Thorax 1994;49:676–683

Characterisation of an oxidative response inhibitor produced by *Streptococcus pneumoniae*

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Abstract

Background **– Pneumonia caused by infection with *Streptococcus pneumoniae* is still a major clinical problem. Reactive oxygen species contribute to the killing of these bacteria by polymorphonuclear leucocytes (PMNs). Defence mechanisms of *Str pneumoniae* which counter reactive oxygen species are characterised.**

Methods **– PMNs were stimulated with phorbol myristate acetate (PMA) in the presence and absence of *Str pneumoniae* and supernatants from them, and superoxide (O_2^-) production was measured by the reduction of ferricytochrome c.**

Results **– *Streptococcus pneumoniae*, but not *Klebsiella pneumoniae* or *Staphylococcus aureus*, inhibited PMA stimulated superoxide production by PMNs. Washed PMNs which had been preincubated with *Str pneumoniae* autolysis phase supernatants also exhibited depressed H_2O_2 production in response to PMA. The inhibitory activity was not attributable to non-specific cytotoxicity as assessed by release of the cytoplasmic enzyme lactate dehydrogenase, nor did the supernatants inhibit PMA stimulated degranulation of PMNs. Fractionation of the autolysis phase supernatants revealed inhibitory activity in both the fractions greater than and less than 10 kD. Like pneumolysin the inhibitory activity was heat sensitive. However, both a parent and pneumolysin negative mutant *Str pneumoniae*, and autolysis phase supernatants from them, inhibited PMN superoxide production. Antisera to pneumolysin failed to abrogate the inhibitory effect of intact *Str pneumoniae* or autolysis phase supernatants from types 1 or 14 *Str pneumoniae*.**

Conclusions **– The inhibitory effect of *Str pneumoniae* on the respiratory burst of PMNs is not shared by two other common lung pathogens. The existence of a novel inhibitor of the PMN respiratory burst, distinct from pneumolysin, has been demonstrated. The inhibitor is specific for the respiratory burst and is active both in the logarithmic phase of growth and during autolysis.**

(*Thorax* 1994;49:676–683)

Despite antibiotics, infections caused by *Streptococcus pneumoniae* continue to cause a high morbidity and mortality. Pneumococcal pneumonia, for example, is the most common form of community acquired pneumonia in hospital[1] with an overall mortality of 5–13%,[2] whilst meningitis caused by *Str pneumoniae* carries a mortality of 30%.[3] Furthermore, the worldwide increase in penicillin resistance and multiple antibiotic resistance among pneumococci,[4–7] and the limited use of pneumococcal vaccine,[8] suggest that pneumococcal infection may cause increasing morbidity and mortality in future years.

In the host's defence against *Str pneumoniae* killing by phagocytes has a key role.[9,10] The mechanism of killing is incompletely understood, but there is evidence that, as with the killing of many other microorganisms,[11–13] the release of oxygen species by phagocytes is important. For example, *Str pneumoniae* is killed by reactive oxygen species;[14,15] polymorphonuclear leucocytes (PMNs) incubated anaerobically have a diminished capacity to kill *Str pneumoniae*,[16] and *Str pneumoniae* can stimulate PMNs to release superoxide under certain conditions.[17,18] Recent studies in our laboratories, however, have shown that *Str pneumoniae* in suspension are able to interfere with the oxidative burst of PMNs.[19] They inhibit both spontaneous superoxide production by PMNs and the respiratory burst stimulated by phorbol myristate acetate (PMA). The effect is associated with no change in viability of the PMNs (assessed by trypan blue exclusion), and is evident within 15 minutes of adding the organism. The inhibitory activity is exhibited by intact organisms in the logarithmic phase of growth and is released on autolysis. Inhibition is dependent on both the dose and viability of the bacteria and was demonstrated for two different serotypes which are among the major causative organisms of serious pneumococcal infections. Given the central role of phagocytes in the host's defence against *Str pneumoniae*, and the importance of reactive oxygen species in the killing of bacteria by phagocytes, this inhibitory activity may be an important defence mechanism of *Str pneumoniae*.

The purpose of the current work was (1) to determine whether the inhibitory activity was specific to *Str pneumoniae* and to the respiratory burst, and (2) to characterise the activity further. Since pneumolysin (a toxin released on autolysis of *Str pneumoniae*) also has been shown to inhibit the respiratory burst of PMNs,[20] we were particularly interested to determine whether the inhibitory activity that we have observed could be accounted for by pneumolysin. Two approaches were taken: the ability of a pneumolysin negative strain of *Str pneumoniae* to inhibit the respiratory burst was investigated and the effect of antipneumolysin serum on the inhibitory activity of *Str pneumoniae* was assessed.

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Received 23 March 1993
Returned to authors
13 August 1993
Revised version received
16 March 1994
Accepted for publication
21 March 1994

Methods

STREPTOCOCCUS PNEUMONIAE

Encapsulated types 1 and 14 *Str pneumoniae* were obtained from patients with bacteraemic pneumococcal pneumonia. The pneumolysin negative *Str pneumoniae* was a mutant of serotype 2 which had been constructed previously by disrupting the gene encoding pneumolysin by insertion-duplication mutagenesis.[21] The bacteria were stored in 1 ml aliquots at −70°C. Before use, thawed *Str pneumoniae* was grown in Todd–Hewitt broth with 10% (v/v) new born calf serum for 3–4 hours so that the organisms were in the logarithmic phase of growth (log phase organisms). The bacteria were collected by centrifugation at 2200 *g* for 10 minutes, washed twice, resuspended in phosphate buffered saline (PBS), and adjusted to 10×10^7/ml. The total bacterial count was determined with a Helber chamber and the viable count assessed by serial dilution and plating onto blood agar, using the method of Miles and Misra.[22] In some experiments *Str pneumoniae* was used which had been grown for 18–24 hours, by which time the organisms had undergone autolysis (autolysis phase).

KLEBSIELLA PNEUMONIAE AND *STAPHYLOCOCCUS AUREUS*

Clinical isolates of *K pneumoniae* and *Staph aureus* were obtained from the Bacteriology Laboratory, Bristol Royal Infirmary. They were stored and prepared as for *Str pneumoniae*, using nutrient broth as the culture medium.

PREPARATION OF CULTURE SUPERNATANTS

The supernatant from the first centrifugation of the bacterial culture was retained for use in some experiments. As appropriate to the experiment, the supernatants were treated in the following ways.

Filtration

Sterilisation of bacterial culture supernatants was achieved by filtration through a 0·2 μm filter (Millipore).

Heat treatment

Culture supernatants were heat treated by incubation in a water bath at 60°C for 30 minutes.

Dialysis

Dialysis of supernatants (3–5 ml) was carried out in Visking tubing with a molecular weight cut off of 10 kD against 500 ml Todd–Hewitt broth overnight at 4°C and again for four hours at room temperature with a magnetic stirrer against 500 ml fresh Todd–Hewitt broth.

Fractionation

Culture supernatants were separated into high and low molecular weight fractions by centrifugation in Millipore Ultrafree-MC filters with 10 kD cut off membranes at 1110 *g* for three hours. The > 10 kD material retained in the filter cup was resuspended to the original volume in Todd–Hewitt broth.

POLYMORPHONUCLEAR LEUCOCYTES

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% (w/v) sodium citrate. In each experiment blood from one donor was used. PMNs were isolated by dextran sedimentation followed by centrifugation through Percoll (Sigma) using a modification of the method of Dooley *et al.*[23] The cells obtained were washed and resuspended in PBS with 4% citrate at 4×10^6/ml. Ethical approval for obtaining blood from healthy volunteers was provided by the ethical committee of the Bristol and Weston Health Authority.

REAGENTS

Ferricytochrome c (type III), superoxide dismutase (SOD, type I, 300 U/mg protein), phorbol myristate acetate (PMA), phenol red, horseradish peroxidase, cytochalasin b (1 mg/ml in DMSO) and Triton X-100 were obtained from Sigma (Poole, Dorset, UK). The PMA was dissolved in DMSO at 1 mg/ml and stored in 10 μl aliquots at −20°C. The cytochrome c and SOD were dissolved in PBS citrate, either made fresh on the day of the experiment or stored at −20°C.

SUPEROXIDE DETECTION

The amount of superoxide (O_2^-) released by PMNs either spontaneously or in response to PMA or *Str pneumoniae* was measured by the microassay of Pick and Mizel[24] which uses SOD inhibitable reduction of ferricytochrome c. The reaction mixture (100 μl) consisted of 160 μmol/l cytochrome c, 1×10^5 PMNs, and the appropriate stimulus (*Str pneumoniae* or 1 μg/ml PMA), with or without 300 U/ml SOD. Six or eight replicates of each condition were set up in vertical rows of a 96-well flat bottomed tissue culture plate (Nunc, 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 produced per well was calculated from the formula[24]:

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

HYDROGEN PEROXIDE DETECTION

Hydrogen peroxide production by PMNs was measured by hydrogen peroxide dependent reduction of phenol red by horseradish peroxi-

dase. Phenol red solution was prepared using 4·64 ml Hank's balanced salt solution + 4% (w/v) bovine serum albumin: 0·09 ml 28 mmol/l phenol red: 0·07 ml 12 mg/ml horseradish peroxidase. Phenol red solution, 0·5 ml, was combined with 10^6 PMNs and PMA in replicate wells of a 24-well plate (final volume 1 ml). The mixtures were incubated for 30 minutes at 37°C and the reaction terminated by the addition of 0·01 ml 1N NaOH. The plates were centrifuged at 110 *g* for five minutes to remove the PMNs and the supernatants transferred to 1 ml cuvettes. The absorbance of the solutions was measured at 610 nm and the data converted to nanomoles of hydrogen peroxide by means of a standard curve which was established for each experiment.

DEGRANULATION ASSAY

Neutrophil degranulation was measured by assaying for the azurophil enzyme myeloperoxidase. Neutrophils were pretreated with cytochalasin b (20 µg/ml) for 10 minutes at room temperature, then incubated (at 10^6/ml), either alone or with PMA (1 µg/ml), or *Str pneumoniae* autolysis phase supernatants (final concentration 1/4 v/v), or both, for 60 minutes at 37°C. Myeloperoxidase activity in the PMN supernatants was measured using an adaptation of the method of Suzuki *et al*[25] utilising 3,3',5,5' tetramethyl benzidine (TMB). Triplicate aliquots (150 µl) of the supernatant to be assayed were added to the assay substrate (20 mmol/l TMB (50 µl), 0·03% hydrogen peroxide (v/v, 20 µl), 0·01 mmol/l sodium acetate buffer pH 5·2 (50 µl) and 150 µl PBS) and incubated for exactly five minutes. The reaction was terminated by placing the reagents on ice. PBS, 1 ml, was added and the absorbance at 655 nm measured against a substrate blank in a Shimadzu spectrophotometer. Enzyme release was expressed as a percentage of the maximum releasable. This was determined by lysing PMNs with 0·05% Triton X-100 for two minutes.

LACTATE DEHYDROGENASE ASSAY

Release of the cytoplasmic enzyme lactate dehydrogenase (LDH) was used as a marker of cell death. The amount of LDH in the supernatants of PMNs (10^6/ml) incubated either alone, with *Str pneumoniae* autolysis phase supernatants (final concentration 1/4 v/v), or with 0·05% Triton X-100 for 90 minutes at 37°C was assayed using a standard kit (Sigma). Spectrophotometric absorbance data were converted to Berga–Broida (BB) units of LDH activity by means of a standard curve.

PNEUMOLYSIN AND ANTIPNEUMOLYSIN SERUM

Recombinant pneumolysin,[26 27] normal rabbit serum, and antipneumolysin rabbit serum were prepared as described elsewhere.[27]

SHEEP RED BLOOD CELL HAEMOLYSIS ASSAY

In order to measure the amount of pneumolysin present in *Str pneumoniae* log phase cultures and autolysis phase supernatants, an adaptation of the method of Steinfort *et al*[28] was used. Doubling dilutions of recombinant pneumolysin, *Str pneumoniae* culture, or autolysis supernatants were prepared and incubated with or without 1:100 antipneumolysin serum for one hour at room temperature. Fresh sheep red blood cells (2% v/v) were added and the plate incubated for 30 minutes at 37°C. The plate was then centrifuged for two minutes at 110 *g* and 50 µl of the supernatant was transferred to a 96-well flat bottomed plate. Absorbance was read at 550 nm and the values obtained expressed as a percentage of total haemolysis measured in sheep red blood cells lysed with distilled water. The pH of autolysis supernatants was adjusted to 7·0–7·2 for use in the haemolysis assays since preliminary experiments had shown that non-pH adjusted supernatants which were 5·3–6·4 had some lytic activity when mixed 1:1 with sheep red blood cells (27·8% (6·3%) haemolysis, $n = 10$), which in only five samples was partially reduced by antipneumolysin rabbit serum (17·8% (4·8%) haemolysis) and which was lost (2·7% (2·1%) haemolysis, $n = 10$) when the supernatants were first diluted with an equal volume of PBS to give the same concentration as used in the superoxide assay. These results suggested that the haemolysis caused by non-pH adjusted supernatants was due to their acidity.

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 (SD) of the combined experiments. Unless otherwise stated, the paired *t* test was used to assess differences between means, $p < 0.05$ being considered significant.

Results

OXIDATIVE RESPONSES OF PMNS TO DIFFERENT RESPIRATORY PATHOGENS

The results of incubating PMNs with either washed log phase *Str pneumoniae* (type 1), *Klebsiella pneumoniae*, or *Staphylococcus aureus* at a PMN:bacteria ratio of 1:20 in the presence of 10% pooled human serum are shown in fig 1. *Streptococcus pneumoniae* reduced the amount of spontaneous superoxide detected as previously reported.[19] By contrast, in each of five experiments both *K pneumoniae* and *Staph aurens* stimulated release of superoxide. It should be noted that the experiments with *Str pneumoniae* were performed at different times from those with the other organisms, accounting for the difference in spontaneous superoxide release. Further experiments were set up to determine whether *K pneumoniae* and *Staph aureus* are able to inhibit PMA stimulated superoxide production. The results are presented in fig 2 and show that, in sharp contrast to *Str pneumoniae*, neither *K pneumoniae* nor *Staph aureus* interfered with the respiratory burst of PMNs.

SPECIFICITY OF THE INHIBITOR

We have previously shown that the inhibitory activity of *Str pneumoniae* is not the result of

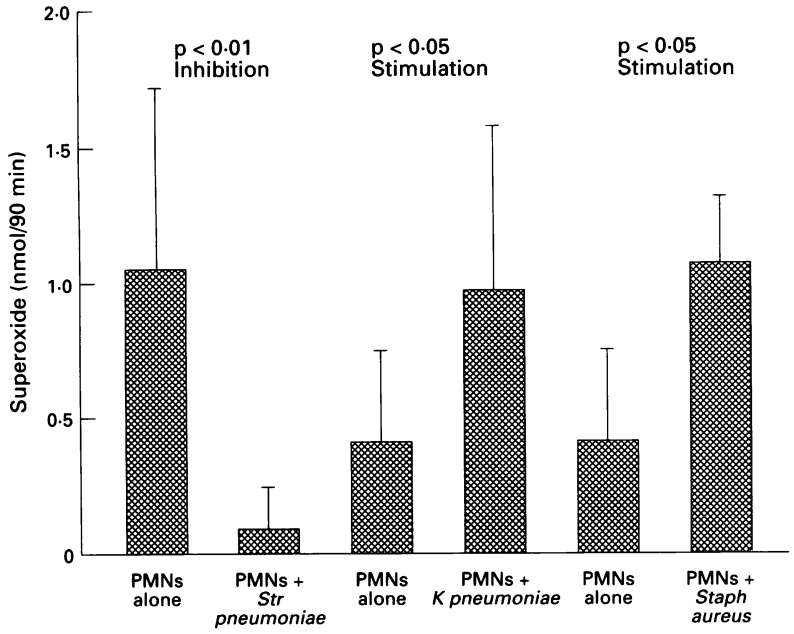

Figure 1 Oxidative response of polymorphonuclear leucocytes (PMNs) to log phase Str pneumoniae, K pneumoniae, and Staph aureus in the presence of 10% pooled human serum. Results are mean (SD) of five experiments.

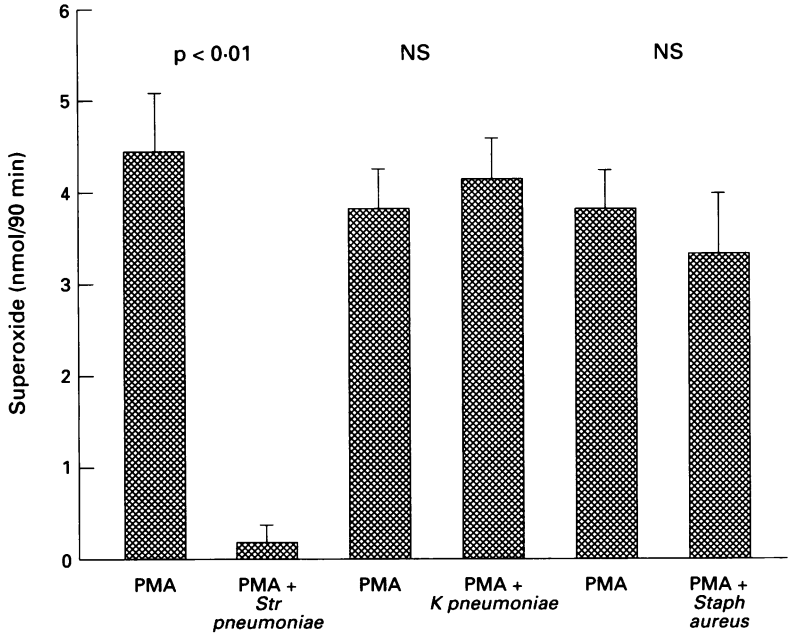

Figure 2 Effect of log phase Str pneumoniae, K pneumoniae, and Staph aureus on phorbol myristate acetate (PMA) stimulated superoxide production by polymorphonuclear leucocytes (PMNs). Results are mean (SD) of five experiments.

non-specific toxicity towards PMNs as assessed by trypan blue exclusion.[19] As an additional test of PMN viability, the more sensitive method of lactate dehydrogenase release was employed. PMNs incubated with *Str pneumoniae* autolysis phase supernatants released no more lactate dehydrogenase than PMNs incubated alone: PMNs alone, 418 (92) BB units LDH/90 minutes; PMNs + autolysis phase supernatant, 402 (83) BB units LDH/90 minutes; lysed PMNs (total LDH), 1220 (115) BB units LDH/90 minutes, n = 7.

To assess whether *Str pneumoniae* autolysis phase supernatants inhibited or affected other PMN functions their effect on PMN degranulation was investigated. The results are shown in table 1 and demonstrate that the supernatants did not inhibit PMA stimulated myeloperoxidase release but, rather, significantly enhanced it. This finding raised the possibility that the supernatants were stimulating PMN degranulation. PMNs incubated with autolysis phase supernatants, however, did not release significantly more myeloperoxidase than PMNs incubated alone.

INHIBITION OF HYDROGEN PEROXIDE PRODUCTION BY *STR PNEUMONIAE*

To reinforce the conclusion that *Str pneumoniae* inhibited the respiratory burst of PMNs, the release of a second product, hydrogen peroxide, was measured. PMN suspensions were preincubated with either Todd–Hewitt broth or pH adjusted (pH 7·2) autolysis phase supernatants (at a volume:volume ratio of 1) for one hour at 37°C and washed twice in PBS before stimulation with PMA. After 30 minutes hydrogen peroxide production was measured. PMNs preincubated with autolysis phase supernatants generated significantly less hydrogen peroxide in response to PMA than control PMNs preincubated with Todd–Hewitt broth: PMNs + Todd–Hewitt broth 28·59 (2·11) nmol hydrogen peroxide/30 minutes, PMNs + autolysis phase supernatant 17·02 (8·02) nmol hydrogen peroxide/30 minutes, n = 3, $p < 0.05$. The viability of the PMNs in these experiments was 96–98% as judged by trypan blue exclusion.

CHARACTERISATION OF THE RESPIRATORY BURST INHIBITOR PRODUCED BY *STR PNEUMONIAE*

We have previously reported[19] that the inhibitory activity of *Str pneumoniae* is associated with washed organisms in the logarithmic phase of growth and is not released into the log phase supernatant, but that it is released into the culture supernatant during autolysis. As a first step in characterising the inhibitory activity its heat sensitivity was investigated. In four experiments autolysis phase supernatants heated for 30 minutes at 60°C lost their ability to inhibit PMA stimulated superoxide production: PMNs + PMA + Todd–Hewitt broth (control) 4·52 (0·64) nmol $O_2^-/10^5$ PMNs/90 minutes, PMNs + PMA + untreated autolysis supernatant 1·51 (1·11) nmol $O_2^-/10^5$ PMNs/90 minutes ($p < 0.01$), PMNs + PMA + heat treated autolysis supernatant 4·33 (0·89) nmol $O_2^-/10^5$ PMNs/90 minutes. Filtered supernatants from the autolysis phase of growth were then dialysed (exclusion pore size 10 000 molecular weight) and their ability to inhibit PMA stimulated superoxide compared with that of non-dialysed supernatants. In four experiments the amount of superoxide detected was as follows: (a) PMNs + PMA 4·71 (0·43); (b) PMNs + PMA + autolysis supernatant 3·21 (0·49); (c) PMNs + PMA + dialysed supernatant 3·82 (0·34) nmol $O_2^-/10^5$ PMNs/90

Table 1 Effect of Streptococcus pneumoniae autolysis phase supernatants on the degranulation response of polymorphonuclear leucocytes (PMNs)

	n	Myeloperoxidase released (% of total)	p
PMNs + PMA	7	27·5% (18·2%)	<0·05
PMNs + PMA + supernatant		64·0% (17·0%)	
PMNs alone	6	1·7% (1·7%)	NS
PMNs + supernatant		3·0% (2·4%)	

PMN = polymorphonuclear leucocyte; PMA = phorbol myristate acetate.

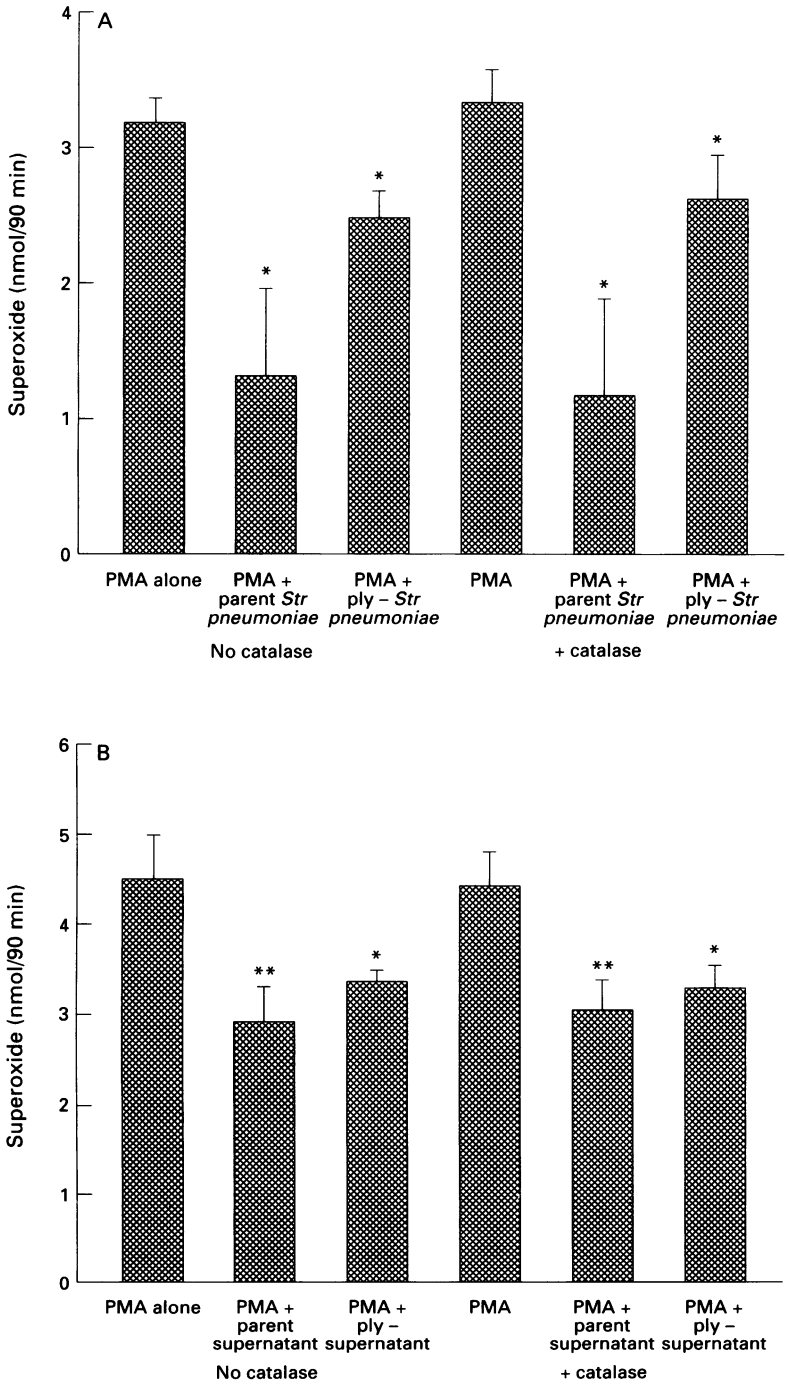

*Figure 3 Inhibition of polymorphonuclear leucocyte (PMN) phorbol myristate acetate (PMA)-stimulated superoxide production by (A) log phase parent and pneumolysin-negative (ply-) Str pneumoniae (n=3) and (B) parent and pneumolysin negative Str pneumoniae autolysis phase supernatants (n=4). *p<0·05, **p<0·01 v PMA alone.*

minutes. Thus, whilst dialysis significantly reduced the inhibitory activity of the supernatant ($p<0·05$, c *v* b), dialysed supernatants still possessed significant inhibitory activity ($p<0·05$, c *v* a). This result suggests that there is a fraction of molecular weight >10 kD with inhibitory activity and possibly another with molecular weight <10 kD. Since hydrogen peroxide produced by *Str pneumoniae* can reoxidise cytochrome c and thus interfere with the inhibition assay,[19 29] experiments were set up to determine whether the inhibitory activity of the fractions was sensitive to catalase. The amount of catalase used (50 µg/ml) was that previously shown to completely block reoxidation of reduced cytochrome c by *Str pneumoniae*.[16] In the four experiments catalase did not reduce the inhibitory activity of the supernatant: 3·10 (0·35) (NS *v* b) or the dialysed supernatant: 4·15 (0·17) (NS *v* c). To determine whether inhibitory activity was present in the low as well as the high molecular weight fractions, autolysis phase supernatants were separated through a 10 kD filter and both fractions assessed for inhibitory activity. The results were as follows: (i) PMNs + PMA 4·42 (0·55); (ii) PMNs + PMA + unfractionated supernatant 3·03 (0·41) ($p<0·01$ *v* i); (iii) PMNs + PMA + >10 kD fraction 4·01 (0·26) ($p<0·05$ *v* i); (iv) PMNs + PMA + <10 kD fraction 3·20 (0·49) ($p<0·01$ *v* i) nmol $O_2^-/10^5$ PMNs/90 minutes, n = 8, all experiments performed in the presence of 50 µg/ml catalase. These findings confirm the presence of inhibitory activity in both fractions.

ROLE OF PNEUMOLYSIN IN THE INHIBITION OF THE RESPIRATORY BURST OF PMNS BY *STR PNEUMONIAE*

To assess the role of pneumolysin in the inhibition two approaches were undertaken. Firstly, the ability of a laboratory mutated strain of *Str pneumoniae*, which lacks a functional pneumolysin gene, to inhibit the respiratory burst was investigated. Secondly, the effect of antipneumolysin serum on the inhibitory activity of type 1 and type 14 *Str pneumoniae* was measured.

Ability of pneumolysin-negative Str pneumoniae to inhibit the respiratory burst of PMNs

The ability of log phase pneumolysin negative *Str pneumoniae* and autolysis phase supernatants to inhibit PMA stimulated superoxide production by PMNs is illustrated in fig 3A. As can be seen, both parent and pneumolysin negative log phase *Str pneumoniae* significantly inhibited superoxide production by PMNs. The same result was obtained in the presence and absence of catalase. The degree of inhibition between the mutant and parent strain was not significantly different. Supernatants from autolysis phase cultures of both parent and pneumolysin negative *Str pneumoniae* caused significant inhibition of the PMN respiratory burst (fig 3B) and the same result was obtained in the presence of catalase. Again the dif-

ference between the extent of inhibition caused by the parent and mutant strains was not significant.

Effect of antipneumolysin serum on the ability of Str pneumoniae to inhibit the respiratory burst of PMNs

Haemolytic assays: before examining the ability of antipneumolysin rabbit serum to reduce the inhibitory effect of *Str pneumoniae* on PMA stimulated PMN superoxide production it was necessary to determine the ability of the antipneumolysin serum to neutralise pneumolysin. The latter was tested by exploiting the ability of pneumolysin to lyse sheep red blood cells. Purified pneumolysin lysed sheep red blood cells at concentrations at or above 0·3 ng/ml; antipneumolysin serum at 1:100 protected sheep red blood cells up to 5 ng/ml pneumolysin.

Additional experiments were set up to determine whether intact log phase *Str pneumoniae* and autolysis phase supernatants produced pneumolysin as judged by their capacity to lyse sheep red blood cells. In three experiments log phase type 1 *Str pneumoniae* caused only slight haemolysis and neither normal rabbit serum nor antipneumolysin serum were protective (*Str pneumoniae* alone 8·5% (1·1%), *Str pneumoniae* + normal rabbit serum 7·9% (1·3%), *Str pneumoniae* + antipneumolysin rabbit serum 6·5% (1·9%) haemolysis). In two of three experiments type 14 *Str pneumoniae* showed substantial haemolytic activity (66·4% and 87·3% haemolysis respectively). Normal rabbit serum partially protected the sheep red blood cells (28·1% and 19·9% haemolysis with normal rabbit serum present) and antipneumolysin rabbit serum protected further (8·0% and 8·3% haemolysis). The third type 14 sample had only a small lytic effect (9·3%); however, both normal rabbit serum and antipneumolysin rabbit serum completely blocked haemolysis (0%).

Ten autolysis supernatants (pH adjusted to 7·0–7·2) had very little lytic activity (2·2% (2·9%) haemolysis).

Inhibition assays: log phase type 1 and type 14 *Str pneumoniae* were incubated at room temperature for one hour either alone, with 1:100 normal rabbit serum or with 1:100 antipneumolysin rabbit serum. The results are summarised in table 2. It can be seen that in the absence or presence of catalase neither normal rabbit serum nor antipneumolysin rabbit serum abrogated the inhibitory effect of log phase types 1 or 14 *Str pneumoniae*.

Table 3 shows the results of similar experiments with autolysis phase supernatants. Again, neither normal rabbit serum nor antipneumolysin rabbit serum had any effect on the inhibition.

Discussion

We have previously reported that *Str pneumoniae* inhibits the respiratory burst of PMNs.[19] The results presented here show that this ability is not shared by two other common lung pathogens, *K pneumoniae* and *Staph aureus*. In contrast to *Str pneumoniae*, these organisms stimulated PMNs to release superoxide. Since reactive oxygen species can cause tissue damage,[30] including lung injury,[31] this observation may help to explain why pneumococcal pneumonia is characterised by a lack of residual lung damage,[32] whereas tissue necrosis and/or lung fibrosis are frequently observed in pneumonias caused by *K pneumoniae* and *Staph aureus*.[32 33]

These results also indicate that the inhibitory activity of *Str pneumoniae* is specific for the oxidative response and is not attributable to a non-specific toxic factor. Autolysis phase supernatants which inhibited the respiratory burst did not kill PMNs as assessed by LDH release – confirming our previous observations using trypan blue exclusion – nor did they inhibit the degranulation response of PMNs.

Table 2 Effect of normal rabbit serum (NRS) and antipneumolysin serum (PnIS) on the ability of log phase Streptococcus pneumoniae (S pn) to inhibit phorbol myristate acetate (PMA)-stimulated superoxide production by polymorphonuclear leucocytes (PMNs) (nmol O_2^-/10^5 PMNs/90 minutes)

	PMA alone	*PMA + S pn*	*PMA + S pn + NRS*	*PMA + S pn + PnIS*
Type 1				
No catalase	3·16	1·57	1·59	1·57
+ 50 µg/ml catalase	3·13	2·65	2·77	2·91
Type 14				
No catalase	3·16	1·70	1·57	1·52
+ 50 µg/ml catalase	3·13	2·59	2·91	2·69

Results are the mean of two experiments for each serotype.

Table 3 Effect of normal rabbit serum (NRS) and antipneumolysin serum (PnIS) on the ability of autolysis phase supernatants to inhibit phorbol myristate acetate (PMA)-stimulated superoxide production by polymorphonuclear leucocytes (PMNs) (nmol O_2^-/10^5 PMNs/90 minutes)

	PMA alone	*PMA + supernatant*	*PMA + supernatant + NRS*	*PMA + supernatant + PnIS*
No catalase	3·85 (0·38)	2·48 (0·43)	2·61 (0·47)	2·70 (0·47)
+ 50 µg/ml catalase	4·09 (0·81)	3·04 (0·88)	3·00 (0·77)	3·10 (0·71)

Results are mean (SD) of four experiments (two using type 1 and two using type 14 *Str pneumoniae*).
The inhibitions caused by the supernatants under the different conditions are not significantly different from each other (analysis of variance).

Rather, the supernatants appeared synergistically to enhance PMA stimulated myeloperoxidase release. It is possible that the enhanced myeloperoxidase release is a consequence of the oxidative response inhibitor since myeloperoxidase is known to be inactivated by reactive oxygen species.[34]

Previous attempts[19] to determine how *Str pneumoniae* interferes with the PMN respiratory burst showed that, although the organism produces SOD, the amounts are insufficient to account for the interference observed. Thus, the interference is not due to a scavenging effect of SOD. Here it was shown that washed PMNs which had been preincubated with autolysis phase supernatants exhibited diminished production of hydrogen peroxide upon stimulation with PMA. This result also renders it unlikely that the interference is due to scavenging, but rather that it results from inhibition of the respiratory burst. Again this effect could not be attributed to death of the PMNs as they retained their viability as judged by trypan blue exclusion. The above results also show that the inhibitory effects are demonstrable using different assays for measuring the respiratory burst.

The ability of *Str pneumoniae* to inhibit the respiratory burst of PMNs may constitute a major counter defence of the microorganism and help to explain its pathogenicity. We therefore sought to characterise the inhibitory factor further. The results indicate that the activity is heat sensitive but that it is not inactivated by acidic conditions. Experiments using dialysed supernatants indicated that a fraction of molecular weight >10 kD contained inhibitory activity. The loss of some activity on dialysis suggested the possibility of an additional low molecular weight inhibitor which is insensitive to catalase. Experiments using filtered supernatants, in which both the fractions >10 kD and <10 kD were retained, confirmed the presence of inhibitory activity in both fractions, in this instance more inhibitory activity being found in the fraction <10 kD.

Some of the attributes of the inhibitory activity we have described are in keeping with the known characteristics of the pneumococcal toxin pneumolysin – for example, pneumolysin is released into the culture supernatant during autolysis, is heat sensitive, and has a molecular weight of approximately 54 kD. We therefore investigated whether, or to what extent, pneumolysin was responsible for the inhibition we have reported. The results clearly distinguish our inhibitor from pneumolysin. Firstly, a pneumolysin negative strain of *Str pneumoniae* was able to inhibit PMA stimulated superoxide production by PMNs. Both log phase organisms and autolysis phase supernatants exerted the inhibitory effect. Secondly, experiments designed to measure the haemolytic activity of *Str pneumoniae* revealed a dissociation between haemolytic activity and the ability to inhibit the respiratory burst; both types 1 and 14 log phase *Str pneumoniae* inhibited PMN oxidative responses,[19] but only type 14 showed substantial haemolytic activity. Furthermore, autolysis phase supernatants were inhibitory despite the fact that they lacked haemolytic activity. A lack of haemolytic activity in itself suggests the absence of active pneumolysin. Such an outcome was not unexpected in the case of the autolysis phase supernatants because those used here were obtained from 18–24 hour cultures and were acidic (pH 5·3–6·4), and it is known that pneumolysin activity declines with prolonged *Str pneumoniae* culture (beyond 16 hours) due to increasing acidity.[28] Finally, in confirmation of the conclusion that the inhibitor described here is distinct from pneumolysin, antipneumolysin serum failed to abrogate the inhibitory effect of either log phase organisms or autolysis phase supernatants from *Str pneumoniae* despite its efficacy in neutralising pneumolysin in haemolytic assays.

It has been shown that pneumolysin inhibits oxidative responses.[20] Our results suggest that the new inhibitor described here also contributes to the overall inhibitory effect of *Str pneumoniae* on the respiratory burst. Moreover, since pneumolysin is inactivated by the respiratory burst,[35] the inhibitor, by interfering with this process, may potentiate the activity of pneumolysin.

Our finding of haemolytic activity in log phase type 14 *Str pneumoniae* was surprising since extracellular pneumolysin activity has not previously been reported for log phase organisms. This may suggest that, although not released, pneumolysin is exposed on log phase type 14 *Str pneumoniae*. Alternatively, it is possible that the activity derives from a minority of organisms undergoing autolysis within the population and not from log phase organisms themselves.

In conclusion, these results demonstrate that *Str pneumoniae* produces a substance other than pneumolysin which inhibits the respiratory burst of PMNs. This substance is biologically active during the logarithmic phase of growth and is manifested by both clinically important and pneumolysin negative strains.

This work was supported by a research grant from the British Lung Foundation. TJM is in receipt of a Royal Society Fellowship.

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