Role of the (Mn)superoxide dismutase of Enterococcus faecalis in the in vitro interaction with microglia

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Enterococcus faecalis is a significant human pathogen worldwide and is responsible for severe nosocomial and community-acquired infections. Although enterococcal meningitis is rare, mortality is considerable, reaching 21%. Nevertheless, the pathogenetic mechanisms of this infection remain poorly understood, even though the ability of E. faecalis to avoid or survive phagocytic attack in vivo may be very important during the infection process. We previously showed that the manganese-cofactored superoxide dismutase (MnSOD) SodA of E. faecalis was implicated in oxidative stress responses and, interestingly, in the survival within mouse peritoneal macrophages using an in vivo–in vitro infection model. In the present study, we investigated the role of MnSOD in the interaction of E. faecalis with microglia, the brain-resident macrophages. By using an in vitro infection model, murine microglial cells were challenged in parallel with the wild-type strain JH2-2 and its isogenic sodA deletion mutant. While both strains were phagocytosed by microglia efficiently and to a similar extent, the ΔsodA mutant was found to be significantly more susceptible to microglial killing than JH2-2, as assessed by the antimicrobial protection assay. In addition, a significantly higher percentage of acidic ΔsodA-containing phagosomes was found and these also underwent enhanced maturation as determined by the expression of endolysosomal markers. In conclusion, these results show that the MnSOD of E. faecalis contributes to survival of the bacterium in microglial cells by influencing their antimicrobial activity, and this could even be important for intracellular killing in neutrophils and thus for E. faecalis pathogenesis.

INTRODUCTION

Enterococcus faecalis is a natural member of the digestive microflora in humans and many other animals but in some instances it becomes a pathogen capable of causing severe human diseases (Gilmore et al., 2002), such as bacteraemia (Suppli et al., 2010), endocarditis (Fernández Guerrero et al., 2007), neonatal sepsis (Sarkar et al., 2006) and meningitis (Pintado et al., 2003). Although enterococcal meningitis is rare, mortality is relatively high and occurs in 21% of cases, posing additional challenges for the treatment of this infection (Pintado et al., 2003). In addition, the emergence of vancomycin- and multidrug-resistant enterococci as important nosocomial pathogens is a cause of concern, as they are very difficult to control once established (Murray, 2000; Willems & Bonten, 2007; Huycke et al., 1998).

Microglia are brain macrophages that share many, if not all, the properties of macrophages in other tissues including the generation of free radicals, such as reactive oxygen species (ROS) during the respiratory burst, which is regarded as an important defence mechanism of the central nervous system against intracellular micro-organisms (Rock et al., 2004). ROS formed during phagocytosis are

Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; MnSOD, manganese-cofactored superoxide dismutase; ROS, reactive oxygen species.
primarily inside the phagolysosome, even though stimuli other than phagosome formation can induce ROS production in intracellular compartments (Bylund et al., 2010). E. faecalis has developed several enzymic and non-enzymic mechanisms to counteract deleterious effects of superoxide anion (O$_{2}^{-}$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (OH$^\cdot$), which range from DNA strand damage to peroxidation of membrane lipids (Imlay, 2003).

An essential element of the primary antioxidant defence system of E. faecalis is the manganese-cofactored superoxide dismutase (MnSOD) SodA encoded by the sodA gene (Verneuil et al., 2006). In particular, we showed that the E. faecalis sodA deletion mutant was more sensitive to treatment with H$_2$O$_2$ than the isogenic parental strain JH2-2, whereas complementation of the ΔsodA mutant restored resistance to this oxidant. Furthermore, the ΔsodA mutant exhibited decreased resistance to intracellular killing by murine peritoneum-resident macrophages compared with its parent JH2-2, suggesting that MnSOD may be considered to be a virulence factor (Verneuil et al., 2006).

As a consequence of renewed interest in microglia, insights are being gained from in vivo and in vitro studies on their physiological and pathological properties, supporting the defensive role of microglia against CNS infections (Rock et al., 2004). Thus, in-depth studies on the interaction of microglial cells with E. faecalis would greatly facilitate a better understanding of bacterial neuropathogenesis. In particular, the initial E. faecalis-to-microglia recognition phase as well as the post-phagocytosis intracellular events should be carefully investigated, since both steps seem to be relevant in the outcome of such pathogen–host cell interplay.

In this study, the JH2-2 wild-type strain and its ΔsodA mutant were compared for the ability to interact with microglia. Moreover, the intracellular fate of the ΔsodA mutant was examined in comparison with the wild-type strain in order to evaluate whether MnSOD might influence phagosome maturation following E. faecalis internalization by microglia.

**METHODS**

**Microglial cells.** The murine microglial cell line BV2, established as described previously (Blasi et al., 1990), was maintained in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) fetal calf serum (heat-inactivated for 30 min at 56 °C, EuroClone), gentamicin (50 µg ml$^{-1}$; Bio Whittaker) and 1-glutamine (2 mM; EuroClone), hereafter referred to as complete RPMI, at 37 °C in a 5% CO$_2$ in air atmosphere. Then, BV2 cells were detached by vigorous shaking biweekly and fresh cultures were started at a concentration of 5 × 10$^5$ ml$^{-1}$ the day before the experiment.

**Bacterial strains, growth conditions and labelling procedure.** E. faecalis JH2-2 wild-type and its derivatives, the ΔsodA mutant and a complemented ΔsodA strain (Verneuil et al., 2006), were used in this study. Briefly, the ΔsodA mutant was constructed by replacement of the wild-type gene with a mutated copy of sodA containing stop codons and a central deletion, whereas the complemented strain was obtained by knocking in the wild-type sodA allele into the ΔsodA mutant (Verneuil et al., 2006). Bacteria were cultivated in brain heart infusion (BHI) medium (Oxoid) at 37 °C with vigorous shaking (150 r.p.m.) to reach OD$_{600}$ 0.2, harvested by centrifugation and suspended at the desired concentration. For all fluorescence-based assays, 2 × 10$^8$ bacterial cells ml$^{-1}$ were labelled with 4.6-diamidino-2-phenylindole (DAPI) (Molecular Probes/Invitrogen; 1 µg ml$^{-1}$).

After incubation at 37 °C for 30 min, bacteria were washed three times with PBS to remove unbound dye, and the bacteria were diluted to the desired concentration in complete RPMI medium without antibiotics. MICs of gentamicin and vancomycin were determined by a standard broth microdilution method (CLSI, 2010).

**Microglia survival assay.** Bacterial microglia survival was quantified utilizing a modified antibiotic-protection assay (Brett et al., 2008). Briefly, BV2 cells (1 × 10$^6$ ml$^{-1}$) were incubated for 1.5 h with each of the bacterial strains in complete RPMI medium without antibiotics at a host : pathogen ratio of 1 : 5. Cells were washed twice with PBS to remove extracellular bacteria and exposed for 2 h to gentamicin (150 µg ml$^{-1}$) and vancomycin (10 µg ml$^{-1}$) in fresh complete RPMI to suppress the growth of residual extracellular bacteria. BV2 cells were then washed twice with PBS and incubated in complete RPMI without antibiotics (time 0). At 0, 2, 4 and 8 h post-infection, cells were lysed with 0.2% (v/v) Triton X (see above) and 100 µl ml$^{-1}$ Triton X-100 for 15 min to release intracellular bacteria, and serial dilutions of the lysates were plated onto BHI agar. The plates were incubated at 37 °C for 48 h and the c.f.u. counts were then used to enumerate bacterial loads. Control experiments were carried out to verify that the Triton X-100 treatment was not toxic for bacteria (data not shown).

**Phagocytosis assay.** To strengthen attachment of BV2 cells to the wells, Lab-Tek II chamber slides (Nalge Nunc International) were pre-treated with poly-L-lysine (Sigma; 10 µg per well) for 30 min, and washed twice with PBS. BV2 cells (2 × 10$^6$ ml$^{-1}$ in complete RPMI, 100 µl per well) were seeded, incubated as described for 24 h and then infected with 100 µl of a suspension of 10$^7$ DAPI-labelled bacteria ml$^{-1}$ (see above) in complete RPMI (host : pathogen ratio of 1 : 5). After incubation for 1.5 or 3 h, cells were then treated with trypsin blue for 5 min to quench the fluorescence of the bacteria which were bound but not internalized by the microglia, washed three times in PBS to remove extracellular bacteria and fixed for 30 min with 4% paraformaldehyde (PFA) (Sigma) in PBS. The remaining fluorescence of the phagocytosed bacteria was visualized by epifluorescence microscopy. At least 200 microglial cells from each sample were examined and the percentage of cells that had intracellular bacteria was estimated as the ratio of the number of BV2 cells containing one or more bacteria to the total number of BV2 cells examined.

**Phagosome acidification assay.** Visualization of the acidic bacteria-containing phagosomes was performed as described previously (Orsi et al., 2009). Briefly, Lab-Tek II chamber slides were prepared and BV2 cells were infected as in the phagocytic assay. Then, the cells were washed to eliminate extracellular bacteria, and were exposed to 4 µl of the acidotrophic dye LysoTracker Red DND-99 (Molecular Probes/Invitrogen) at a final concentration of 5 µM ml$^{-1}$. Thirty minutes before the end of incubation, an additional volume (4 µl) of the same dye was added. Finally, 5 min before the end of incubation, 100 µl trypsin blue was dispensed into each well. After PFA fixing, cells with acidic phagolysosomes were washed and immediately visualized by epifluorescence microscopy. Successful acidification of phagosomes containing DAPI-labelled bacteria was indicated by the appearance of both LysoTracker Red DND-99 (red) and DAPI (blue) fluorescence within the phagosomes, so that merging of images of the different fluorescence channels resulted in purple fluorescence. For quantitative analysis, the percentage of acidic
(purple) phagosomes per image was determined by counting purple phagosomes within the phagocytic cells.

**Colocalization studies.** Phagosome-maturation markers (Rab5, Rab7 and Rab9) and lysosome-associated membrane protein 2 (LAMP2) (Kinchen & Ravichandran, 2008) were assessed in the colocalization studies. Briefly, BV2 cells were prepared as in the phagocytosis assay and allowed to take up DAPI-labelled bacteria. At 30 and 45 min post-infection, cells were washed, fixed with 4% formaldehyde in PBS for 30 min and permeabilized for 10 min with 0.2% Triton X-100 in PBS. Cells were then incubated (1 h at room temperature) with the primary antibodies (anti-Rab5, -Rab7, -Rab9 or -LAMP2; Sigma), washed twice with PBS, and incubated (1 h at room temperature) with the secondary Chromo 642 goat anti-rabbit IgG antibody (Active Motif). The overlay signals of DAPI-labelled bacteria (blue fluorescence) with the red fluorescence of secondary antibody Chromo 642 were interpreted as colocalization by using epifluorescence microscopy. Association of DAPI with Rab5-, Rab7-, Rab9- or LAMP2-positive vacuoles was quantified by scoring the colocalization phenotypes of at least 100 individual bacteria from each well in the chamber slides.

**Epifluorescence microscopy.** Prior to visualization, Lab-Tek II chamber slides were washed with PBS and then treated with prolong gold antifade reagent (Invitrogen) to suppress photo-bleaching and preserve the fluorescence signal. Epifluorescence and differential interference contrast microscopy were performed with a Nikon Eclipse 90i imaging system equipped with Nomarski DIC optics (Nikon Instruments). At each time point, samples were photographed with a DS-2MV Nikon digital camera, and the resulting photographs were analysed by using the Nikon NIS-ELEMENTS version D3.1 software.

**Statistical analysis.** Results are reported as mean ± SD of three independent experiments and triplicates were prepared for each time point and experiment. In all epifluorescence studies at least 200 cells per group were scored. To evaluate the statistical significance of the results, Student’s unpaired t-test or analysis of variance (one-way ANOVA) with a Bonferroni correction post-test was performed using JMP software. P-values <0.05 were considered significant.

**RESULTS**

In the present study, *E. faecalis* wild-type strain JH2-2, its ΔsodA mutant and the complemented ΔsodA mutant were used to investigate the role of MnSOD in the interaction between *E. faecalis* and microglia. Upon infection with each strain, microglial cells were assessed for phagocytosis, antimicrobial activity and phagosome maturation.

**MnSOD does not influence the susceptibility of *E. faecalis* to phagocytosis by microglia**

We performed fluorescence studies to characterize the interaction of the ΔsodA mutant with BV2 murine microglia. Previously, we had established a quantitative phagocytosis assay, in which the fluorescence only from attached bacteria, and not from those internalized by microglial cells, was quenched by trypan blue (Peppoloni et al., 2010). Thus, we could reliably detect internalized enterococci alone and perform time-course studies of the phagocytic activity of the BV2 cells against JH2-2, ΔsodA mutant and the complemented ΔsodA strain. Fig. 1 depicts the number of phagocytic cells that ingested one or more bacteria at 1.5 and 3 h post-infection. The phagocytic uptake of the three strains followed a similar pattern with time, as the percentages of BV2 cells with internalized JH2-2, ΔsodA mutant or the complemented ΔsodA strain were slightly, but not statistically significantly, different. These data show that MnSOD does not affect the phagocytic activity of murine microglia against *E. faecalis*.

![Fig. 1. Phagocytic activity of microglial cells towards JH2-2 (white bars), the ΔsodA mutant (black bars) and the complemented ΔsodA strain (grey bars). BV2 cells were incubated with DAPI-labelled bacteria for the indicated times. Fluorescence of non-phagocytosed bacteria was quenched via treatment with trypan blue before fluorescence of ingested bacteria was measured. Values represent the means ± SD of three to five independent experiments.](image-url)
First, JH2-2, the D. great importance to the response to bacterial pathogens. GTPases) followed by fusion with acidic lysosomes, is of sequential acquisition of different proteins (i.e. the Rab Ravichandran, 2008). The phagosome maturation, through containing phagosomes within microglia.

MnSOD delays the maturation of E. faecalis-containing phagosomes within microglia

It is known that the killing of internalized pathogens is also due to the fusion of phagosomes with lysosomes, which contain degradative enzymes in an acidic environment. To investigate the events following E. faecalis internalization by microglia, the fate of phagosomes with the ingested bacterial strains was evaluated in terms of acidification and expression of endolysosomal markers (Kinchen & Ravichandran, 2008). The phagosome maturation, through sequential acquisition of different proteins (i.e. the Rab GTPases) followed by fusion with acidic lysosomes, is of great importance to the response to bacterial pathogens.

First, JH2-2, the ΔsodA mutant and the complemented ΔsodA strain labelled with DAPI were exposed to BV2 cells and LysoTracker Red DND-99, a marker for phagosome acidification, was added. Representative overlapping pictures of the different fluorescence channels are shown in Fig. 3. A red colour indicates the presence of an acidic organelle, i.e. the phagolysosomes, whereas a blue fluorescence the presence of bacteria. If bacteria were found in acidic organelles, i.e. a red and a blue colour were present in the same site, this resulted in purple fluorescence and was taken as an indicator of successful phagosome maturation. To quantify the phenomenon, we determined the percentage of acidic phagosomes by counting purple phagosomes within the phagocytic cells. At 1.5 h post-infection, the percentage of ΔsodA mutant-harbouring phagosomes that showed an accumulation of the acid-specific fluorescent dye was significantly higher than that of JH2-2- or complemented ΔsodA-containing phagosomes (Fig. 4). Similar results were observed at 3 h post-infection (Fig. 4).

Finally, BV2 cells infected with the ΔsodA mutant and JH2-2 strains were quantitatively analysed for the appearance of endolysosomal markers, i.e. Rab5, Rab7, Rab9 and LAMP2. The ΔsodA mutant and JH2-2 showed a different degree of colocalization with most of the antibody-stained compartments (Fig. 5). At 30 min post-infection, Rab5-positive phagosomes, and even more Rab7-, Rab9- and LAMP2-positive phagosomes, were present in significantly higher numbers in the ΔsodA- than in JH2-2-infected BV2 cells. At 45 min post-infection, the ΔsodA-containing phagosomes retained a higher percentage of staining, in terms of endosomal markers, yet such differences were not statistically significant (Fig. 5).

**DISCUSSION**

As part of the innate control system of brain infection, microglia encompassing up to 15% of brain cells are located within the brain parenchyma, just behind the blood–brain barrier, and constitute the main phagocytic population of the central nervous system (Rock et al., 2004; Hauwel et al., 2005). Usually recognized as resident ‘amateur’ macrophages, they are responsible for the early control of infections as well as for the recruitment of cells of the adaptive immune system required for pathogen clearance (Neglia et al., 2006; Lehnardt, 2010). Like ‘professional’ macrophages, microglial cells promptly respond to invading micro-organisms through their uptake and internalization into phagosomes, in which they face a hostile environment, characterized by not only a low pH but also the presence of a number of toxic compounds, e.g. ROS, defensins and lysosomal hydrolases, all potentially contributing to killing and digestion of ingested pathogens (Desjardins et al., 2005).

At present, little is known regarding the molecular mechanisms used by E. faecalis to persist within eukaryotic cells or how the bacterium specifically evades innate and acquired host immune defences (Gilmore et al., 2002). The presence of MnSOD confers to the wild-type strain JH2-2 strong resistance to early killing by BV2 cells, which was indeed significantly decreased in the ΔsodA mutant. The similarity of results between the present and other studies (Orsi et al., 2009; Peppoloni et al., 2010) implies that in microglial cells, as well as in ‘professional’ macrophages, phagocytosis and intracellular killing are not strictly related phenomena, even though the biological function of MnSOD was sufficient for us to rule out any involvement of MnSOD in the process of taking up by BV2 cells.
Killing of internalized pathogens is attributed to not only the release of reactive oxygen and nitrogen species but also the fusion of phagosomes with lysosomes, as mentioned above. This process, called phagolysosome biogenesis or phagosome maturation, implies that, after their formation, phagosomes undergo a series of changes, by sequentially fusing with endocytic organelles, such as early endosomes, late endosomes and lysosomes, and by modulating the sequential appearance and disappearance of key phagosome proteins (Desjardins, 1995). A long-standing paradigm is that ROS formed during phagocytosis are primarily inside the phagolysosome, and this fits their definition for being intracellular, i.e. they are detected in the presence of extracellular scavengers (Bylund et al., 2010). Thus, while it is clear that phagolysosome ROS are imperative for effective killing of microbes, our data argue that their removal by the intracellular scavenger activity of MnSOD should result in enhanced survival of the bacterium.

In addition, we examined differences in the fate of \( E.\ faecalis \) JH2-2 phagosomes compared with the \( \Delta sodA \) mutant strain to determine whether MnSOD could somehow alter phagolysosome biogenesis. As acidification is one of the major steps of phagosome maturation, we first analysed the percentage of acidic phagosomes bearing both \( E.\ faecalis \) strains. Interestingly, we demonstrated that the deletion of \( sodA \) enhances the acidification process, such that only \( \sim 5\% \) of phagosomes harbouring JH2-2 were fully acidified, with respect to \( \sim 20\% \) seen with the \( \Delta sodA \) mutant. Next, we analysed the pathway for phagosome maturation in terms of recruitment of endolysosomal markers and we demonstrated that the early endosome marker Rab5 was detectable in \( \sim 24\% \) of the microglia infected with the \( \Delta sodA \) mutant and JH2-2 strains, respectively. More striking differences between the two strains were observed with Rab7, which is known to traffic from early to late endosome or lysosome, and with the late endosome marker Rab9 (Kinchen & Ravichandran, 2008). The increased maturation of phagosomes housing the \( \Delta sodA \) mutant has been further confirmed by the finding that they were labelled for the LAMP2 marker, a glycoprotein that is specifically localized to acidic lysosome structures (Eskelinen et al., 2003), to an extent that exceeded that seen with the parental strain JH2-2. Although LAMP proteins are required for fusion of lysosomes with phagosomes (Huynh et al., 2007), the precise molecular functions of these proteins remain to be determined (Kinchen & Ravichandran, 2008). However,
defects in LAMP2 by blocking phagosome maturation have been linked to decreased neutrophil-mediated bacterial killing (Beertsen et al., 2008).

Remarkably, our results indicate that the wild-type strain JH2-2 is more effective than the ΔsodA mutant in preventing its targeting to compartments displaying late endosomal/lysosomal features and this could impair the bacterium’s digestion by microglia to a limited extent and thus favour its survival in the phagocytic cells. A similar effect could also be observed in neutrophils, which are an essential component of the human innate immune system, thus contributing to the pathogenesis of E. faecalis. Further investigations are also required to assess the significance of this finding through elucidation of the intracellular mechanisms triggered by MnSOD or by accumulation of O$_2^-$ to critical levels. An unresolved question is whether ROS directly react with and kill microbes or if they are indirect mediators of killing (Kinchen & Ravichandran, 2008). Recent evidence suggests that SodA is central to the intrinsic ability of E. faecalis to withstand drug-induced killing and O$_2^-$ is the key effector of bacterial death (Bizzini et al., 2009). Thus, the identification of the O$_2^-$-sensitive targets or specific superoxide dismutase inhibitors may provide novel insights into the molecular mechanisms of E. faecalis death and novel potential targets for antimicrobial intervention.

In conclusion, we have shown for the first time to our knowledge that MnSOD significantly affects the ability of E. faecalis to cope with the oxidative damage in microglia, as the mutant defective in this enzyme exhibited marked susceptibility to macrophages in vitro. However, the contribution of oxygen-dependent microbicidal activity against E. faecalis in microglia studied outside the native context of host organ, i.e. the brain, may differ from that occurring in vivo. Studies using a mouse model of E. faecalis meningitis will be undertaken to test this hypothesis.

ACKNOWLEDGEMENTS

We wish to acknowledge Mr Antonio Martino for excellent informatic support. Very special thanks are also given to Dr Andrea Ardizzoni for helpful suggestions and a critical review of the manuscript.

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Edited by: P. C. F. Oyston

S. Peppoloni and others