Interference with the oxidative response of neutrophils by *Streptococcus pneumoniae*

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Abstract

Background—Pneumococcal infections are still a major clinical problem. Polymorphonuclear leucocytes (neutrophils) are considered to have a key role in the host's defence against *Streptococcus pneumoniae* but the mechanisms by which they kill the pneumococcus remain unclear. As reactive oxygen species are regarded as a major antimicrobial defence of phagocytes an attempt has been made to establish their role in the response of neutrophils to *S pneumoniae*.

Methods—*S pneumoniae* isolated from patients with bacteraemic pneumococcal pneumonia were incubated with neutrophils in suspension and superoxide production was measured by reduction of ferricytochrome c.

Results—*S pneumoniae* did not stimulate superoxide production alone or in the presence of normal human serum. Spontaneous superoxide production by neutrophils was actually abrogated by *S pneumoniae*, as was the powerful respiratory burst stimulated by phorbol myristate acetate. This phenomenon depended on both the dose and the viability of the bacteria. With *S pneumoniae* in the logarithmic phase of growth inhibitory activity was confined to the organisms themselves but with organisms undergoing autolysis it was also present in filtered supernatants, suggesting that the inhibitory activity can be attributed to a factor released during autolysis.

Conclusions—*S pneumoniae* can interfere with the respiratory burst of neutrophils. This property may help to explain the pathogenicity of the organism.

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Although penicillin has greatly reduced the number of deaths from pneumococcal infection, *Streptococcus pneumoniae* remains a major cause of morbidity and mortality. Pneumococcal pneumonia, for example, is the most common form of community acquired pneumonia[1] and still has an overall mortality of 5–13%,[2] though patients who survive almost invariably make a full recovery without detectable lung damage.[3] In pneumococcal meningitis the mortality is 30%.[4]

Furthermore, the worldwide increase in penicillin resistance and multiple antibiotic resistance among pneumococci[5-8] and the limited use of pneumococcal vaccine[9] suggest that pneumococcal infection may cause increasing morbidity and mortality in the future.

In the host's defence against *S pneumoniae* polymorphonuclear leucocytes (neutrophils) are considered to have a key role. Immunological defences against *S pneumoniae* include the production of specific anticapsular antibody and activation of complement, both of which function opsonically.[10-12] Complement is not lytic[11] and studies with nude mice have indicated that T cells are not necessary for normal host defence.[13] These results, and the finding that *S pneumoniae* is killed by phagocytes in vitro,[14] suggest the primacy of phagocytosis as an effector defence mechanism. The mechanisms by which phagocytes kill the pneumococcus, however, are incompletely understood. These mechanisms are of increasing clinical importance for two main reasons. Firstly, various cytokines, including interferon gamma, tumour necrosis factor, granulocyte-macrophage colony stimulating factor, and interleukin-8, have been shown to enhance the function of neutrophils;[15-18] thus the potential now exists for modulating microbial killing by neutrophils. Secondly, the reactive oxygen species that neutrophils produce during microbial killing have been implicated in causing tissue damage,[19] including lung damage,[20] which has led to the investigation of a therapeutic role for antioxidants.[21] If oxidative mechanisms are crucial for killing antioxidants may predispose patients to pneumonia. On the other hand, inhaled microorganisms that stimulate neutrophils to produce large amounts of toxic oxygen species may contribute to lung damage.

Previous studies of microbial killing by phagocytes have indicated the importance of reactive oxygen species. For example, the susceptibility of *Toxoplasma gondii* and *Leishmania donovani* promastigotes to killing by phagocytes has been shown to be related to their ability to trigger release of oxygen species[22 23] and the killing of *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus viridans* by neutrophils has been shown to be inhibited by scavengers of reactive oxygen species.[24] It is not known, however, whether *S pneumoniae* is killed by reactive oxygen species or whether it stimulates phagocytes to generate an oxidative response. The purpose

of the current work was thus to determine whether *S pneumoniae* stimulates or suppresses the ability of neutrophils to generate superoxide.

Methods

NEUTROPHILS

Peripheral blood was collected from healthy volunteers attending the outpatients department (mainly for elective surgery) at Bristol Royal Infirmary. Blood was obtained by venepuncture and collected in 3·8% sodium citrate. For each experiment blood was pooled from four volunteers. Neutrophils were isolated by dextran sedimentation followed by centrifugation through Percoll (Sigma), with a modification of the method of Dooley *et al.*[25] The cells obtained were washed and resuspended in phosphate buffered saline with 4% citrate at 5 × 10⁶/ml. Permission for taking blood from healthy volunteers was provided by the Bristol and Weston District ethical committee and informed consent was obtained from all patients.

STREPTOCOCCUS PNEUMONIAE

Encapsulated *S pneumoniae* organisms of types 1 and 14 were obtained from patients with bacteraemic pneumococcal pneumonia and stored in 1 ml aliquots at −70°C. Before use *S pneumoniae* were grown in Todd Hewitt broth with 10% newborn calf serum for three to four hours so that they were in the logarithmic phase of growth (log phase organisms). The bacteria were collected by centrifugation at 2200 *g* for 10 minutes, washed twice, and resuspended in phosphate buffered saline and the concentration was adjusted to 10 × 10⁷/ml. Their total concentration was determined by a Helber chamber count and the count of viable organisms was determined by serial dilution and plating on to blood agar by the method of Miles and Misra.[26] In some experiments *S pneumoniae* were used that had been grown for 18–24 hours to the autolysis phase of growth (autolysis phase organisms).

THERMAL KILLING

In some experiments *S pneumoniae* organisms were heat killed before use by incubation in a water bath at 60°C for 30 minutes. The efficacy of this procedure was confirmed by the lack of growth observed 48 hours after the heat treated pneumococci had been plated on to blood agar.

REAGENTS

Ferricytochrome c (type III), superoxide dismutase (type I, 300 U/mg protein), and phorbol myristate acetate were obtained from Sigma, Poole. The phorbol myristate acetate was dissolved in dimethylsulphoxide at 1 mg/ml and stored in 10 μl aliquots at −20°C. The cytochrome c and superoxide dismutase were dissolved in phosphate buffered saline citrate, either made fresh on the day of the experiment or stored at −20°C.

SUPEROXIDE DETECTION

The amount of superoxide released by neutrophils either spontaneously or in response to phorbol myristate acetate or *S pneumoniae* was measured by the microassay of Pick and Mizel,[27] which uses superoxide dismutase inhibitable reduction of ferricytochrome c. The reaction mixture (100 μl) consisted of 160 μmol cytochrome c, 1·25 × 10⁵ neutrophils, and the appropriate stimulus (*S pneumoniae* or 1 μg/ml phorbol myristate acetate), with or without 300 U/ml superoxide dismutase. Six or eight replicates of each condition were set up in vertical rows of a 96 well, flat bottomed, tissue culture plate (Nunc, Roskilde, Denmark). The plate was covered and placed in a humidified incubator gassed with 95% air and 5% carbon dioxide for 90 minutes. The plate was then read on a Multiskan MCC/340 plate reader (Titertek) fitted with a 550 nm wavelength interference filter. The amount of superoxide (O_2^-) produced per well was calculated from the formula:[27]

$$\text{nmoles } O_2^- \text{ per well} = \frac{(\text{absorbance at 550 nm} \times 100)}{6·51}.$$

CHEMICAL REDUCTION OF CYTOCHROME C

Cytochrome c solution was reduced with sodium dithionite and the reductant removed by passage over a sephadex G-25 column. Its concentration was determined spectrophotometrically and adjusted to the desired starting concentration. Oxidation of reduced cytochrome c was measured by an adaptation of the superoxide assay. The reaction mixture (100 μl/well) consisted of reduced cytochrome c and *S pneumoniae* (final concentration 2 × 10⁷/ml). The plate was read at the beginning and end of the 90 minute incubation and the amount of cytochrome c oxidised was calculated in the same way as superoxide production.

MEASUREMENT OF SUPEROXIDE DISMUTASE ACTIVITY

Superoxide dismutase activity associated with *S pneumoniae* was measured according to the method of Metcalf *et al*,[28] in which the ability of known concentrations of superoxide dismutase to inhibit superoxide catalysed reduction of cytochrome c is used to measure its activity in unknown samples. Superoxide is generated by the oxidation of xanthine by xanthine oxidase. To establish the standard curve the following were added to a cuvette: 0·675 ml 20 mmol/l Na_2CO_3, 0·1 mmol/l EDTA, 1 mmol/l NaN_3; 0·1 ml 50 μmol/l xanthine; 0·1 ml 5 μmol/l ferricytochrome c; 0·1 ml superoxide dismutase standard (0·025–1·0 U/ml). Xanthine oxidase 0·025 ml 1 U/ml was added to start the reaction and the reduction of cytochrome c was monitored at 550 nm for five minutes by means of a spectrophotometer linked to a chart recorder. The linear regression was established for the standards. The superoxide dismutase was then substituted with 0·1 ml *S pneumoniae* suspension (final concentration 15 × 10⁶/ml) or 0·1 ml *S pneumoniae* culture supernatant,

Table 1   Oxidative response of neutrophils (PMNs) to Streptococcus pneumoniae (mean(SD) values for five experiments*)

	nmol $O_2^-/1.25 \times 10^5$ PMNs/90 min
Neutrophils + PMA	4.07 (0.62)
Neutrophils alone	0.84 (0.62) ⎫ p < 0.01
Neutrophils + S pneumoniae	0.02 (0.09) ⎭

*Each experiment had six or eight replicates per condition. PMA—phorbol acetate; O_2^-—superoxide.

the change in absorbance at 550 nm measured over five minutes, and the superoxide dismutase activity determined from the linear regression.

STATISTICAL ANALYSIS
Each experiment had either six or eight replicates per condition. For comparisons between experiments the mean value of the replicates was used. Results are expressed as
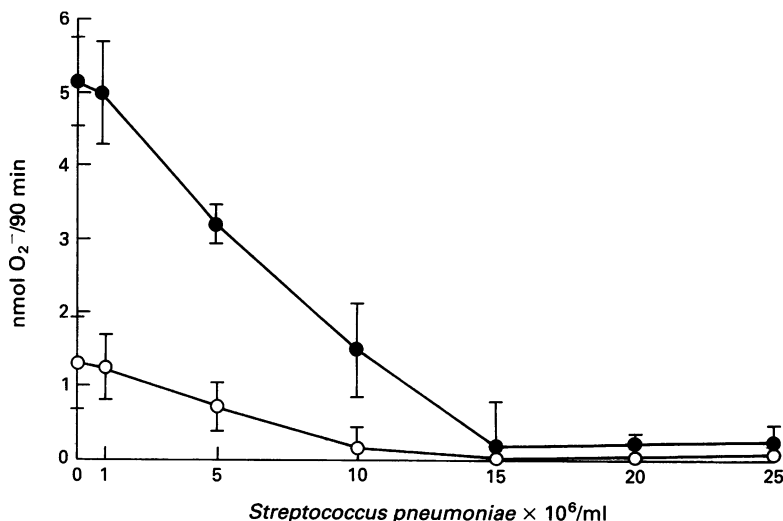
the mean and standard deviation (SD) of the combined experiments. Unless otherwise stated, the Behrens-Fisher test was used to assess differences between means, $p < 0.05$ being considered significant.

Results
OXIDATIVE RESPONSE OF NEUTROPHILS TO
S PNEUMONIAE
The results of incubating neutrophils with either type 1 *S pneumoniae* at a ratio of 1:20 or phorbol myristate acetate are shown in table 1. As expected, phorbol myristate acetate stimulated a powerful respiratory burst, generating on average 4.07 nmol of superoxide per well over 90 minutes, about five times more than was generated spontaneously by neutrophils alone. In contrast, neutrophils exposed to *S pneumoniae* (in the absence of serum) generated significantly less superoxide than neutrophils alone. The experiments were repeated in the presence of normal human serum but again the oxidative response of neutrophils exposed to *S pneumoniae* in the presence of serum was less than that of neutrophils alone: neutrophils alone 1.06 (0.61), neutrophils + *S pneumoniae* 0.05 (0.02), neutrophils + *S pneumoniae* + 0.6–20% normal human serum 0.00 (0.00)–0.17 (0.21) nmol $O_2^-/1.25 \times 10^5$ neutrophils/90 min (n = 3).

Figure 1   Inhibition of neutrophil superoxide production by increasing concentrations of type 1 Streptococcus pneumoniae. The results are the means and standard deviations from four experiments. Closed symbols denote the oxidative response of neutrophils to phorbol myristate acetate in the presence of different concentrations of S pneumoniae. Open symbols denote oxidative response of neutrophils to S pneumoniae alone.

EFFECT OF *S PNEUMONIAE* ON PHORBOL MYRISTATE ACETATE INDUCED OXIDATIVE RESPONSES
In six experiments neutrophils stimulated with phorbol myristate acetate alone generated 4.47 (0.62) nmol of superoxide per 1.25×10^5 neutrophils over 90 minutes, whereas neutrophils stimulated with both phorbol myristate acetate and type 1 *S pneumoniae* generated only 0.20 (0.17) nmol of superoxide per 1.25×10^5 neutrophils over 90 minutes. This reduction was highly significant (p < 0.01).
Similar results were obtained with type 14 *S pneumoniae*: neutrophils + phorbol myristate acetate 4.06 (0.45), neutrophils + phorbol myristate acetate + type 14 *S pneumoniae* 0.72 (0.45) nmol $O_2^-/90$ min (n = 5; p < 0.01).
Experiments were set up to determine whether the inhibition of phorbol myristate acetate induced superoxide production depended on the dose and viability of the pneumococci. As can be seen from fig 1, the greatest amounts of superoxide were observed in the absence of *S pneumoniae* (far left). As the concentration of *S pneumoniae* increased the levels of superoxide detected fell, total inhibition of superoxide production being seen at 15×10^6 ml⁻¹.
Figure 2 shows that this inhibitory effect also depends on the viability of *S pneumoniae*. Viable pneumococci inhibited both phorbol myristate acetate induced and spontaneous superoxide production by neutrophils whereas heat killed *S pneumoniae* failed to inhibit either at any dose.
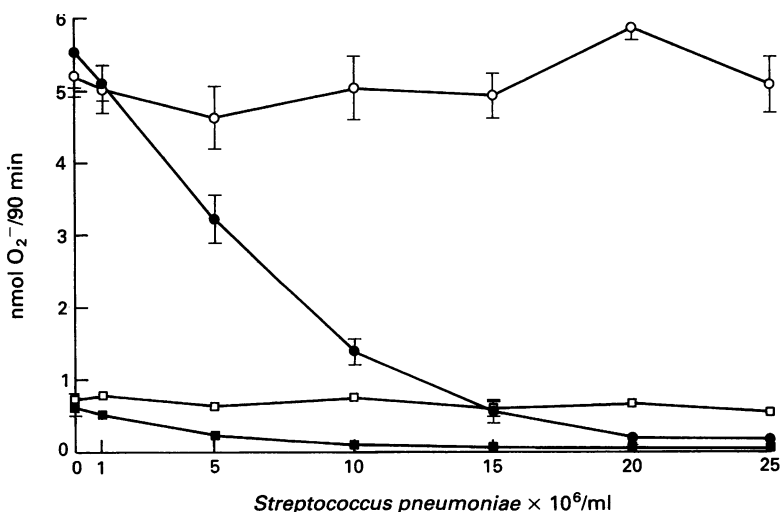

Figure 2   Effect of heat treatment on the ability of type 1 Streptococcus pneumoniae to inhibit superoxide production. The circles denote the oxidative response of neutrophils to phorbol myristate acetate in the presence of viable (closed circles) or heat killed (open circles) S pneumoniae. The squares denote the spontaneous oxidative response of neutrophils to viable (closed squares) or heat killed (open squares) S pneumoniae.
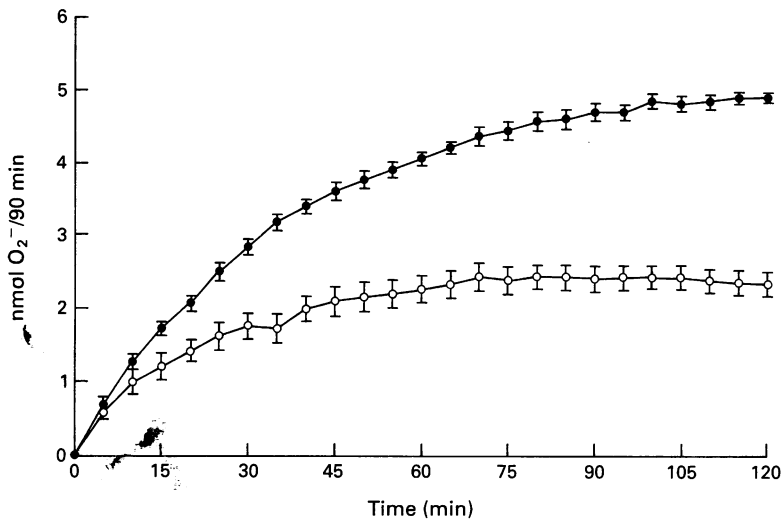
Figure 3   Time course of inhibition of neutrophil production of superoxide by Streptococcus pneumoniae. Closed circles denote superoxide release by neutrophils in response to phorbol myristate acetate alone; open circles denote superoxide release by neutrophils in response to both phorbol myristate acetate and S pneumoniae.

The results described above refer to end point assays (at 90 minutes). An incubation time of 90 minutes was chosen because the response of neutrophils to phorbol myristate acetate was essentially complete at this point and the superoxide detection system appeared to be stable. To determine how *S pneumoniae* affects the kinetics of superoxide production by neutrophils the time course of phorbol myristate acetate stimulated production of superoxide and its inhibition by type 1 *S pneumoniae* was investigated (fig 3). As can be seen, inhibition was observed throughout the period studied and was evident at the first time point monitored (5 minutes).

Table 2   Effect of Streptococcus pneumoniae culture supernatants on the oxidative response of phorbol myristate acetate stimulated neutrophils (PMNs)

	nmol $O_2^-/1.25 \times 10^5$ PMNs/90 min	
Supernatant treatment	Log phase supernatant	Autolysis phase supernatant
Todd-Hewitt broth (control)	5·07 (0·70)	4·52 (0·64)
Untreated	2·47 (1·70)*	1·51 (1·11)**
Filtered	4·79 (1·06)	2·21 (1·07)**
Centrifuged (16 000 g × 2 min)	5·04 (0·54)	2·69 (0·83)*
Autolysis phase S pneumoniae		3·33 (2·46)

*p < 0·05; **p < 0·01 in comparison with control. No other results were significantly different from control values. O_2^-—superoxide.

Table 3   Ability of Streptococcus pneumoniae to oxidise reduced cytochrome c in the presence and absence of catalase (mean (SD) values for six experiments)

Initial concentration of reduced cytochrome c (nmol)	3·97 (0·73)
Spontaneous oxidation (nmol/90 min)	0·67 (0·23)
Oxidation (nmol/90 min)	
by S pneumoniae	
Type 1	3·79 (1·42)
Type 14	3·70 (1·51)
by S pneumoniae + 50 µg/ml catalase	
Type 1	0·67 (0·37)**
Type 14	0·68 (0·33)**

**p < 0·01 compared with oxidation in the absence of catalase.

The viability (as judged by trypan blue exclusion) of neutrophils incubated with *S pneumoniae* remained greater than 98%, the same as that of neutrophils incubated alone.

To determine whether any superoxide dismutase produced by *S pneumoniae* might interfere with the detection of superoxide anions, the superoxide dismutase activity in *S pneumoniae* was measured and the effect of this concentration of superoxide dismutase on superoxide production by neutrophils investigated. Both washed *S pneumoniae* and *S pneumoniae* supernatants each contained less than 0·1 U/ml superoxide dismutase activity. This dose had no effect on spontaneous superoxide detection and reduced phorbol myristate acetate stimulated detection of superoxide by an average of only 8% (n = 3).

The ability of *S pneumoniae* culture supernatants to exert the inhibitory effect was also investigated (table 2). Whereas untreated supernatants possessed substantial inhibitory activity, this was lost if the supernatants were either filtered (through a 0·2 µm bacterial filter) or centrifuged, suggesting that the inhibitory activity of log phase supernatants depends on the presence of viable *S pneumoniae* within the supernatant. Supernatants from 18 hour (autolysis phase) cultures were also examined and again showed inhibitory activity (table 2). In contrast to the log phase of growth, filtering or centrifuging did not remove the activity. *S pneumoniae* from 18 hour cultures varied widely in their ability to inhibit stimulation of superoxide by phorbol myristate acetate but overall showed less inhibitory activity than log phase organisms.

Experiments were set up to determine whether *S pneumoniae* can oxidise reduced cytochrome c. The results are presented in table 3. *S pneumoniae* was able to oxidise reduced cytochrome c but the oxidation did not show the same dose dependency as inhibition of neutrophil superoxide production since all but the lowest concentration of *S pneumoniae* (1 × 10^6/ml) oxidised cytochrome c to the same extent. A further difference between the ability of *S pneumoniae* to oxidise cytochrome c and inhibit superoxide production was that both filtered supernatants from log phase cultures and heat killed organisms retained their ability to oxidise reduced cytochrome c. As it has been reported that hydrogen peroxide (H_2O_2) can reoxidise cytochrome c[29] and H_2O_2 is a product of *S pneumoniae*,[30] the effect of the H_2O_2 scavenger catalase on the ability of *S pneumoniae* to oxidise cytochrome c was examined. It was found that 50 µg/ml catalase blocked the reoxidation of cytochrome c by *S pneumoniae* (table 3). Experiments were then set up to determine the effect of catalase on the ability of *S pneumoniae* to inhibit superoxide production by neutrophils (table 4). As can be seen, catalase only partially removed the inhibitory effect of *S pneumoniae* on neutrophil superoxide detection. The reduction was significant (p < 0·05) for type 1 but not for type 14 (paired *t* test)—in contrast to the effect of

Table 4 Effect of catalase on the ability of Streptococcus pneumoniae types 1 and 14 to inhibit superoxide (O_2^-) production by phorbol myristate acetate stimulated neutrophils (mean (SD) values for seven (type 1) and 6 (type 14) experiments)

	Type 1	Type 14
Inhibition (nmol O_2^-/90 min)		
by S pneumoniae	2·02 (1·25) ⎱ p < 0·05	3·02 (0·52) ⎱ NS
by S pneumoniae + 50 μg/ml catalase	0·96 (0·74) ⎰	2·34 (0·78) ⎰
Removal of inhibition by catalase (%)	54·0 (31·0)	23·5 (28·6)

Inhibition = nmol O_2^- generated by neutrophils + phorbol myristate acetate (with or without catalase) − nmol O_2^- generated by neutrophils + phorbol myristate acetate + S pneumoniae (with or without catalase).

catalase on reoxidation. Thus whereas catalase blocked reoxidation almost completely it only partially removed the inhibitory effect of S pneumoniae on production of superoxide by neutrophils.

Discussion

These results show that S pneumoniae isolated from patients with bacteraemic pneumococcal pneumonia interferes with the respiratory burst of neutrophils. S pneumoniae appear to inhibit not only the spontaneous production of superoxide by neutrophils but also the strong respiratory burst stimulated by phorbol myristate acetate. The effect was seen with two different serotypes, which are among the major causative organisms of serious pneumococcal infections,[29] and depended on both the concentration and the viability of the organisms.

It could be argued that S pneumoniae merely kills neutrophils, either rendering them unable to mount a respiratory burst or causing release of neutrophil products that might interfere with the assay. The pneumococci were, however, shown not to affect the viability of the neutrophils.

Another possible explanation for the results was that the S pneumoniae organisms were interfering with the assay by reoxidising cytochrome c. We found that S pneumoniae was capable of oxidising chemically reduced cytochrome c, indicating a pitfall of this method we have not seen discussed elsewhere. Blocking reoxidation did not, however, abrogate the inhibitory effect of S pneumoniae because the organisms retained about 50% (type 1) and over 75% (type 14) of their capacity to inhibit the respiratory burst of neutrophils when reoxidation was blocked by catalase.

Thus S pneumoniae exerts an inhibitory effect on the oxidative response of neutrophils, suggesting that they must either scavenge superoxide or inhibit its production. The production of antioxidant enzymes or scavenging substrates is a recognised defence tactic of microorganisms against phagocytic oxidative activity.[31] Endogenous production of superoxide dismutase and catalase, for example, has been described in many bacterial species and for certain of these, including Staphylococcus aureus, Listeria monocytogenes and Nocardia asteroides, the virulence of different strains correlates with their superoxide

dismutase or catalase production.[31-33] We examined the possibility that S pneumoniae produces superoxide dismutase as this could destroy any superoxide produced. Although S pneumoniae samples and supernatants were shown to possess superoxide dismutase activity the amount produced had minimal effect on the respiratory burst of neutrophils. Whether S pneumoniae possesses other substrates that might scavenge superoxide remains to be elucidated.

The second possibility is that S pneumoniae prevents the generation of reactive oxygen species. Such inhibitory activity has been described for Legionella micdadei,[34] Aspergillus fumigatus,[35] Histoplasma capsulatum,[36] and Yersinia pestis[37] and is thought to be associated with their pathogenicity. Inhibition of the respiratory burst of neutrophils is also one of the effects of purified pneumolysin, a toxin produced by S pneumoniae.[38] Experiments were set up to determine whether the inhibitory activity was associated with the pneumococci themselves or with a released factor. Untreated log phase supernatants inhibited both spontaneous and neutrophil stimulated superoxide production; treatments that removed any residual bacteria from the supernatant, however, also removed the inhibitory activity. By contrast, the inhibitory activity present in the supernatants of S pneumoniae cultures in the autolysis phase of growth was not removed by filtration or centrifugation, suggesting that it can be attributed to a soluble factor released during autolysis. As pneumolysin is predominantly a product of autolysis phase S pneumoniae,[39] and, in purified form, is known to inhibit the respiratory burst of neutrophils, it may be the factor responsible for the inhibitory activity that we have observed. Results to be published elsewhere, however, show that a pneumolysin negative strain of S pneumoniae and its autolysis phase supernatant are also able to inhibit the respiratory burst of neutrophils, showing that pneumococci must produce an inhibitory factor other than pneumolysin.

The ability of S pneumoniae to interfere with the respiratory burst of neutrophils may constitute a major counter defence of the microorganism and help to explain its pathogenicity. Moreover, as toxic oxygen species produced by neutrophils are often implicated in lung damage the inhibitory effect on the respiratory burst may account for the lack of residual lung damage that characterises recovery from pneumococcal pneumonia. Finally, as S pneumoniae can abrogate a powerful respiratory burst it may reduce the capacity of neutrophils to kill other organisms in mixed infections.

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