in the absence of MPO. Taken together, these data suggest a major role of MPO in the response to cryptococcal infection.

INTRODUCTION

*Cryptococcus neoformans* is an encapsulated fungus that causes life-threatening infections, particularly in patients with impaired cell-mediated immunity (Chuck & Sande, 1989). The lung is the initial site of *C. neoformans* infection and elicits an influx of macrophages, lymphocytes and neutrophils. Although the infection is typically restricted to the lung, *C. neoformans* disseminates to the central nervous system in the immunosuppressed patient and occasionally in the normal host (Murphy, 1996), and leads to a potentially life-threatening meningoencephalitis (Kozel, 1993). Successful eradication of *C. neoformans* is largely mediated by T cells (Buchanan & Doyle, 2000), and is dependent on protective Th1 cell-derived cytokines (Kawakami et al., 1996; Yuan et al., 1997).

In addition to cell-mediated immunity, neutrophils have been implicated in protection against *C. neoformans* (Graybill et al., 1997; Miller & Mitchell, 1991; Retini et al., 1996; Vecchiarelli et al., 1998). Whereas normal human neutrophils kill *C. neoformans*, those isolated from patients with chronic granulomatous disease lack fungicidal activity (Diamond et al., 1980). Since chronic granulomatous disease is associated with a defect in the phagocyte NADPH oxidase, this suggests that normal neutrophil anti-cryptococcal activity depends on an intact NADPH oxidase. However, the contribution of neutrophil-derived reactive oxygen species (ROS) to host defence against *C. neoformans* has not been fully characterized.

Abbreviations: H&E, haematoxylin and eosin; IFN, interferon; IL, interleukin; KC, keratinocyte-derived cytokine; MPO, myeloperoxidase.
Myeloperoxidase (MPO) (Hansson et al., 2006; Klebanoff, 2005) is found mainly in neutrophils and to a lesser degree in monocytes. In the presence of hydrogen peroxide (H₂O₂), MPO oxidizes chloride to produce the potent microbialidal agent hypochlorous acid. MPO also catalyses the oxidation of bromide, iodide and the pseudohalide ion thiocyanate. MPO deficiency (Lehrer & Cline, 1969; Nauseef, 1998) is the most common inherited neutrophil defect, with an estimated incidence of 1 in 2000–4000 in Europe and the USA (Parry et al., 1981), and of 1 in 55 000 in Japan (Nunoi et al., 2003). Phagocytes deficient in MPO express a mild to moderate defect in bacterial killing but a marked defect in fungicidal activity in vitro (Diamond et al., 1980; Parry et al., 1981). Using MPO-deficient (MPO−/−) mice, we have previously demonstrated that the MPO-dependent oxidative system is important for in vivo host defence against a variety of micro-organisms (Aratani et al., 1999, 2000, 2002a, b). The aim of the present study was to define the contribution of the MPO-dependent antimicrobial system to the in vivo host defence against C. neoformans.

METHODS

Animals. Animal experimentation was carried out in accordance with the guidelines of Kihara Institute for Biological Research, Yokohama City University. All mice used were 10- to 12-week-old females. C57BL/6 mice were purchased from Japan SLC (Hamamatsu). Homozygous mutant mice for MPO (Aratani et al., 1999, 1997) were backcrossed more than ten times with C57BL/6 to ensure similar genetic backgrounds. Before infection, all animals were housed under specific-pathogen-free conditions.

Experimental infection with C. neoformans. C. neoformans (ATCC 24067) was cultured on agar slants containing 2-1% YM broth for 4 days at 27°C. Fungi were enumerated with a haemocytometer, and the viable number determined in c.f.u. For pulmonary infection, wild-type C57BL/6 and MPO−/− mice were intranasally challenged with 0.04 ml fungal suspension. Thirty minutes later, the lungs of two wild-type and two MPO−/− mice were removed aseptically and homogenized in 1 ml sterile saline to determine the initial number of organisms. At various time points after the challenge, selected organs (lungs, brain, spleen) were harvested aseptically and homogenized in sterile saline in the presence of protease inhibitors (Complete Protease Inhibitor Cocktail Tablets; Roche). Appropriate dilutions of the homogenates were plated in duplicate onto tryptophan case soy agar plates (Eiken Chemical). After 2 days incubation at 37°C, the number of viable organisms was determined in c.f.u. Data were recorded as the mean log(c.f.u.) per organ. The remaining homogenates of the lungs and brain were centrifuged at 19 000 g for 10 min to remove cell debris and filtered with 0.45 μm pore-size filters (MILLEX-HV; Millipore). The final supernatants were frozen at −20°C until assayed for cytokines. For intravenous infection, mice were warmed under a heat lamp to vasodilate the tail vein, and fungi were injected in a 0.1 ml volume through a 27-gauge needle. The spleen was removed and homogenized in 1 ml sterile saline and the aliquots were diluted and plated onto the agar plates.

Quantification of cytokine and chemokine levels. The lung and brain supernatants were analysed using the Bio-Plex system and a Lumienx 100TM analyser (Bio-Rad) according to the manufacturer’s instructions. Results were expressed as the mean ± SD. The detection limit of the assay was 1.95 pg ml−1 for all cytokines and chemokines, as stated by the manufacturer.

Preparation of sections and slides. Five wild-type and five MPO−/− mice were sacrificed for histopathologic examination at the same time points as those for c.f.u. measurement. The whole lungs and brain were removed and fixed in a buffered 4% paraformaldehyde solution, dehydrated in ethanol, and embedded in paraffin for sectioning. The sections of these organs were processed for haematoxylin and eosin (H&E) and Grocott staining using standard protocols. Five well-separated cross sections of each organ were obtained and all available fields were observed by light microscopy.

Statistical analysis. Survival curves were analysed by the Kaplan–Meier log-rank test. Differences in the number of c.f.u. were examined by the Mann–Whitney U test. P<0.05 was considered significant.

RESULTS AND DISCUSSION

To examine the contribution of MPO to immunological defence against pulmonary infection with C. neoformans, wild-type and MPO−/− mice were infected intranasally or intravenously with 3·6×10⁵ or 8·7×10⁵ viable yeast, respectively. After 6 months intranasal infection, there was a 94% survival rate among infected wild-type mice (Fig. 1a), and all of the intravenously infected wild-type mice remained viable after 3 months (Fig. 1b). Thus, the dose of C. neoformans induced minimal mortality in C57BL/6 control mice. In contrast, there was rapid death in MPO−/− mice infected with C. neoformans, with a 100% death rate before 1 month and 4 months of intravenous and intranasal infection, respectively (Fig. 1a, b). The difference in survival rates between wild-type and MPO−/− mice was highly significant (P<0.001).

We reasoned that the most likely explanation for the increased mortality in MPO−/− mice following intranasal inoculation of C. neoformans was an impaired clearance of the initial inoculum from the airway. To assess this possibility, we compared the lung fungal burden in MPO−/− mice to that of wild-type controls (Fig. 2). Nearly half of the inoculum, 1·7×10⁵ fungi, was recovered from the lung of wild-type and MPO−/− mice 30 min after infection. The number of C. neoformans in the lungs increased simultaneously for the first 7 days in both groups and peaked on day 19, with a slightly but significantly higher number of c.f.u. in the MPO−/− mice. In the wild-type mice, a gradual elimination of the fungi occurred between days 19 and 34, but the MPO−/− mice failed to eliminate C. neoformans from the lung. Consequently, by day 60, MPO−/− mice contained nearly 200-fold more fungi than the infected control group (P<0.01).

Minimal inflammation was observed histopathologically on day 7, irrespective of genotype, and similar degrees of inflammation were observed on day 19 in both groups (Fig. 3). The lungs of infected wild-type mice showed only localized areas of leukocyte infiltration at days 34 and 60, whereas MPO−/− mice at day 34 exhibited extensive areas of inflammatory infiltration, and all of the airway spaces were filled with the inflammatory cells at day 60. Thus, the lung c.f.u. and the development of pulmonary inflammation correlated with the increased mortality in MPO−/− mice.
Our previous report also demonstrated that the numbers of *C. neoformans* in the lungs of wild-type and MPO−/− mice 48 h after infection do not differ significantly (Aratani et al., 2000). Taken together, these results strongly suggest that the MPO-dependent oxidative system plays an important role in the *in vivo* host defence against *C. neoformans*, although this system is inefficient in the early period after the infection.

Since Th1 cytokines have been shown to be critical for effective host defences in murine models of cryptococcosis (Decken et al., 1998; Hoag et al., 1997; Kawakami et al., 1996) and there is accumulating evidence that neutrophils play an important role in modulating the balance of Th1 and Th2 responses (Mednick et al., 2003; Tateda et al., 2001), we suspected that the higher fungal burden observed in the infected MPO−/− mice could be caused by a reduction in Th1 cell activity. Interestingly, levels of interleukin (IL)-2, IL-12p70 and interferon (IFN)-γ in the lungs of MPO−/− mice at day 7 of infection were significantly lower than those in infected wild-type mice (Fig. 4), suggesting a role for neutrophil-derived hypohalous acids in driving Th1-type host responses.

Previous studies have shown that the constitutive production of IL-4 in transgenic mice results in an increased susceptibility to infection with *Leishmania major* (Leal et al., 1993), and that resistance to infection occurs when the endogenously synthesized IL-4 is neutralized in the susceptible mice (Chatelain et al., 1992). Consistently, in our study, the level of IL-4 was significantly higher in the MPO−/− mice at day 7 compared to the wild-type controls (Fig. 4), although there was no significant difference between the mice with different genotypes in the levels of IL-5 and IL-10 (data not shown). The levels of these cytokines were not significantly different between the mice with different genotypes before infection and at day 34 of infection (Fig. 4). These results indicate that MPO deficiency affects Th1 and Th2 immune response in the early stage of pulmonary cryptococcosis infection.

In murine models, production of IL-1 in the airways is required for full neutrophil migratory responses to LPS or diesel exhaust particles (Ulich et al., 1991; Yang et al., 1997). It is of interest that the lung concentration of IL-1β was significantly higher in the MPO−/− mice than in the wild-type at days 7 and 34 post-infection, and that IL-1α was also significantly higher in the MPO−/− mice at day 34 (Fig. 4), since these data suggest that higher concentrations of these pro-inflammatory cytokines enhanced the pulmonary inflammation of the MPO−/− mice. Overexpression of IL-1β in the lung epithelium leads to pulmonary inflammation, with an increase in the level of keratinocyte-derived cytokine (KC) (Lappalainen et al., 2005). Indeed, in our model, we have observed a time-dependent increase in the level of KC in the lung (Fig. 4). However, there was no difference in the level between the wild-type and MPO−/− mice, suggesting that this chemotactic mediator for neutrophils is not a limiting factor for the higher inflammation observed in the MPO−/− mice. It is well accepted that leukocyte migration from the vasculature occurs by a multistep process, and that this process is dictated by the sequential activation of adhesive proteins and their ligands on both leukocytes and endothelial cells (Wagner & Roth, 2000). Exposure of endothelial cell monolayers or neutrophils *in vitro* to IL-1 causes expression of selectins and integrins (Schleimer & Rutledge, 1986; Scholz et al., 1996). In addition, IL-1 treatment *in vivo* induces intercellular adhesion molecule-1 (ICAM-1) in lung (Komatsu et al., 1997). Taken together, it is possible that the elevated IL-1α/β levels in MPO−/− mice could facilitate the transmigration of circulating neutrophils into tissues.

**Fig. 1.** Survival of wild-type and MPO−/− mice following infection with *C. neoformans*. (a) Wild-type (*n* = 32) and MPO−/− (*n* = 29) mice were intranasally infected with 3·6 × 10⁶ c.f.u. *C. neoformans*, and survival was observed over the course of 180 days. (b) Wild-type (*n* = 10) and MPO−/− (*n* = 10) mice were intravenously infected with 8·7 × 10⁶ c.f.u. *C. neoformans*. *P* < 0·001 (MPO−/− versus wild-type mice) for both intranasal and intravenous infections. The data are representative of two independent experiments.
The recovery of \textit{C. neoformans} from the brain of infected wild-type and MPO\textsuperscript{-/-} mice was compared (Fig. 2). Whereas cryptococci were nearly undetectable in the brain of wild-type mice at all time points, significant numbers of fungi were found in the MPO\textsuperscript{-/-} mice on days 34 and 60 post-infection. We carried out histological analysis of the brain in five control and five MPO\textsuperscript{-/-} mice on day 60 after infection. One MPO\textsuperscript{-/-} mouse showed a cellular infiltrate including neutrophils in the meningeal area (Fig. 5a, b). Another mouse showed cyst-like cavities in the medulla.
considered the possibility that the large fungal burden was the cause of death of the MPO-deficient mice. In contrast, no pathological change was observed in all five of the wild-type mice (Fig. 5f). Since dissemination of the fungi into the cerebrum is often fatal in humans, we observed the slight but consistent accumulation of inflammatory cells, and a subset of these cells could be morphologically identified as neutrophils (Fig. 5). This accumulation of neutrophils suggests that a higher brain fungal burden occasionally stimulates a neutrophil mobilization into the brain across the blood–brain barrier in response to KC. In mice of both genotypes, the brain concentrations of Th1-associated (IL-2, IL-12p70, IFN-γ), Th2-associated (IL-4, IL-5, IL-10) and pro-inflammatory (IL-1β, IL-1β) cytokines did not change during the course of infection (data not shown).

Although the fungal burdens in the spleen were similar in both groups for the first 7 days, those of MPO−/− mice on and after day 19 were significantly higher (P<0.05) than those of wild-type mice (Fig. 2). However, the blood fungal burden of the mice was equivalent to that in the wild-type (Fig. 2), suggesting that the higher distributions of fungi from the lungs to the spleen were due to an increased systemic dissemination from the lungs, but rather due to a decreased local resistance in the spleen. To more rigorously examine the role of MPO during systemic infection without an ongoing localized pulmonary infection, an intravenous infection experiment was performed. Wild-type and MPO−/− mice were infected intravenously with 8.7 × 10^5 viable yeast. To determine fungal dissemination kinetics from the bloodstream, wild-type and MPO−/− mice were analysed on days 1 and 5 post-infection. The fungal burden in the spleen of wild-type mice on day 5 was equivalent to that on day 1. In contrast, the burden in MPO−/− mice on day 5 was significantly higher than that on day 1 (Fig. 7), indicating that the control of fungal growth within the spleen was dependent on MPO production. Since intravenous infection experiments result in a systemic infection of blood-borne fungi in the absence of an ongoing pulmonary infection, the results indicate that MPO was important for host defence against C. neoformans not only in the lungs but also in peripheral organs.

C. neoformans is acquired via the respiratory tract and occasionally disseminates to the central nervous system in some immunocompromised patients (Lee et al., 1996). So far, MPO deficiency is not known to be associated with human cryptococcosis. Since mouse neutrophils are devoid of the cause of death of the MPO-deficient mice. In contrast, no pathological change was observed in all five of the wild-type mice (Fig. 5f). Since dissemination of the fungi into the cerebrum is often fatal in humans, we observed the slight but consistent accumulation of inflammatory cells, and a subset of these cells could be morphologically identified as neutrophils (Fig. 5). This accumulation of neutrophils suggests that a higher brain fungal burden occasionally stimulates a neutrophil mobilization into the brain across the blood–brain barrier in response to KC. In mice of both genotypes, the brain concentrations of Th1-associated (IL-2, IL-12p70, IFN-γ), Th2-associated (IL-4, IL-5, IL-10) and pro-inflammatory (IL-1β, IL-1β) cytokines did not change during the course of infection (data not shown).

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Fig. 4. Cytokine and chemokine levels in the lungs of wild-type and MPO−/− mice after infection with C. neoformans. Wild-type (black bars) and MPO−/− mice (open bars) were intranasally infected with 3·6 × 10^6 c.f.u. C. neoformans. Mice were killed before infection, or at 7 or 34 days post-infection, and the lungs were harvested and homogenized. Cytokines and chemokines were measured in triplicate using the Bio-Plex system. Data are expressed as pg cytokine per lung. Results are presented as the mean ± SD of four infected or uninfected mice. *, P < 0·05 compared to wild-type infected mice.
Fig. 5. Histopathology of brain of MPO<sup>−/−</sup> mice 60 days after C. neoformans infection. Brain sections of wild-type (f) and MPO<sup>−/−</sup> mice (a–e) 60 days after intranasal challenge with 3-6 x 10<sup>6</sup> c.f.u. are shown. C. neoformans were stained with H&E (a, b, d and f) or Grocott methamine silver (c and e). Panel (b) is a higher magnification of the cellular infiltrate observed in the box shown on panel (a). Arrows indicate neutrophils. Bars, 1 mm.

Fig. 6. KC levels in the brains of wild-type and MPO<sup>−/−</sup> mice after infection with C. neoformans. Wild-type (black bars) and MPO<sup>−/−</sup> mice (open bars) were intranasally infected with 3-6 x 10<sup>6</sup> c.f.u. C. neoformans. Mice were killed before infection, or at 7 or 34 days post-infection. The KC levels of brain homogenates were measured in triplicate using the Bio-Plex system. Data are expressed as pg KC per organ. Results are presented as the mean ± SD of four infected or uninfected mice. *, P<0.05 compared to wild-type infected mice.

Fig. 7. Cultures from spleen after intravenous infection with C. neoformans. Wild-type (black bars) and MPO<sup>−/−</sup> mice (open bars) were inoculated intravenously with 8-7 x 10<sup>5</sup> c.f.u. C. neoformans per mouse and analysed on days 1 and 5. Aliquots of homogenized organs were plated on agar plates, and total c.f.u. per organ was determined. Five mice were used for each group. Results represent mean log(c.f.u.) per organ ± SD of the means for five animals. *, P<0.05.
of defensins (Eisenhauer & Lehrer, 1992), prominent non-oxidative contributors to human anticryptococcal defence (Mambula et al., 2000), impaired oxidative systems in mice may permit a more severe infection than in humans. Whereas cell-mediated immunity, non-oxidative systems and reactive nitrogen intermediates (Lovchik et al., 1997) likely contribute to microbial clearance, our data strongly suggest that impairment of the MPO-mediated antimicrobial system could be one of the risk factors for the infection.

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