Catalase negative *Staphylococcus aureus* retain virulence in mouse model of chronic granulomatous disease

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Abstract Myeloperoxidase-mediated chlorination is thought to be a necessary microbicidal mechanism. The H_2O_2 required for this process is generated by the NADPH oxidase. *Staphylococcus aureus* can also produce H_2O_2, which is not broken down by catalase negative organisms. It has been thought that this bacterial H_2O_2 can substitute for cellular H_2O_2 in the halogenation reaction in chronic granulomatous disease (CGD) where neutrophils are lacking the NADPH oxidase. We have readdressed this issue in a mouse model of CGD using clinical isolates of catalase positive and negative strains of *S. aureus*. The results showed these organisms to be equally virulent and that the H_2O_2 they produced is insufficient to cause significant iodination, a marker for chlorination, thereby contradicting the accepted views on this subject. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Superoxide; Chronic granulomatous disease; *Staphylococcus*; Catalase; Infection; Leukocyte

1. Introduction

The NADPH oxidase is missing in the syndrome of chronic granulomatous disease (CGD), a human condition characterised by a profound predisposition to bacterial and fungal infection [1]. This system has been thought to exert it's effect by direct killing of the organisms by superoxide (O_2^-) [2], the primary product of the oxidase, or through halogenation by myeloperoxidase (MPO) using H_2O_2 [3], the dismutation product of O_2^-, as substrate.

Catalase negative organisms rarely infect these patients [4] and an explanation proposed for this was that these bacteria generated enough H_2O_2 to catalyse their own MPO-mediated halogenation within the vacuole of the phagocytosing neutrophil [5,6]. This theory was supported by a study in which in vitro mutagenesis was used to generate strains of *Staphylococcus aureus* containing varying levels of catalase. Their virulence in mice was found to be inversely proportional to their catalase content [7].

Recently however doubts have been cast upon this theory. Catalase deficient *Aspergillus nidulans* [8] was shown to be as virulent as the catalase positive variety in a mouse model of CGD. We have therefore readdressed the issue of whether catalase is an important determinant of microbial virulence in vivo. We chose *S. aureus* because it is the commonest cause of infection in patients with CGD [1]. A mouse model of CGD in which the gene for $p47^{phox}$ had been targeted [9] was infected with two naturally occurring clinical isolates of staphylococci; *S. aureus* NCTC 12981, a catalase positive (cat (+ve)) variety, and *S. aureus* NCTC 12035 which is catalase negative (cat (−ve)). Experiments were also preformed to investigate if the hydrogen peroxide produced by these organisms could be used for MPO-mediated halogenation.

2. Materials and methods

2.1. Phagocytosis and oxidative iodination of staphylococci

Neutrophil rich peritoneal exudate was harvested by peritoneal lavage with RPMI 1640 containing heparin at 5 U/ml 16 h after intraperitoneal injection of 0.7 ml/20 g body weight thioglycollate broth 3% [10]. 1×10^7 exudate cells were incubated at 37°C in 1 ml of phosphate-buffered saline (PBS) (140 mM NaCl, 10 mM KCl, 10 mM NaH_2PO_4, 5 mM glucose, pH 7.3) containing 5% immune mouse serum as opsonin [11].

Phagocytosis was measured by incubating (1×10^7) cells with (5×10^7) fluorescein isothiocyanate (FITC)-labeled *S. aureus* in a rapidly stirred chamber at 37°C. An aliquot was removed immediately after the addition of the bacteria to the cells ($t=0$) and after a further 6 min. Fluorescence of extracellular organisms was quenched by trypan blue (0.04% solution). One hundred cells were examined and the associated fluorescent *S. aureus* counted using a fluorescent microscope.

Iodination by 1×10^7 mouse neutrophils was carried out in PBS containing 50 nM KI, 10 μCi ^{125}I, and 8×10^8 *S. aureus* as described [11]. 50 μl samples were transferred into 10% TCA, 200 mM KI and centrifuged at $400\times g$, 4°C for 20 min. The pellet was resuspended in 5 ml of 10% TCA and recentrifuged six times, before counting in a γ counter.

2.2. Characterisation of bacterial strains

Catalase activity of staphylococcal strains NCTC 12035 (catalase negative (cat (−ve)) or NCTC 12981 (catalase positive (cat (+ve)) was determined as described [12] using liver bovine catalase (Sigma) as a standard. H_2O_2 production was determined using the Amplex Red Hydrogen Peroxide Assay kit (Molecular Probes). Fluorescence was measured using excitation in the range 530–560 nm and emission at 590 nm.

Susceptibility of bacteria to H_2O_2 was determined by incubation in 10 and 100 mM H_2O_2 at 37°C. Survival was determined by counts of colony forming units (CFU) after plating on LB (EZMix, Sigma) agar plates. All studies were performed in triplicate and colonies counted on three aliquots taken at each time point.

Southern and Western blot analyses were carried out following standard procedures. Rabbit polyclonal antiserum specific for *S. aureus* catalase (supplied by de la Fuente [13]) was used to detect catalase.

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Abbreviations: CGD, chronic granulomatous disease; cat, catalase; MPO, myeloperoxidase; CFU, colony forming units

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2.3. Infection experiments

Staphylococci were grown overnight at 37°C in LB broth and washed in PBS. Cell density was determined photometrically and confirmed by plating onto LB agar plates. Age- and sex-matched mice, all on the strain 129 genetic background, maintained in microisolator cages, were injected intravenously with 4×10^7 CFU *S. aureus* and survival was monitored for up to 3 weeks. For determination of bacterial load in the kidneys, animals were killed 48 h after infection and kidneys homogenised in 1 ml sterile PBS. CFU were determined by serial dilution on LB agar plates.

2.4. Statistical analysis

Survival of mice after intravenous injection was analysed by Kaplan–Meier survival analysis. In vitro phagocytosis of bacteria, iodination and bacterial survival within the kidneys of CGD mice were analysed by Student's two-tailed *t*-test.

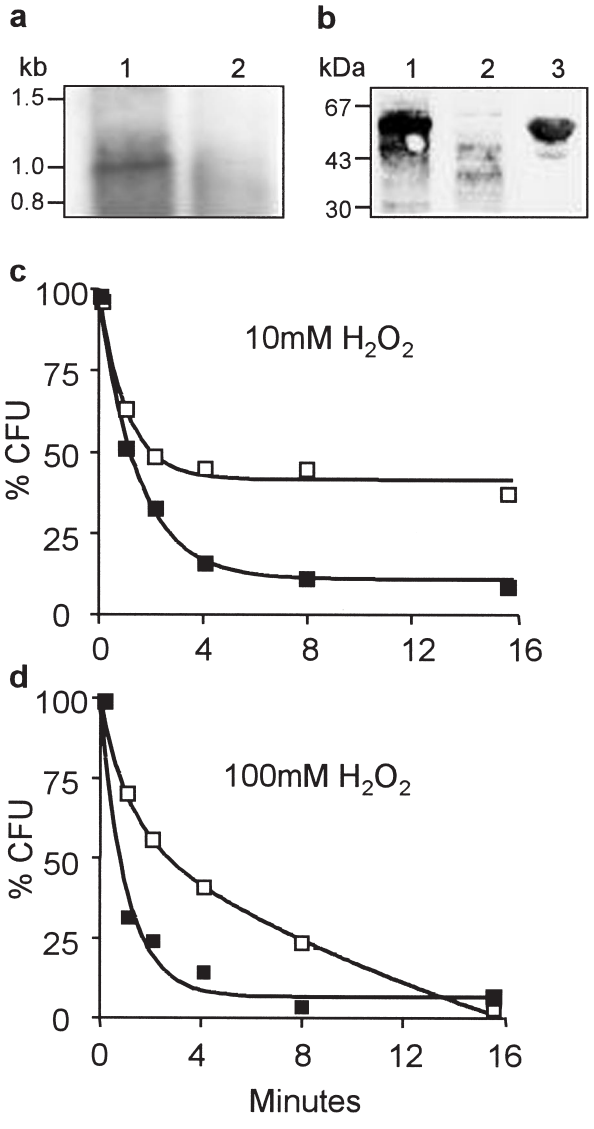

Fig. 1. Characterisation of catalase positive and negative strains of *S. aureus*. a: Southern blot of total genomic DNA, digested with *Nco*I and *Hin*dIII and probed with *S. aureus* catalase gene, yielded a 1 kb band in cat (+ve) (lane 1) but not in cat (−ve) organisms (lane 2). b: Western blot of cell lysates from cat (+ve) (lane 1) or cat (−ve) (lane 2) bacteria was probed with a catalase specific antiserum. 1 μg of bovine liver catalase served as control (lane 3). c,d: cat (+ve) (□) and cat (−ve) (■) *S. aureus* were incubated in 10 mM (c) or 100 mM (d) H_2O_2 and their viability determined. All results are the mean ± S.E.M. of at least three measurements.

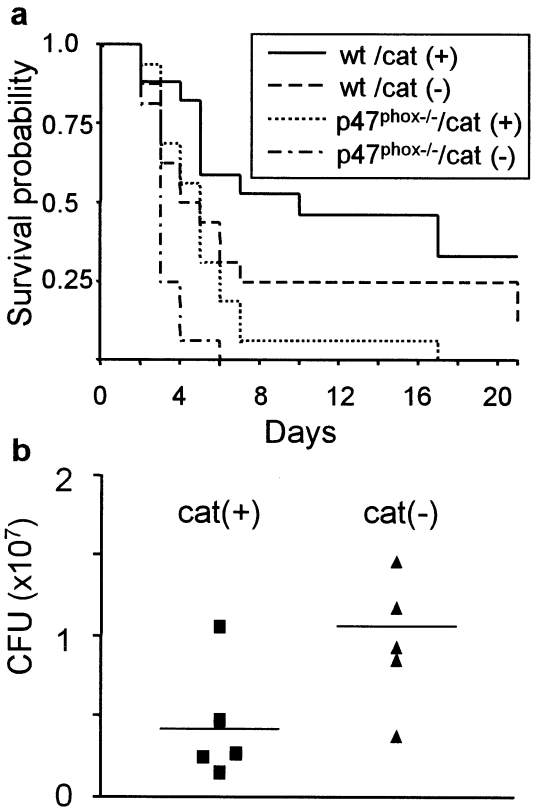

Fig. 2. Susceptibility of wild-type and $p47^{phox}$ deficient mice to infection with *S. aureus*. a: Survival probability plot (Kaplan–Meier) of mice injected intravenously with 4×10^7 viable *S. aureus* (16 mice in each group). Survival was decreased in the $p47^{phox-/-}$ mice infected with cat (+ve) ($P=0.005$) and cat (−ve) ($P=0.011$) strains. In the wild-type the survival was not significantly different after infection with the two strains ($P=0.15$). However the $p47^{phox-/-}$ mice were more susceptible to the cat (−ve) organisms ($P=0.0037$) b: Bacterial load in kidneys of $p47^{phox-/-}$ mice 48 h after infection with *S. aureus* cat (+ve) or cat (−ve). Horizontal line depicts mean number of CFU. Significantly more cat (−ve) organisms survived than cat (+ve), ($n=6$, $P=0.038$).

3. Results

The *S. aureus* catalase negative NCTC 12035 strain (cat (−ve)) was shown to be completely defective in catalase. Quantification of enzyme activity showed the catalase positive NCTC 12981 *S. aureus* (cat (+ve)) produced more than 300 IU of catalase activity per mg of protein extract (31.6 ± 0.5 IU), whereas no activity was detected in the cat (−ve) strain (< 10 IU/mg, $n=6$).

To see whether the absence of catalase activity was a result of a mutation or the absence of a gene, a PCR reaction was carried out employing primers, corresponding to bases 337–357 and 1524–1540. This gave a product of the expected size (1.2 kb) from cat (+ve) NCTC 12981. This product was partially sequenced and the sequence corresponded exactly with that of the *S. aureus* catalase gene, while no such product was detected in cat (−ve) NCTC 12035 (result not shown).

A Southern blot of restriction fragments obtained from total genomic DNA cut with *Nco*I and *Hin*dIII and probed with the 1.2 kb PCR product revealed a 1 kb band identified in cat (+ve) NCTC 12981 but not in cat (−ve) NCTC 12035, confirming the absence of the catalase gene (Fig. 1a). The absence of catalase protein in cat (−ve) NCTC 12035 was

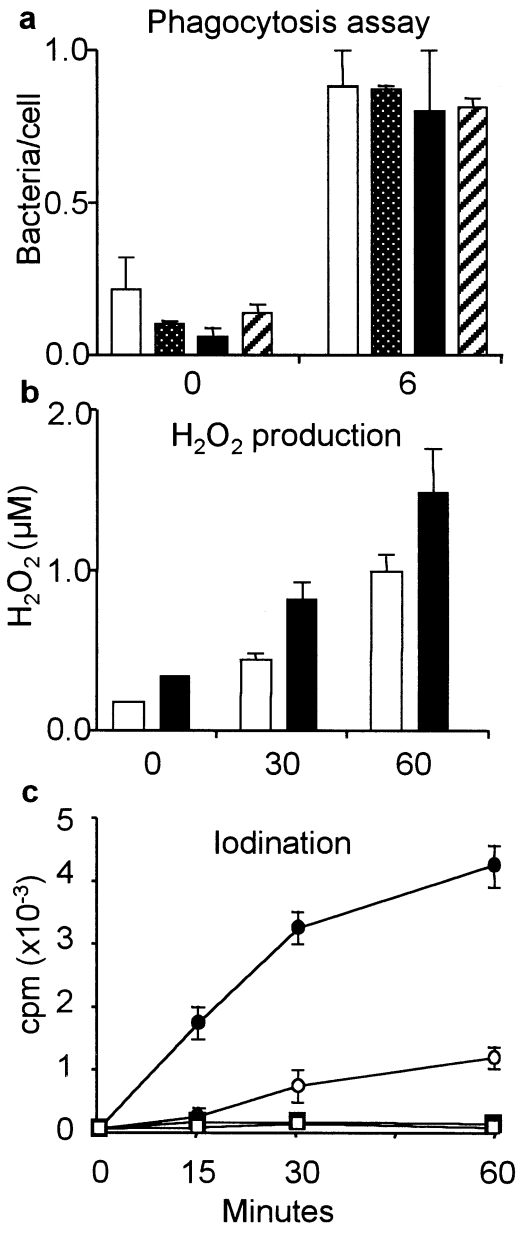

Fig. 3. Phagocytosis and iodination of, and H_2O_2 production by *S. aureus*. a: In vitro phagocytosis of live FITC-labeled *S. aureus* by neutrophils. At time 6 min, CGD ($p47^{phox-/-}$) neutrophils (■, hatched square) showed no significant difference ($P > 0.1$) from control wild-type (□, cross-hatched square) on the phagocytosis of cat (−ve) (□, ■) or cat (+ve) (hatched square, cross-hatched square) *S. aureus* (each point = mean ± S.E.M. of 100 measurements). b: Significantly more H_2O_2 was produced by cat (−ve) (■) than by cat (+ve) (□) *S. aureus* ($n = 8$, mean ± S.E.M., $P < 0.003$). c: Time course of iodination by peritoneal exudate cells from control (●, ○) or $p47^{phox}$ deficient mice (■, □) exposed to opsonised cat (+ve) (○, □) or cat (−ve) (●, ■) *S. aureus* (mean ± S.E.M. of at least three separate measurements).

confirmed by Western blot analysis (Fig. 1b) and lack of catalase activity led to increased sensitivity of *S. aureus* to the toxic effects of H_2O_2 added in vitro (Fig. 1c,d).

Control wild-type and CGD $p47^{phox}$ deficient mice ($p47^{phox-/-}$) were infected with cat (+ve) or cat (−ve) strains of *S. aureus*. The survival data (Fig. 2a) illustrate the increased susceptibility of $p47^{phox-/-}$ mice to infection. While control mice show a median survival time of 10 days, 50% of the mutants have succumbed to the infection with cat (+ve) *S. aureus* by day 5.

The CGD mice were shown to be susceptible to the two strains of *S. aureus*, as shown by the shortened median survival time of either group. Bacterial counts in the kidneys after 2 days revealed that neither organism had been eliminated (Fig. 2b). If anything the $p47^{phox-/-}$ mice appeared to be more susceptible to the cat (−ve) organisms.

Both strains of bacteria were phagocytosed equally by both neutrophil cell types in vitro (Fig. 3a). H_2O_2 was produced by both organisms with slightly more being detected in the cat (−ve) bacteria (Fig. 3b). Phagocytosis of live organisms by normal mouse neutrophils resulted in iodination, which was higher with the catalase negative organisms (Fig. 3c), presumably because the catalase positive bacteria were able to partially degrade the H_2O_2 in the vacuole. Virtually no iodination was detected when the CGD cells phagocytosed either of the organisms, indicating that the H_2O_2 produced by the bacteria (Fig. 3b) was insufficient to act as substrate for significant MPO halogenation activity in the vacuole.

4. Discussion

The initial concept that the H_2O_2 produced by some microbes could result in their own destruction by acting as substrate for MPO-mediated halogenation seemed attractive. In previous studies a single strain of *S. aureus* [7] or *A. nidulans* [8] was modified to produce catalase deficient mutants. We show here, supporting the observation of others [8], that this theory is incorrect. The absence of catalase did not in itself make staphylococci any less virulent. The lack of bacterial catalase was seen to significantly increase the levels of iodination by neutrophils (Fig. 3c). However the absence of this enzyme per se does not lead to reduced virulence of *S. aureus* in $p47^{phox}$ deficient mice (Fig. 2).

In this study, unlike in the other two in which mutants of the same strain of organism were used, we have studied two totally unrelated clinical isolates. Because we found the catalase negative strain to be virulent it could be argued that virulence factors other than catalase could be responsible in these organisms. However the current view is that chlorination is a potent antimicrobial mechanism and that catalase negative organisms produce enough H_2O_2 to provide the substrate for their own destruction by this system. We demonstrate here that although the cat (−ve) organisms produce H_2O_2 and iodination is increased after they are phagocytosed, this is not the case

The suggestion that bacteria could produce H_2O_2 as substrate for iodination did not take into account the major differences in the amounts of H_2O_2 produced by bacteria and neutrophils. Bacteria produce about 170 pmol/10^8 organisms/min (Fig. 3b) whereas when the same number of particles are phagocytosed by neutrophils [14] approximately 100 nmol of H_2O_2 is produced. It is known from studies on patients with variant CGD [15], in whom residual amounts of NADPH oxidase are present, that 10% of normal oxidase levels are insufficient to cause efficient microbial killing. In this case the cells are producing approximately two orders of magnitude more H_2O_2 than would be generated by the bacteria. Thus the possibility that CGD phagocytes could utilise microbial H_2O_2 to generate significant microbicidal activity, by

MPO-mediated conversion into hypohalous acid, seems remote.

These results should be viewed in the light of recent work, which has thrown light on the relationship between the NADPH oxidase and microbial killing. It was shown that oxygen free radicals and MPO-mediated halogenation do not kill *S. aureus* or *Candida albicans*. The primary killing effectors are granule proteases, which are activated by hypertonic K^+ driven into the vacuole to compensate the charge produced across the membrane by the action of the oxidase [16]. This shows that the theory of reduced virulence of catalase negative organisms by producing the substrate for their own destruction does not have a sound theoretical basis, as has been demonstrated by the studies described here.

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