HOST RESPONSE TO INFECTION

Nitric oxide-induced potentiation of the killing of *Burkholderia cepacia* by reactive oxygen species: implications for cystic fibrosis

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Burkholderia (formerly *Pseudomonas*) *cepacia* has emerged as an important pulmonary pathogen in cystic fibrosis, and survives within the lung despite a vigorous neutrophil-dominated immune response. Nitric oxide (NO) contributes to the antimicrobial activity of reactive oxygen species in the normal lung, but recent evidence suggests that inducible NO synthase is not expressed in the airway epithelial cells of cystic fibrosis (CF) patients. This may explain the failure of the neutrophil response to eliminate *B. cepacia*. To test this hypothesis, the present study examined the combined effect of NO, superoxide and H₂O₂ against *B. cepacia*. There was no killing of a highly transmissible strain by either superoxide or NO alone, but their combination reduced the bacterial count by >1000-fold over 75 min. This bactericidal activity was not sensitive to addition of superoxide dismutase, but was abrogated completely by catalase, suggesting that NO and hydrogen peroxide were the bactericidal mediators. Increased killing by NO in combination with H₂O₂ was seen for seven of a further 11 strains examined. The lack of NO in the lungs of CF patients may contribute to the survival of *B. cepacia*.

Introduction

Cystic fibrosis (CF) is characterised by abnormal mucosal secretions at several sites. The dehydrated mucus in the lungs supports growth and chronic colonisation by bacterial pathogens, notably *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Another major characteristic is inflammation, with a massive infiltration of neutrophils into the lung. Indeed, the persistence of pulmonary bacterial infection despite a vigorous host response is a classical feature of CF disease [1].

In recent years *B. cepacia* has emerged as an important pulmonary pathogen in CF. There is evidence of person-to-person transmission of *B. cepacia* among CF patients and epidemic strains have emerged in North America and the UK [2, 3]. CF patients colonised with the same *B. cepacia* strain can have dramatically different responses, ranging from chronic asymptomatic carriage to a rapidly fatal decline in lung function. In either case, however, *B.*

cepacia persists in the lungs despite a vigorous neutrophil-dominated inflammatory response.

The principal antibacterial agents from neutrophils are reactive oxygen species and granule products such as the cationic peptide molecules called defensins. Interestingly, one of the few other patient groups susceptible to *B. cepacia* infection comprises those suffering from chronic granulomatous disease (CGD). These patients lack NADPH oxidase and are unable to generate an oxidative respiratory burst. Speert and co-workers have shown that phagocytes from CGD patients killed *P. aeruginosa* whereas *B. cepacia* is resistant [4]. These data indicate that *B. cepacia* is resistant to the cationic peptides of neutrophils and, therefore, that any killing by phagocytes must be mediated by reactive oxygen species. Several reports have indicated that nitric oxide (NO) contributes to the toxicity of reactive oxygen species, perhaps by combination with superoxide anions to form peroxynitrite [5–7]. Its role in the human lung is controversial, as resting human macrophages are reported not to express inducible nitric oxide synthase (iNOS); however, macrophages from patients with inflammatory conditions or infectious diseases consistently do express iNOS [7] and recent work indicates NO production by neutrophils during phagocytosis [8]. The major source of iNOS and NO in the lung is

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likely to be the airway epithelium, where iNOS is expressed constitutively [9]. Studies measuring NO and its breakdown products in CF patients have yielded conflicting data, indicating increased nitrite in sputum [10, 11], a decrease in exhaled NO [12], or no difference in exhaled NO between CF patients and controls [13, 14]. Very recently, it has been reported that iNOS may not be expressed in the bronchial epithelium of CF patients and this deficiency may contribute to the susceptibility of these patients to bacterial colonisation [15, 16].

This study examined the effect of NO generated *in vitro* on the sensitivity of *B. cepacia* strains to killing by superoxide and H₂O₂.

Materials and methods

Bacterial strains and culture conditions

B. cepacia strains J2315 and J2552 were obtained from Professor J. R. W. Govan (University of Edinburgh). *B. cepacia* J2315 is a highly virulent strain belonging to genomovar III, and has been associated with person-to-person transmission in several centres in the UK and North America. All the other strains were either from the Birmingham (UK) CF clinic or our culture collection. Cultures were grown in succinate minimal medium [17] supplemented with casamino acids (Difco Laboratories) 0.1% w/v at 37°C until they reached an OD₄₇₀ of 0.8 late in the logarithmic phase. The bacteria were harvested by membrane filtration, washed and suspended in M9 salts solution [18] to an OD₄₇₀ of 1.0, corresponding to 1×10^9 cfu/ml.

Generation of reactive oxygen and nitrogen species

Superoxide (O₂⁻) was generated from a mixture comprising 10 mM hypoxanthine and xanthine oxidase (Sigma) 100 mU/ml in M9 salts solution. Its generation was confirmed by the reduction of ferricytochrome C [19]. The NO donor compound *S*-nitroso-*N*-acetyl penicillamine (Calbiochem, Nottingham) was dissolved in dimethyl sulphoxide to produce a 500 mM stock solution immediately before use; 8.82 M H₂O₂, human haemoglobin, bovine erythrocyte superoxide dismutase and bovine liver catalase were all purchased from Sigma.

Bacterial killing assays

B. cepacia cells were added to the reactive oxygen and nitrogen-generation systems to a final density of 1×10^7 cfu/ml. Samples were removed over a 90-min period and put into an inactivation medium comprising catalase 10 000 U/ml in M9 salts solution. A minimum of a 1 in 10 dilution in inactivation medium was performed. After appropriate serial dilution in this inactivation medium, 0.1-ml samples were spread in

triplicate on nutrient agar plates, which were incubated for 48 h at 37°C. Statistical significances of difference between groups were determined by Student's paired *t* test on log₁₀ count data or by two-way analysis of variance followed by the Student-Newman-Keules procedure for multiple comparison of group means.

Results

A cell-free system was chosen to model the role of the reactive oxygen species released during the 'respiratory burst' by activated neutrophils and macrophages, but in the absence of other neutrophil granule products. Time-course studies indicated no killing of *B. cepacia* J2315 by O₂⁻ alone over 90 min (Fig. 1). The NO-generating compound *S*-nitroso-*N*-acetyl penicillamine (5 mM) alone similarly exhibited no antibacterial activity, whereas their combination was rapidly bactericidal (Fig. 1). Addition of haemoglobin 1.5 mg/ml completely abrogated this bactericidal effect, whereas addition of superoxide dismutase 5000 U/ml had no effect (Fig. 1). The activity of the superoxide dismutase was confirmed by its ability to inhibit reduction of ferricytochrome C by O₂⁻ (data not shown).

Following the failure of superoxide dismutase to reduce killing, the effects of catalase 10 000 U/ml

Fig. 1. Survival of *B. cepacia* J2315 treated with 10 mM hypoxanthine/xanthine oxidase 0.1 U/ml (◆), 5 mM *S*-nitroso-*N*-acetyl penicillamine (□), 10 mM hypoxanthine/xanthine oxidase 0.1 U/ml + 5 mM *S*-nitroso-*N*-acetyl penicillamine (■), 10 mM hypoxanthine/xanthine oxidase 0.1 U/ml + 5 mM *S*-nitroso-*N*-acetyl penicillamine + superoxide dismutase 5000 U/ml (●) and 10 mM hypoxanthine/xanthine oxidase 0.1 U/ml + 5 mM *S*-nitroso-*N*-acetyl penicillamine + haemoglobin 1.5 mg/ml (▲). Data are representative of three similar experiments.

and 5 mM H_2O_2 were examined (Fig. 2). Catalase completely abolished the killing mediated by O_2^- /NO mixtures, suggesting that the antibacterial activity is *via* peroxide and NO. H_2O_2 (5 mM) alone was not antibacterial, whereas its combination with 5 mM *S*-nitroso-*N*-acetyl penicillamine achieved a >1000-fold decrease in cell viability over 30 min (Fig. 2). The effects of H_2O_2 in combination with NO on a further 11 *B. cepacia* strains are shown in Fig. 3; in no case did NO alone achieve any killing (data not shown). A CGD isolate (strain 32) and an environmental isolate (J2552) belonging to genomovar I were killed so efficiently by H_2O_2 alone that it was not possible to measure any synergic killing with NO; otherwise, only strains 34 and 40 (both from CF patients) failed to show any significant synergic killing between hydrogen H_2O_2 and NO.

Discussion

B. cepacia is an important pulmonary pathogen in CF patients. Several survival strategies have been proposed for the organism in this patient group, including resistance to non-oxidative neutrophil defence mechanisms [4] and invasion of respiratory epithelial cells [20].

The present study showed that NO could potentiate the toxicity of H_2O_2 , which is one of the major

neutrophil-derived reactive oxygen species. Coupled with the recent observation that there is little or no iNOS activity in CF airway epithelial cells [15], these results may partly explain the predilection of *B. cepacia* for the lungs of CF patients. It is noteworthy that the only other major group of patients susceptible to *B. cepacia* infection comprises those with CGD, whose neutrophils do not mount an oxidative respiratory burst. These data are consistent with the proposal that reactive oxygen and nitrogen species must both be present for successful eradication of the bacterium.

The nature of the reactive antibacterial species formed is unclear. Data from the present study suggest that the combined effects of NO and H_2O_2 are more important than products formed by reaction between NO and O_2^- , such as peroxynitrite ($ONOO^-$). The failure of superoxide dismutase to abrogate the antibacterial activity of O_2^- and NO could be attributed to the faster reaction kinetics between O_2^- and NO than that between O_2^- and superoxide dismutase [21]; however, the high concentrations of exogenous superoxide dismutase used in this study would be sufficient to compete with NO for O_2^- and argue against O_2^- being the cytotoxic species. Pacelli *et al.* [22] also found that NO greatly potentiated O_2^- -mediated killing of *Escherichia coli*, and that this killing was abolished by catalase but not by superoxide dismutase. Other in-vitro studies have failed to demonstrate synergy between O_2^- and NO; indeed, NO

Fig. 2. Survival of *B. cepacia* J2315 treated for 30 min with (1) 10 mM hypoxanthine/xanthine oxidase 0.1 U/ml; (2) 5 mM *S*-nitroso-*N*-acetyl penicillamine; (3) 10 mM hypoxanthine/xanthine oxidase 0.1 U/ml + 5 mM *S*-nitroso-*N*-acetyl penicillamine; (4) 10 mM hypoxanthine/xanthine oxidase 0.1 U/ml + 5 mM *S*-nitroso-*N*-acetyl penicillamine + haemoglobin 1.5 mg/ml; (5) 10 mM hypoxanthine/xanthine oxidase 0.1 U/ml + 5 mM *S*-nitroso-*N*-acetyl penicillamine + superoxide dismutase 5000 u/ml; (6) 10 mM hypoxanthine/xanthine oxidase 0.1 U/ml + 5 mM *S*-nitroso-*N*-acetyl penicillamine + catalase 10 000 U/ml; (7) 5 mM H_2O_2 ; (8) 5 mM H_2O_2 + 5 mM *S*-nitroso-*N*-acetyl penicillamine. Control cells held in buffer did not show any loss in viability over the 30-min period. Data are mean log₁₀ surviving fraction and SEM from three independent experiments, each performed in triplicate. Significant differences between treatments: **p* < 0.05; ***p* < 0.01.

Fig. 3. Survival of a panel of *B. cepacia* strains treated for 30 min with 5 mM H₂O₂ (■) and 5 mM H₂O₂ + 5 mM *S*-nitroso-*N*-acetyl penicillamine (□). Data are mean log₁₀ surviving fraction and SEM from three independent experiments, each performed in triplicate. *Significantly reduced compared with cells treated with hydrogen peroxide alone, $p < 0.05$; ** $p < 0.01$.

decreased O₂⁻-mediated killing of *Staphylococcus aureus* at early time points and only enhanced or stabilised killing over prolonged periods of incubation [23]. Whilst numerous in-vivo studies have demonstrated that iNOS and NO contribute to host defence, there are reports of improved outcomes when NOS inhibitors were administered, which have been attributed to the inflammatory effects of NO [7].

To date, there are no reports of the contribution of NO to the survival or killing of *B. cepacia* in the CF mouse model. Ex-vivo experiments reported low recovery of *P. aeruginosa* following inoculation of 500 cfu into the lungs of wild-type mice, whereas there was a 20-fold greater recovery from CF (Δ F508/ Δ F508) mice [16]. The low recovery from normal lungs was abolished by administration of a NOS inhibitor. Similarly, Gosselin *et al.* [24] found that administration of the NOS inhibitor, aminoguanidine, to BALB/c mice significantly increased the number of *P. aeruginosa* detectable in the lungs 3 days after infection. Both these experiments are consistent with the view that NO protects the lungs from infection.

The targets for the antibacterial activity of NO and O₂⁻/H₂O₂ are poorly defined, but candidates include the oxidation of sulphhydryl groups and lipids, transition metals, DNA damage and nitration of tryptosines. *S*-Nitrosothiol compounds, such as the *S*-nitroso-*N*-acetyl penicillamine used in this study, are conventionally viewed as NO donors and are believed to

achieve this effect by spontaneous homolytic cleavage, although heterolytic transfer of nitrosium to other sulphhydryl centres is also a possible mechanism [25]. The quenching of the *S*-nitroso-*N*-acetyl penicillamine effects by the NO scavenger, haemoglobin [26], supports the notion that NO itself is the key intermediary. Breakage of double-stranded DNA and glutathione depletion by NO have been reported in *E. coli* [22], and the workers responsible for this finding also noted that iron-cofactored superoxide dismutase was susceptible to inactivation by NO and H₂O₂, or H₂O₂ alone. Cytoplasmic superoxide dismutase activity is unlikely to protect against extracellular O₂⁻, which does not cross biological membranes. However, it may be a target for freely diffusible species such as NO and H₂O₂ and hence render cells susceptible to O₂⁻ generated within themselves. A recently described periplasmic superoxide dismutase with copper and zinc as cofactors protected *Salmonella typhimurium* [27] and *Neisseria meningitidis* [28] from the toxic effects of O₂⁻ generated outside the cell. Mutants lacking this enzyme showed attenuated virulence in animal models. However, periplasmic CuZn superoxide dismutase seems unlikely to have had any effect in the present study, as exogenously added superoxide dismutase failed to reduce O₂⁻ toxicity. We are currently examining catalase levels in the strains used, particularly those that failed to be killed by NO and H₂O₂. Preliminary experiments have demonstrated marked evolution of gas from the resistant strains JL34 and 40 on addition of H₂O₂, an intermediate response from strain J2315 and very little gas

production from strains JL32 and J2552. These data suggest that differences in sensitivity to H_2O_2 are inversely correlated with catalase activity.

In summary, the study showed that NO acts synergistically with reactive oxygen species to kill *B. cepacia* *in vitro*. The observation that iNOS activity may be absent from cells in CF patients provides insight into why this intriguing pathogen survives within the lung despite a vigorous neutrophil-dominated host response.

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References

- Ramsey BW. Drug therapy – management of pulmonary disease in patients with cystic fibrosis. *N Engl J Med* 1996; **335**: 179–188.
- Govan JRW, Brown PH, Maddison J *et al.* Evidence for transmission of *Pseudomonas cepacia* by social contact in cystic fibrosis. *Lancet* 1993; **342**: 15–19.
- Sun L, Jiang R-Z, Steinbach S *et al.* The emergence of a highly transmissible lineage of *cbl*⁺ *Pseudomonas* (*Burkholderia*) *cepacia* causing CF centre epidemics in North America and Britain. *Nat Med* 1995; **1**: 661–666.
- Speert DP, Bond M, Woodman RC, Curnutte JT. Infection with *Pseudomonas cepacia* in chronic granulomatous disease: role of nonoxidative killing by neutrophils in host defense. *J Infect Dis* 1994; **170**: 1524–1531.
- Zhu L, Gunn C, Beckman JS. Bactericidal activity of peroxynitrite. *Arch Biochem Biophys* 1992; **298**: 452–457.
- Brunelli L, Crow JP, Beckman JS. The comparative toxicity of nitric oxide and peroxynitrite to *Escherichia coli*. *Arch Biochem Biophys* 1995; **316**: 327–334.
- MacMicking J, Xie Q-W, Nathan C. Nitric oxide and macrophage function. *Annu Rev Immunol* 1997; **15**: 323–350.
- Evans TJ, Buttery LDK, Carpenter A, Springall DR, Polak JM, Cohen J. Cytokine-treated human neutrophils contain inducible nitric oxide synthase that produces nitration of ingested bacteria. *Proc Natl Acad Sci USA* 1996; **93**: 9553–9558.
- Guo FH, De Raeve HR, Rice TW, Stuehr DJ, Thunnissen FB, Erzurum SC. Continuous nitric oxide synthesis by inducible nitric oxide synthase in normal human airway epithelium *in vivo*. *Proc Natl Acad Sci USA* 1995; **92**: 7809–7813.
- Linnane SJ, Keatings VM, Costello CM *et al.* Total sputum nitrate plus nitrite is raised during acute pulmonary infection in cystic fibrosis. *Am J Respir Crit Care Med* 1998; **158**: 207–212.
- Francoeur C, Denis M. Nitric oxide and interleukin-8 as inflammatory components of cystic fibrosis. *Inflammation* 1995; **19**: 587–598.
- Grasemann H, Michler E, Wallot M, Ratjen F. Decreased concentration of exhaled nitric oxide (NO) in patients with cystic fibrosis. *Pediatr Pulmonol* 1997; **24**: 173–177.
- Balfour-Lynn IM, Laverty A, Dinwiddie R. Reduced upper airway nitric oxide in cystic fibrosis. *Arch Dis Child* 1996; **75**: 319–322.
- Lundberg JON, Nordvall SL, Weitzberg E, Kollberg H, Alving K. Exhaled nitric oxide in paediatric asthma and cystic fibrosis. *Arch Dis Child* 1996; **75**: 323–326.
- Meng Q-H, Springall DR, Bishop AE *et al.* Lack of inducible nitric oxide synthase in bronchial epithelium: a possible mechanism of susceptibility to infection in cystic fibrosis. *J Pathol* 1998; **184**: 323–331.
- Kelley TJ, Drumm ML. Inducible nitric oxide synthase expression is reduced in cystic fibrosis murine and human airway epithelial cells: possible involvement in CF-related sodium hyperabsorption and susceptibility to bacterial infection. *J Clin Invest* 1998; **6**: 1200–1207.
- Meyer JM, Abdallah MA. The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. *J Gen Microbiol* 1978; **107**: 319–328.
- Miller JH. Experiments in molecular genetics. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press. 1972.
- Pick E, Mizel D. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *J Immunol Methods* 1981; **46**: 211–226.
- Burns JL, Jonas M, Chi EY, Clark DK, Berger A, Griffith A. Invasion of respiratory epithelial cells by *Burkholderia* (*Pseudomonas*) *cepacia*. *Infect Immun* 1996; **64**: 4054–4059.
- Beckman JS, Chen J, Ischiropoulos H, Crow JP. Oxidative chemistry of peroxynitrite. *Methods Enzymol* 1994; **233**: 229–240.
- Pacelli R, Wink DA, Cook JA *et al.* Nitric oxide potentiates hydrogen peroxide-induced killing of *Escherichia coli*. *J Exp Med* 1995; **182**: 1469–1479.
- Kaplan SS, Lancaster JR, Basford RE, Simmons RE. Effect of nitric oxide on staphylococcal killing and interactive effect with superoxide. *Infect Immun* 1996; **64**: 69–76.
- Gosselin D, DeSanctis J, Boulé M, Skamene E, Matouk C, Radzioch D. Role of tumor necrosis factor alpha in innate resistance to mouse pulmonary infection with *Pseudomonas aeruginosa*. *Infect Immun* 1995; **63**: 3272–3278.
- Fang FC. Mechanisms of nitric oxide-related antimicrobial activity. *J Clin Invest* 1997; **99**: 2818–2825.
- Rioux F, Petitclerc E, Audet R, Drapeau G, Fielding RM, Marceau F. Recombinant human hemoglobin inhibits both constitutive and cytokine-induced nitric oxide-mediated relaxation of rabbit isolated aortic rings. *J Cardiovasc Pharmacol* 1994; **24**: 229–237.
- Farrant JL, Sansone A, Canvin JR *et al.* Bacterial copper- and zinc-cofactored superoxide dismutase contributes to the pathogenesis of systemic salmonellosis. *Mol Microbiol* 1997; **25**: 785–796.
- Wilks KE, Dunn KRL, Farrant JL *et al.* Periplasmic superoxide dismutase in meningococcal pathogenicity. *Infect Immun* 1998; **66**: 213–217.