

Polymorphonuclear leucocytes consume oxygen in sputum from chronic *Pseudomonas aeruginosa* pneumonia in cystic fibrosis

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ABSTRACT

Background: Chronic lung infection with *Pseudomonas aeruginosa* is the most severe complication for patients with cystic fibrosis (CF). This infection is characterised by endobronchial mucoid biofilms surrounded by numerous polymorphonuclear leucocytes (PMNs). The mucoid phenotype offers protection against the PMNs, which are in general assumed to mount an active respiratory burst leading to lung tissue deterioration. An ongoing respiratory burst by the PMNs has, however, not been demonstrated previously in endobronchial secretions from chronically infected patients with CF.

Objective: Based on the accumulating evidence for depletion of molecular oxygen (O₂) in the mucus in infected CF bronchi, it was hypothesised that the O₂ depletion in the mucus in infected CF bronchi may be accelerated by the respiratory burst of the PMNs due to the reduction of O₂ to the superoxide anion (O₂⁻) by the phagocyte NADPH oxidase (Phox).

Methods: Methods were established to isolate the O₂ consumption by the respiratory burst from aerobic respiration in freshly expectorated sputum from chronically infected patients with CF.

Results: Inhibition of the Phox with diphenylene iodonium (DPI) delayed O₂ depletion, nearly abolished staining of O₂-producing PMNs with hydroethidine and inhibited the rapid luminol-enhanced chemiluminescence in sputum. Furthermore, the total O₂ consumption was correlated to the concentration of PMNs in the sputum samples.

Conclusion: The results demonstrate that CF sputum contains PMNs with an active consumption of O₂ for O₂ production and suggest that the respiratory burst is ongoing and causes accelerated O₂ depletion due to formation of O₂⁻ in the lungs of chronically infected patients with CF.

Chronic lung infection with *Pseudomonas aeruginosa* is the most severe complication in patients with cystic fibrosis (CF).¹ The thick viscous endobronchial mucus hampers mucociliary clearance, which renders the CF lungs susceptible to chronic infections.² The chronic *P aeruginosa* lung infection in patients with CF is characterised by endobronchial mucoid biofilms surrounded by numerous polymorphonuclear leucocytes (PMNs).^{3,4} In spite of the bactericidal mechanisms of the PMNs and aggressive antibiotic treatments, the biofilms persist. *P aeruginosa* may obtain protection against the PMNs by formation of mucoid biofilm.¹ Thus, instead of clearing the bacteria the PMNs are suspected to promote progressive lung tissue deterioration by releasing proteolytic enzymes

and reactive oxygen species (ROS) responsible for premature death in CF.¹⁻⁵ Even though PMNs with evidence of priming and phagocytic activity have been isolated from CF airways, the activity of PMNs in the lungs of patients with CF with chronic *P aeruginosa* lung infection is far from being resolved.⁵ Interestingly, anaerobic conditions in the mucus have recently been demonstrated in non-obstructed bronchi of patients with CF with chronic *P aeruginosa* lung infection.³ The cause of this O₂ depletion may be O₂ consumption by the epithelia and by *P aeruginosa*.³ We hypothesised that the respiratory burst of the PMNs contributes to the O₂ consumption in the mucus of the infected CF airways. The interaction between bacteria and the PMNs may increase O₂ consumption as a consequence of the extra respiration during phagocytosis.⁶ This process is caused by the respiratory burst resulting from the one-electron reduction of O₂ by the phagocyte NADPH oxidase (Phox) to O₂⁻.⁷ To evaluate the capability of the respiratory burst to deplete O₂, we constructed a simple reaction chamber that allowed real-time measurement of the O₂ concentration during phagocytosis of *P aeruginosa* by PMNs. The respiratory burst was isolated from aerobic respiration by blocking the respiratory chain with KCN, which previously has been employed to demonstrate the resistance of the respiratory burst to inhibition of the aerobic respiration in PMNs.⁸ The aerobic respiratory component was exposed by inhibiting the Phox with diphenylene iodonium (DPI). These in vitro findings were utilised to estimate the O₂ consumption and the ROS production by the respiratory burst of the PMNs in freshly expectorated sputum from patients with CF with chronic *P aeruginosa* lung infection.

METHODS

Sputum samples

The study was performed on leftover sputum expectorated for routine bacteriology from 29 patients with CF with chronic *P aeruginosa* infections after obtaining permission by informed consent.

Chronic *P aeruginosa* infection was defined as the presence of *P aeruginosa* in the lower respiratory tract at each monthly culture for ≥6 months, or for a shorter time in the presence of increased antibody response to *P aeruginosa* (≥2 precipitating antibodies, normal: 0–1).⁹ Before measurement of luminol-enhanced chemiluminescence (LEC) and dissolved O₂, the samples were brought into

Table 1 Characteristics of the 29 patients with cystic fibrosis

Characteristic	
Sex (M/F)	15/14
Age (years)*	32 (22–47)
Duration of chronic infection (years)*	19 (1–35)
FEV ₁ (%)*	48 (23–117)
FVC (%)*	89 (58–135)
During intravenous antibiotic treatment	21
In between intravenous antibiotic treatment	8

*Values are medians (range).

F, female; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; M, male.

homogenous suspensions by gentle resuspension with a pipette. Normoxic conditions were achieved by diluting 10 times with Krebs–Ringer buffer equilibrated in ambient air. The concentration of leucocytes in the diluted sputum was determined as previously described.^{10–11} The estimation of the frequency of PMNs is described in online supplementary fig 5.

Preparation of PMNs

Human blood samples were obtained from normal healthy volunteers by venous puncture and the PMNs were isolated by gradient sedimentation and centrifugation, as previously described, before suspending the PMNs in Krebs–Ringer buffer with 10 mM glucose and 5% (v/v) normal human AB-positive sera at a density of 5×10^9 PMNs/L.^{10–11}

Bacteria

Inoculum for phagocytosis by PMNs was prepared from exponentially growing cultures of the widely studied *P aeruginosa* wild-type strain (PAO1) (online supplementary fig 1).

Estimation of the respiratory burst by LEC

The LEC from aliquots of 100 µl of isolated PMNs or sputum was determined as outlined in online supplementary fig 3.

The concentration of dissolved O₂

Measurement of dissolved O₂ in sputum and during phagocytosis is described in online supplementary fig 2.

Visualisation of O₂ production

Hydroethidine (HE) (D7008, Sigma, St Louis, Missouri, USA) at 1 µM was added to 100 µl of sputum and the samples were incubated for 15 min at 37°C before examination with a fluorescence microscope (BX40, Olympus, Ballerup, Denmark). To verify the origin of O₂, sputum was pretreated for 10 min with 50 µM DPI before addition of HE. The interaction between PMNs and bacteria in the sputum samples was visualised by adding HE (1 µM) together with the green permeable DNA dye SYTO 9 (Molecular Probes, Eugene, Oregon, USA) (10 µM), and the samples were incubated for 15 min at 37°C before examination.

Statistical methods

Statistical significance was evaluated by two-way analysis of variance (ANOVA) with Bonferroni post-tests for observations with parametric data. Non-parametric data were analysed by Wilcoxon signed rank test and Spearman rank correlation test. A p value <0.05 was considered statistically significant. The tests were performed with Prism 4.0c (GraphPad Software, La Jolla, California, USA).

RESULTS

O₂ consumption by PMNs and *P aeruginosa* during phagocytosis

Estimations of the O₂ consumption during phagocytosis were initiated at atmospheric O₂ concentration. Accelerated initial O₂ consumption with a reduced O₂ consumption rate at low O₂ concentrations was seen when mixing PMNs with PAO1 (fig 1A). Nevertheless, the O₂ concentration decreased below the level of detection (0.01 mg O₂/l). This was considered as an indication of anaerobic conditions, since similar recordings were obtained in buffer equilibrated in an anaerobic bench with growing strict anaerobes as control. Low O₂ consumption by unstimulated PMNs was also seen (fig 1A). Evidence of phagocytosis was provided by the reduced number of surviving *P aeruginosa* in the presence of PMNs (online supplementary fig 1) and by the green fluorescent protein (GFP)-tagged *P aeruginosa* being ingested by or attached to the PMNs in the reaction chamber (online supplementary fig 2).

The O₂ consumption of *P aeruginosa* resulted in anaerobic conditions within 2 h (fig 1B) and indicated that *P aeruginosa* may consume a considerable part of the O₂ during phagocytosis.

Inhibition of the respiratory burst with DPI during phagocytosis of *P aeruginosa* by PMNs

Treatment with 50 µM DPI reduced the chemiluminescence, which predominantly reflects O₂ production during phagocytosis, to the level of unstimulated PMNs for up to 50 min (online supplementary fig 3A). Thus, inhibition of the respiratory burst with 50 µM DPI during phagocytosis resulted in reduced O₂ consumption and prevention of anaerobic conditions (fig 1A). Since treatment with 50 µM DPI did not affect bacterial O₂ consumption (fig 1B), growth, and survival after phagocytosis (online supplementary fig 1) significantly, 50 µM DPI was selected for estimating the O₂ consumption by the respiratory burst of the PMNs.

Inhibition of the respiratory chain with KCN during phagocytosis of *P aeruginosa* by PMNs

Unexpectedly, the LEC was increased (online supplementary fig 3B) without affecting the O₂ concentration (fig 1A) during phagocytosis inhibited with KCN. This could not be ascribed to *P aeruginosa* since the bacteria ceased to consume O₂ shortly after addition of 2 mM KCN (fig 1B). Instead, the surprisingly high O₂ consumption and LEC during KCN treatment may result from a higher amount of O₂ being available for the respiratory burst due to the bacteria failing to consume O₂. In addition, KCN repression of aerobic respiration may decrease bacterial motility and facilitate capture of *P aeruginosa* by the PMNs, resulting in a stronger respiratory burst. Though KCN treatment may overestimate the O₂ consumption by the respiratory burst, it was decided to use 2 mM KCN for the study of sputum. Lower concentrations may be insufficient to block the pseudomonal cyanide-independent terminal oxidase.¹²

O₂ consumption by the respiratory burst in sputum from patients with CF with chronic *P aeruginosa* lung infection

To ensure similar initial O₂ concentrations, the expectorated sputum was diluted 10 times in Krebs–Ringer buffer equilibrated in ambient air. O₂ consumption was observed in all sputum samples by the decreasing O₂ concentration, and anaerobic conditions were established in 10 of the 23 sputum samples (fig 2A). The chemiluminescence was inhibited >95% after 10 min of treatment with DPI whereas KCN treatment had no effect (online supplementary fig 4). Thus, the sputum

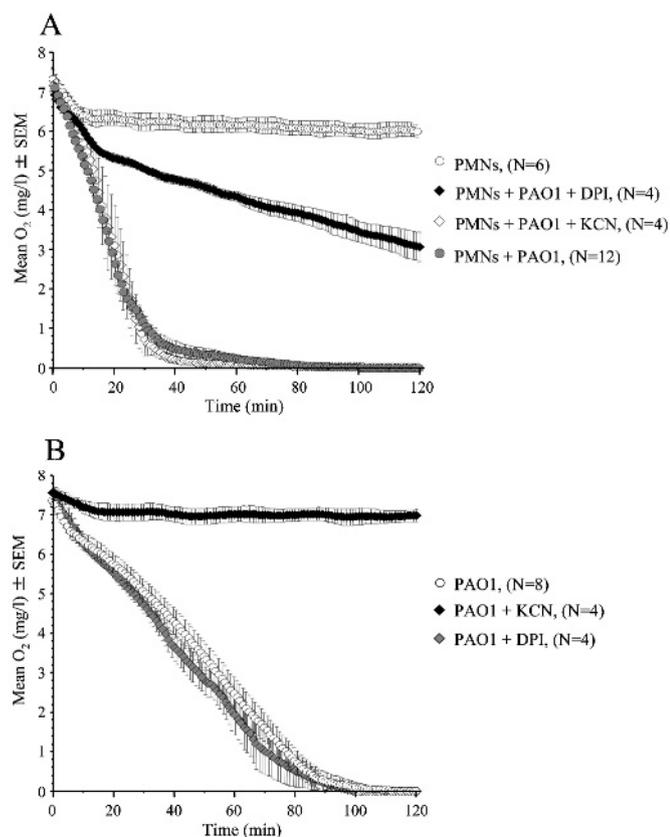


Figure 1 O₂ consumption by the respiratory burst and aerobic respiration in the reaction chamber. (A) Increased O₂ consumption during phagocytosis ($p < 0.001$), inhibition of the respiratory burst during phagocytosis by treatment with 50 μM diphenylene iodonium (DPI) ($p < 0.001$), and unchanged O₂ consumption during phagocytosis by treatment with 2 mM KCN during phagocytosis of strain PAO1 (15×10^9 /l) by normal polymorphonuclear leucocytes (PMNs) (5×10^9 /l). (B) Decreased O₂ consumption by PAO1 (15×10^9 /l) during treatment with 2 mM KCN ($p < 0.001$) and unaffected bacterial O₂ consumption during treatment with 50 μM DPI. Statistical analysis was performed by two-way analysis of variance.

was pretreated with DPI and KCN for 10 min to estimate the O₂ consumption rate during the following 30 min before the O₂ was depleted. The O₂ consumption rate was reduced by DPI treatment, indicating an active respiratory burst in the sputum samples, and resulted in a mean inhibition of the O₂ consumption rate by 56% (SD 20%) (fig 2B), suggesting that the respiratory burst consumed the major part of the O₂. Active O₂ consumption by the respiratory chain was also observed by the reduced O₂ consumption rate caused by addition of KCN (fig 2C). The consequence of inhibiting the respiratory burst with DPI was a delayed generation of anaerobic conditions (fig 2D) whereas inhibition of the respiratory chain with KCN was insufficient to delay the depletion of O₂ significantly. DPI treatment allowed us to detect comparable levels of the O₂ consumption rate by the burst per PMN in CF sputum and in the phagocytosis experiment using normal PMNs and PAO1. In the sputum we found 1.7 nmol O₂/10⁶ PMNs/min (SD 1.6 nmol O₂/10⁶ PMNs/min) and in the experimental phagocytosis assay we found 1.5 nmol O₂/10⁶ PMNs/min (SD 0.4 nmol O₂/10⁶ PMNs/min). These data make it unlikely that the O₂ consumption rate of sputum PMNs is a CF-specific phenomenon.

PMNs with respiratory burst in sputum from patients with CF with chronic *P aeruginosa* lung infection

The mean fraction of PMNs constituted 90% (7%) (SD) of the host cells in the sputum samples. Therefore, the concentration of PMNs was compared with the O₂ consumption rate in the diluted sputum samples. A positive correlation was observed, supporting the involvement of PMNs in the O₂ consumption (fig 3A). Positive correlations were also observed between the concentration of colony-forming units (CFUs) and the O₂ consumption rate (fig 3B) and between the concentration of PMNs and the concentration of CFUs (fig 3C). No significant correlation was observed between the O₂ consumption rate and forced expiratory volume in 1 s (FEV₁) or forced vital capacity (FVC).

To avoid unintended effects of the Krebs–Ringer buffer, the respiratory burst in undiluted sputum samples was estimated by LEC. As in diluted sputum, DPI treatment resulted in >95% inhibition after 10 min in undiluted sputum (fig 4A), indicating an active respiratory burst that persisted during the 2 h of observation. Furthermore, the LEC was resistant to KCN, suggesting an origin that may function independently of the respiratory chain such as the Phox of the PMNs.⁸ To verify further the PMNs as the source of ROS in sputum, O₂ production was visualised by staining with HE, which is oxidised by O₂ to generate 2-hydroxyethidium that emits intensified red fluorescence when complexing with DNA.¹⁵ In the untreated sputum, several fluorescent objects were seen at low magnification. Higher magnification revealed these objects to be PMNs (fig 4B). In DPI-treated sputum, only faint fluorescence was seen (fig 4C). By applying the validated HE to fresh, expectorated sputum together with the permeable DNA stain, SYTO 9, we found bursting PMNs with ingested planktonic bacteria (fig 4D) and bursting PMNs failing to engulf bacteria from a biofilm (fig 4E).

DISCUSSION

PMNs in bronchoalveolar lavage fluids from chronically infected patients with CF and in sputum from healthy individuals have the potential to mount a respiratory burst upon stimulation.^{14 15} In contrast, the presence of PMNs with an active respiratory burst was demonstrated in fresh sputum from patients with CF with chronic *P aeruginosa* lung infection in this study. Both the inhibition of O₂ consumption and ROS generation with DPI as well as the correlation of the PMN concentration to the O₂ consumption rate are indications of an ongoing respiratory burst by the PMNs in sputum from chronically infected patients with CF. PMNs with an active respiratory burst were not demonstrated directly in the lungs of the patients in this study. Therefore, our findings do not necessarily reflect the situation of all PMNs in the endobronchial mucus of infected CF lungs since the pO₂ may differ at various sites in the sputum plugs. However, the rapid, high chemiluminescence, observed in undiluted sputum ex vivo, may exclude the possibility that adding Krebs–Ringer buffer, equilibrated to ambient air, causes the oxidative burst. In this context, the initial, high O₂ consumption and ROS generation seen in untreated sputum samples within 30 min after expectoration suggest that the respiratory burst of the PMNs was already active in the mucus in the lungs. Thus, our results indicate that the respiratory burst of the PMNs is the main contributor to the intensive O₂ depletion inside the mucus of the bronchi.³ The respiratory burst of the PMNs in the sputum may be activated by direct contact with *P aeruginosa*^{5 16} and by the binary signalling from the engagement of the integrins and binding to inflammatory cytokines.¹⁷ Interestingly, we have recently found levels of

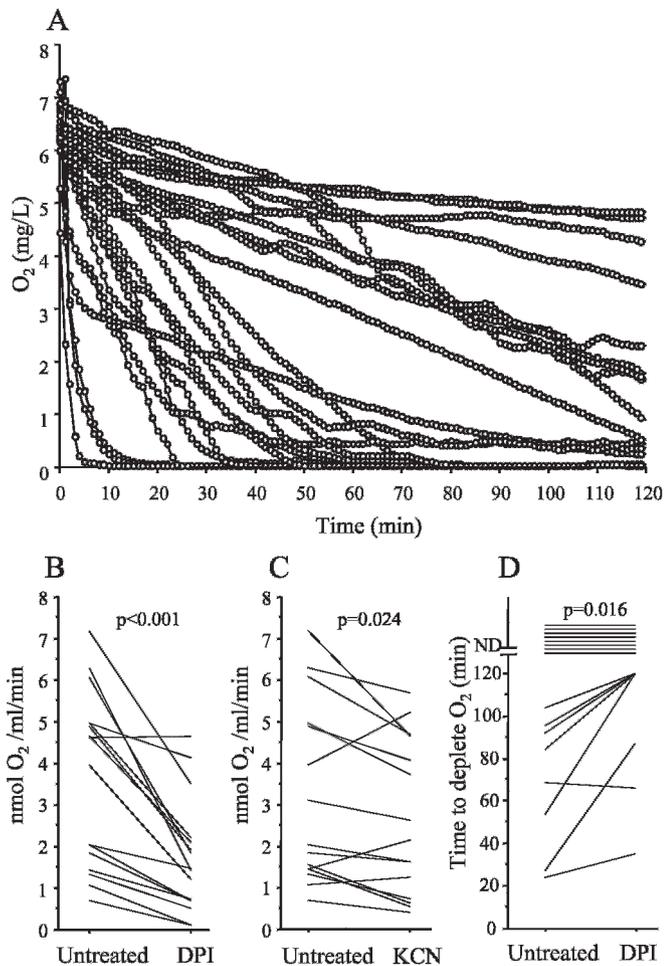


Figure 2 O_2 consumption and reactive oxygen species generation in diluted sputum from patients with cystic fibrosis with chronic *P aeruginosa* lung infection. (A) The decreasing O_2 concentration as a function of incubation time measured in the reaction chamber ($n = 23$). (B) Reduced O_2 consumption rate in sputum treated with 50 μ M diphenylene iodonium (DPI) ($n = 16$). (C) Reduced O_2 consumption rate in sputum treated with 2 mM KCN ($n = 15$). (D) Postponing of O_2 depletion in sputum treated with 50 μ M DPI. In B–D, data were analysed by Wilcoxon signed rank test.

tumour necrosis factor α (TNF α) with the potential to augment the respiratory burst in sputum from several infected patients with CF attending the Copenhagen CF Centre, as in the patients in the present study.¹⁸ In addition, comparable levels of TNF α in sputum from infected patients with CF are reported from other CF centres.¹⁹ Besides TNF α , platelet-activating factor, leukotriene B4, interleukin-8, lipopolysaccharide, antibodies and alginate have been suggested to be major triggers for the induction of the respiratory burst of airway PMNs.^{1, 5, 18, 19} In contrast, the respiratory burst of the PMNs may be repressed by quorum sensing-regulated secretion of rhamnolipid, by exoprotease from *P aeruginosa*, by antibiotic treatment and by the high levels of apoptotic or necrotic cells in the sputum samples.^{10, 11, 20–22} These opposing mechanisms may contribute to the variation observed in the correlation between the concentration of PMNs and the O_2 consumption in the sputum. Our results imply that at least in some parts of the CF mucus O_2 is present, which allows PMNs to create a respiratory burst. This is in accordance with the steep pO_2 gradients in CF mucus.⁵ Furthermore, based on the substantial fraction of PMNs able to create a significant respiratory burst found in CF sputum in this study, our previous demonstrations of

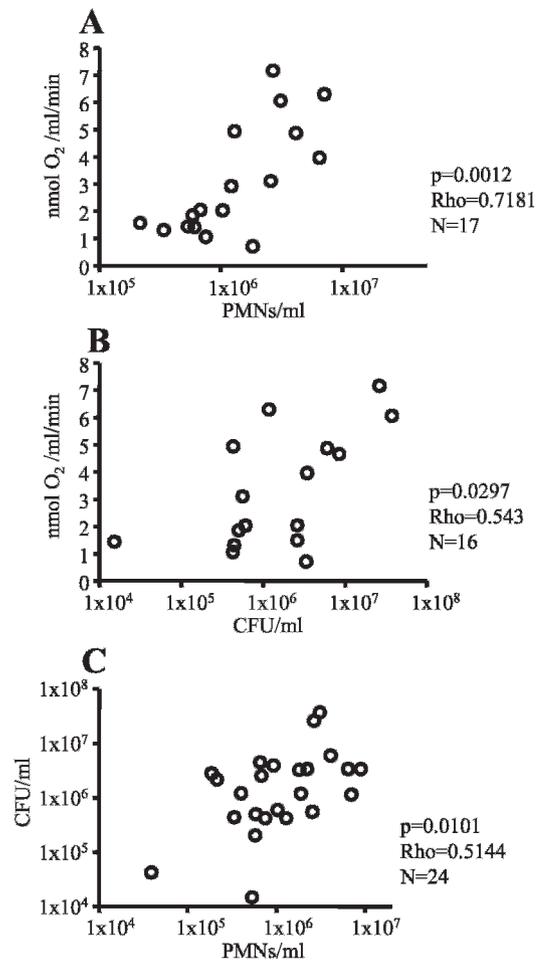


Figure 3 Correlations between the O_2 consumption rate, the concentration of polymorphonuclear leucocytes (PMNs) and colony-forming units (CFUs) in diluted sputum from patients with cystic fibrosis with chronic *P aeruginosa* lung infection. The O_2 consumption rate was determined in the reaction chamber, the concentration of PMNs was estimated by flow cytometry, and the CFUs were counted by plating of serial dilutions. (A) O_2 consumption rate vs the concentration of PMNs. (B) O_2 consumption rate vs the concentration of CFUs. (C) The concentration of PMNs vs the concentration of CFUs. Statistical analysis was performed by Spearman rank correlation test.

the toxic effects of *P aeruginosa* rhamnolipid on PMNs may not apply to the entire population of PMNs in the sputum.^{10, 11}

The demonstration of PMNs with an active respiratory burst in sputum from chronically infected patients with CF may have been anticipated due to the ROS lesions in the infected CF lungs.^{5, 23} However, it could not have been taken for granted. In fact, the ROS in the CF lungs may be derived from sources other than the Phox of the PMNs, including epithelial cells, alveolar macrophages, and from mitochondrial respiration.^{23–25} Due to the predominance of PMNs in the sputum samples the contribution of other cells to O_2 metabolism was regarded to have low impact on the results in this study. In contrast to the Phox, mitochondrial activity is unlikely to influence significantly our observations in the sputum samples. PMNs contain only few and poorly developed mitochondria with a low activity of the respiratory chain.²⁶ In addition, sputum from chronically infected patients with CF may contain *P aeruginosa* producing pyocyanine, which may also cause O_2 formation.²⁷ Thus, O_2 formation by pyocyanine may be a reason why DPI failed to block ROS generation completely in the sputum samples.

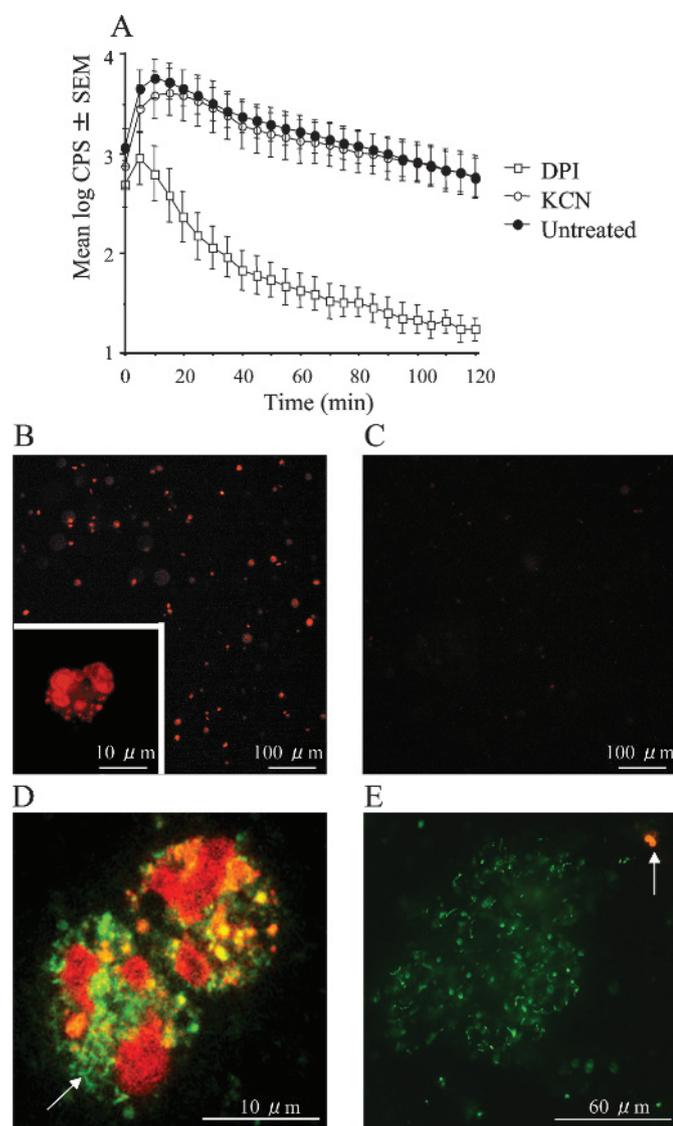


Figure 4 Reactive oxygen species (ROS) generation in sputum from patients with cystic fibrosis with chronic *P aeruginosa* lung infection. (A) The effect of treatment with KCN and inhibition with diphenylene iodonium (DPI) ($p < 0.001$) on ROS generation shown as counts per second (CPS) from luminol-enhanced chemiluminescence ($n = 10$). Statistical analysis was performed by two-way analysis of variance ($n = 10$). (B–E) Visualisation of O_2 production by fluorescence microscopy of sputum stained with hydroethidine. (B) Untreated sputum. (C) Sputum treated with $50 \mu\text{M}$ DPI to inhibit the phagocyte NADPH oxidase. (D) Polymorphonuclear leucocytes (PMNs) with ingested planktonic bacteria (arrow) with green fluorescence from SYTO 9. (E) A bursting PMN (arrow) engaged with a biofilm with bacteria emitting green fluorescence from SYTO 9.

Previously, faster O_2 depletion has been achieved when PMNs were added to *P aeruginosa* growing in experimental biofilms due to increased O_2 consumption of both the bacteria and the PMNs.¹⁶ However, in the present study the ability of the PMNs per se to create anaerobic conditions by an active respiratory burst was observed in the KCN-resistant O_2 depletion during the phagocytosis of PAO1 and in the sputum samples. This may, however, be expected, since PMNs are able to produce a substantial part of the energy needed for the respiratory burst by anaerobic glycolysis resulting in L-lactate formation.²⁸ Accordingly, our suggestion of an active respiratory burst by the PMNs in the CF lungs is supported by the increased glucose uptake in PMNs in CF lungs

and the high concentration of L-lactate in sputum from patients with CF with chronic *P aeruginosa* lung infection.^{29–30}

The demonstration of PMNs with an active respiratory burst in sputum samples from patients with CF with chronic *P aeruginosa* lung infection verifies the frequent assumption that the PMNs induce ROS lesions in the lungs. Sufficient O_2 to produce O_2^- by the respiratory burst of the PMNs in sputum may be supplied by the daily inhalation of 8000 litres of O_2 -rich air.²³ The result of the fast dismutation of O_2^- is H_2O_2 , which may lead to the formation of myeloperoxidase-derived oxidising species found in CF airways.⁵ Considering the short half-lives of the ROS generated by PMNs, the tissue adjacent to the mucus is most likely to be exposed to PMN-derived ROS. Therefore, ROS generated in the mucus may be a reason for the affected bronchi in patients with CF with chronic *P aeruginosa* lung infection.³¹ However, it is less obvious how ROS generated in the mucus may reach more distant lung tissue. Since the included patients with CF show a broad range of FEV₁ values, one may suggest that the O_2 consumption rate might be linked to pulmonary function in these patients. However, no significant correlation was observed between the O_2 consumption rate and FEV₁ or FVC. This may be due to the condition of the infected patients, which has been stabilised for years by elective antibiotic treatment, resulting in a slower decline of lung function and fewer exacerbations. Thus, the lung function of the included patients may primarily result from slowly accumulated deteriorations of the lung tissue rather than the ongoing inflammation in the endobronchial mucus. Our results provide arguments to control the extent of ROS by antibiotic treatment, since O_2 consumption was mainly caused by ROS formation of the PMNs and was correlated to the concentration of *P aeruginosa* in the sputum samples. The latter correlation probably results from a balanced accumulation of PMNs in response to the concentration of *P aeruginosa* in the sputum samples.

As indicated in fresh sputum samples, the ROS from the respiratory burst of the PMNs in the mucus may reach the bacteria, due to their ingestion by PMNs, with a respiratory burst and the intimate surrounding of the endobronchial biofilms by the PMNs.⁴ Though the killing of *P aeruginosa* by PMNs was apparently independent of ROS formation in this study, the surviving bacteria may experience ROS lesions, which may affect virulence. In particular, PMN-derived ROS may cause mutations in the *muca* gene, leading to formation of the crucial mucoid phenotypes.³² In fact, mutations in the *muca* gene were found in the majority of the mucoid isolates from 91 Scandinavian patients with chronic *P aeruginosa* infection.³³ In addition, the PMN-derived ROS may induce highly mutagenic lesions in the bacterial DNA that are associated with resistance to antibiotics and accumulation of hypermutable isolates.³⁴

The accelerated O_2 depletion by the PMNs probably affects the O_2 distribution in the endobronchial mucus. Though the precise O_2 profile of the endobronchial mucus has not been determined, the activity of the PMNs is sensitive to the local O_2 tension. In particular, the accumulation of PMNs in the lung secretions may be increased due to the postponing of apoptosis by hypoxia.³⁵

Sputum from chronically infected patients with CF may also contain biomarkers for the bacterial response to O_2 depletion such as slow growth and cyanide secretion.^{12–36}

This study demonstrates an ongoing respiratory burst of the PMNs in sputum from patients with CF with chronic *P aeruginosa* lung infection that may induce environmental changes in the lungs with the potential to generate virulent and resistant bacterial phenotypes, to facilitate colonisation by anaerobic bacteria and to cause oxidative lesions in the lung tissue of the patients.

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