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## Reassessment of the microbicidal activity of reactive oxygen species and hypochlorous acid with reference to the phagocytic vacuole of the neutrophil granulocyte

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### Abstract

During phagocytosis, neutrophils undergo a burst of respiration in which oxygen is reduced to superoxide ( $O_2^-$ ), which dismutates to form  $H_2O_2$ . Myeloperoxidase (MPO) is discharged from the cytoplasmic granules into the phagosome following particle ingestion. It is thought to utilize  $H_2O_2$  to oxidize halides, which then react with and kill ingested microbes. Recent studies have provided new information as to the concentration of  $O_2^-$  and proteins, and the pH, within the vacuole. This study was conducted to examine the antimicrobial effect of  $O_2^-$ ,  $H_2O_2$  and hypochlorous acid under these conditions and it was found that the previously described bactericidal effect of these agents was reversed in the presence of granule proteins or MPO. To establish which cellular proteins were iodinated by MPO, cellular proteins and bacterial proteins, iodinated in neutrophils phagocytosing bacteria in the presence of  $^{125}I$ , were separated by 2D gel electrophoresis. Iodinated spots were detected by autoradiography and the oxidized proteins were identified by MS. The targets of these iodination reactions were largely those of the host cell rather than those of the engulfed microbe.

### INTRODUCTION

Phagocytosis results in assembly and activation of the respiratory burst NADPH oxidase at the membrane of the phagocytic vacuole. The respiratory burst is required for optimal antimicrobial function by neutrophils, and its importance is demonstrated by the syndrome of chronic granulomatous disease (CGD), a rare condition in which its absence predisposes patients to severe infection (Thrasher *et al.*, 1994). Activation of the oxidase is associated with the generation of various reduced oxygen species (ROS) (Root *et al.*, 1975). These have widely been thought to be responsible for the killing of phagocytosed micro-organisms, either directly (Babior *et al.*, 1973, 1974) or by acting as substrates for myeloperoxidase (MPO)-mediated halogenation (Klebanoff, 1975).

The first product of the oxidase is  $O_2^-$ , the product of the univalent reduction of oxygen (Babior *et al.*, 1973).  $O_2^-$  has minimal antibacterial activity (Rosen & Klebanoff, 1979; Hurst & Barrette, 1989) and dismutates to produce  $H_2O_2$ .  $H_2O_2$  is thought to be acted upon by

MPO, and released into the vacuole from the cytoplasmic granules, to produce hypochlorous acid (HOCl), a potent antimicrobial oxidant (Klebanoff, 1967a, 1968).

Recently an attempt was made to define the conditions that pertain within the phagocytic vacuole at the time at which the respiratory burst takes place (Reeves *et al.*, 2002). It was found that large amounts of  $O_2^-$ , of approximately 4 mols  $l^{-1}$ , are produced, that the concentration of granule proteins is as much as 500 mg  $ml^{-1}$ , and the pH is between 7.4 and 8.0. This study was undertaken to examine the antibacterial action of  $O_2^-$  and  $H_2O_2$ , and products of chloride oxidation (HOCl), under these conditions.  $^{125}I$  was used to identify the protein targets of MPO-induced iodination.

## METHODS

### *In vitro* killing of *Staphylococcus aureus* and *Escherichia coli* by human neutrophils

Neutrophils were purified from fresh human blood by dextran sedimentation and centrifugation through Ficoll/Hypaque as described previously (Segal & Jones, 1980). Cells ( $5 \times 10^8$ ) were incubated at 37 °C in 1 ml PBS (140 mM NaCl, 10 mM KCl, 10 mM  $NaH_2PO_4$ , 5 mM glucose, pH 7.3) in a rapidly stirred chamber. IgG opsonized *S. aureus* (NCTC 12981) or *E. coli* (ATCC 11775) ( $1 \times 10^8$  c.f.u.  $ml^{-1}$ ) was added and killing was measured as described by Segal *et al.* (1981) omitting lysostaphin. Results were calculated as the mean ( $\pm$ SE) from at least three experiments with colony counts performed in triplicate for each sample and expressed as a percentage of the original numbers at time zero.

### Preparation of granules and MPO purification

Diisopropyl fluorophosphate (DIFP; 1 mM) was added to  $1 \times 10^{10}$  neutrophils, which were mixed and left on ice for 10 min. The cells were then resuspended in Break Buffer (10 mM KCl, 3 mM NaCl, 4 mM  $MgCl_2$ , 10 mM PIPES, pH 7.3) containing protease inhibitors [ $10 \mu g ml^{-1}$  leupeptin, *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), pepstatin A and aprotinin] and disrupted by nitrogen cavitation after 400 p.s.i. (2760 kPa) for 20 min. A post-nuclear supernatant was prepared (400 g, °15 min, 4 8C) and layered on top of a discontinuous gradient of 30, 43 and 55% sucrose (w/w in Break Buffer) and centrifuged (1 h/100 000 g/4 °C) in a Beckman SW41 head. The azurophil and specific granules were collected from the interfaces of the 55 and 43% sucrose, respectively. The granules were washed in PBS, pelleted and stored at -80 °C at a final concentration of 25 mg  $ml^{-1}$ .

For the preparation of MPO, azurophilic granules of  $1 \times 10^{10}$  neutrophils were homogenized in 120 ml Break Buffer containing 0.75% cetyltrimethylammonium bromide (CETAB) and centrifuged (30 000 g/10 min/4 °C). Ammonium sulphate was added to the supernatant to 50% final concentration. The precipitate was removed by centrifugation at 30 000 g/10 min/4 °C. Further ammonium sulphate (100 mg  $ml^{-1}$ ) was added to the supernatant, at which concentration the MPO came out of solution and was pelleted by centrifugation (30 000 g/10 min/4 °C). The pellet was resolubilized in 50 mM phosphate buffer (pH 7.2). The MPO was purified by ion-exchange chromatography on Fast Flow S-Sepharose (1.5  $\times$  10 cm column, flow rate of 1 ml  $min^{-1}$ , 1 ml fractions collected, eluted with a 100 ml linear gradient of 50-500 mM phosphate buffer, pH 7.2, at 4 °C). Peak fractions were pooled, the ionic concentration was diluted to 50 mM and the pH was adjusted to pH 6.5. The sample was then loaded onto a Mono S column (1 ml column, flow rate of 0.5 ml  $min^{-1}$ , eluted with a linear gradient of 50-500 mM phosphate buffer, pH 6.5, 1 ml fractions collected). The peak fractions of MPO with a purity index ( $A_{430}/A_{280}$ ) of 0.82 (Olsen & Little, 1982) were pooled and stored at -80 °C.

### Killing by, and oxidative capacity of, purified MPO

Bacteria ( $2 \times 10^7$  c.f.u. ml<sup>-1</sup>) from an overnight culture were washed twice and resuspended in PBS at pH 7.5, 6.5 or 5.5 together with purified MPO (5 mg ml<sup>-1</sup>). H<sub>2</sub>O<sub>2</sub> was then added at concentrations of 0.1, 1.0, 10 or 100 mM. After mixing at 37 °C for 0, 1, 2, 4, 8 and 16 min, aliquots were removed and diluted 1/10 in ice-cold LB broth (Difco). Serial tenfold dilutions were then made, and plated in triplicate on LB agar plates. Colony counts were performed in triplicate or quadruplicate for each sample and results were calculated as the mean ( $\pm$ SE) from at least three experiments. The pH remained stable during assays to within 0.15 pH units of the starting pH.

The oxidative capacity (COX) of purified MPO was determined in the presence of 10 mM taurine by the addition of potassium iodide in molar excess, to detect the reaction product *N*-chlorotaurine. The product triiodide ( $>N - Cl + 3I^- + H^+ \leftrightarrow I_3^- >N - H$ ) was measured by its  $A_{350}$ , which is the wavelength of maximum absorption ( $\lambda_{max}$ ) of I<sub>3</sub><sup>-</sup> ( $\epsilon = 22\,900$  mol<sup>-1</sup> cm<sup>-1</sup>) (Nagl *et al.*, 2000). Values measured from 1, 10 and 100 mM H<sub>2</sub>O<sub>2</sub> in PBS at pH 5.5, 6.5 and 7.5 without MPO were subtracted from these values to ensure determination of HOCl and *N*-chlorotaurine, respectively.

### *In vitro* killing of *S. aureus* and *E. coli* by H<sub>2</sub>O<sub>2</sub>, HOCl and superoxide (KO<sub>2</sub>).

From an overnight culture, bacteria ( $2 \times 10^7$  c.f.u. ml<sup>-1</sup>) were washed twice in PBS and resuspended in PBS at pH 7.5, 6.5 or 5.5. To investigate the pH-dependency of killing, the suspended bacteria were incubated with gentle mixing at 37 °C for 32 min. Aliquots were removed periodically and surviving bacteria were counted by serial dilution and colony counting.

Increasing concentrations of H<sub>2</sub>O<sub>2</sub> (1.0, 10 or 100 mM) or HOCl (1 or 5  $\mu$ M) were added and incubated at 37 °C for 0, 1, 2, 4, 8, 16 and 32 min. Aliquots were removed and plated out as described above. The pH remained stable during assays to within 0.2 pH units of the starting pH.

This experiment was repeated with 100 mM H<sub>2</sub>O<sub>2</sub> and up to 1 mM HOCl in the presence of a mixture of azurophil and specific demembrated granules (25 mg ml<sup>-1</sup>). Membranes were removed by Percoll granule disruption as described by Vita *et al.* (1997). The granules were purified in the presence of protease inhibitors to prevent killing of bacteria by these enzymes. Due to the viscosity of the granule protein at high concentration, for technical handling purposes the concentration used was 25 mg ml<sup>-1</sup>. Bacteria ( $2 \times 10^7$  c.f.u. ml<sup>-1</sup>) were added to the granule protein prior to the addition of H<sub>2</sub>O<sub>2</sub> or HOCl.

Killing of bacteria by O<sub>2</sub><sup>-</sup> was performed similarly to that described for H<sub>2</sub>O<sub>2</sub> and HOCl. As a source of O<sub>2</sub><sup>-</sup>, KO<sub>2</sub> was employed and added as a powder to the reactions. Since concentrations of KO<sub>2</sub> greater than 50 mM elevated the pH, bacteria were suspended in PBS at a pH of 6.5 prior to the addition of 100 mM KO<sub>2</sub>, which resulted in a rise in the pH to approximately 7.5.

### Iodination studies

Iodination studies were performed as described by Klebanoff & Clark (1976). Neutrophils ( $1 \times 10^7$ ) were resuspended in 1 ml PBS supplemented with 40 nM KI and 100  $\mu$ Ci (3700 kBq) <sup>125</sup>I. The cell mixture was placed in a magnetically stirring oxygenated chamber at 37 °C and IgG opsonized *S. aureus* was added at a ratio of 10 : 1. After 4 min, the mixture was taken into 1 ml cold PBS containing 10% trichloroacetic acid (TCA). This experiment was also carried out with IgG opsonized *E. coli*, added at a ratio of 100 : 1.

## Two-dimensional electrophoresis of proteins using immobilized pH gradients

Samples were centrifuged (8000 g, 5 min, 4 °C). The pellet was washed three times with ice-cold 80% acetone and air-dried. The pellet was resuspended in 300 µl IEF sample buffer (8 M urea, 2 M CHAPS, 1% Triton X-100, 65 mM DTT, 10 mM Tris base, 0.8% Ampholyte), sonicated briefly and centrifuged (10 000 g/5 min/4 °C). Samples (0.5 mg/270 µl) were pipetted on top of IPG (3–10) strips, and IEF was performed for 40 000 V h at 16 °C.

Prior to electrophoresis in the second dimension, IPG strips were equilibrated twice in 10 ml equilibration buffer [30% (v/v) glycerol, 2% (w/v) SDS, 6 M urea, 50 mM Tris/HCl, pH 6.8] for 20 min. The first equilibration was in 10 ml equilibration buffer containing 2% (w/v) DTT and the second contained 2.5% (w/v) iodoacetamide. The IPG gel strips were electrophoresed into a 12% SDS-PAGE gel and stained with Coomassie R-250.

## MALDI-TOF MS

The protein bands/spots of interest were excised from the SDS gel and digested according to the protocol described by Rosenfeld *et al.* (1992).

The following peptides were used as external standards for MALDI spectra calibration: human angiotensin I and II, ACTH (clip 18–39), [Glu]-fibrinopeptide B, renin substrate tetradecapeptide and insulin B chain. The amount of each peptide was 25 pmol per spot. MALDI-TOF mass spectra of the peptides were obtained using a Biflex III mass spectrometer (Bruker). All spectra were acquired in a positive-ion reflector. Typically 200 shots were recorded. Proteins were identified by comparing mass fingerprints to NCBI's database using Matrix Science, Mscfit and PeptIdent searching machines (<http://www.matrixscience.com/>).

## Statistical analysis

Statistical comparisons were made with Student's *t* test.

## RESULTS AND DISCUSSION

### Vacuolar conditions

The kinetics of bacterial killing by neutrophils is illustrated in Fig. 1(a). Killing occurred quickly, with over 50% killed after just 2 min and 20% remaining after 4 min as described previously (Segal *et al.*, 1981).

Fig. 1(c, d) shows that pH did not affect the viability of *E. coli* or *S. aureus*, except after prolonged exposure approximately 50% of *S. aureus* was killed at pH 5.5 ( $P < 0.05$ ) after 32 min.

### Bactericidal effects of $O_2^-$ , $H_2O_2$ and HOCl

The bactericidal effect of increasing concentrations of  $O_2^-$  was investigated at pH 7.5 after 6 min. Fig. 1(b) shows that  $O_2^-$  itself is relatively non-toxic.

Fig. 2(a–f) shows the killing of *S. aureus* and *E. coli* exposed to increasing concentrations of  $H_2O_2$  at pH values of 7.5, 6.5 or 5.5. The bactericidal effect of  $H_2O_2$  was both dose- and pH-dependent. As the pH was elevated to 7.5, a concentration of 100 mM  $H_2O_2$  was required to reduce the survival of *S. aureus* by 50%. The effect of 100 mM  $H_2O_2$  on *S. aureus* and *E. coli* was totally eliminated in the presence of granule protein (Fig. 2g, h).

The result of incubating *S. aureus* and *E. coli* in the presence of 1 and 5 µM HOCl at pH 7.5, 6.5 or 5.5 is illustrated in Fig. 3(a–d). This agent was rapidly lethal. However, when added

to bacteria in the presence of granule proteins no killing was evident (result not shown). No killing was seen even when 1 mM HOCl was used at pH 7.5 and 5.5 in the presence of granule protein (Fig. 3e–h).

### Bactericidal effects of the MPO system

Bacterial killing by the MPO/H<sub>2</sub>O<sub>2</sub>/Cl<sup>-</sup> system was assessed using purified neutrophil granule MPO. Bacteria were washed and suspended in PBS at pH 5.5, 6.5 and 7.5 containing MPO (5 mg ml<sup>-1</sup>) and reactions were started by the addition of 0.1, 1, 10 or 100 mM H<sub>2</sub>O<sub>2</sub>. The bactericidal effect proved to be dependent upon the concentration of H<sub>2</sub>O<sub>2</sub> and the pH. In the presence of 0.1 (result not shown) and up to 1 mM H<sub>2</sub>O<sub>2</sub> no bacterial killing was observed despite the low pH of 5.5 and the presence of Cl<sup>-</sup> (Fig. 4a–c). With the use of 10 mM H<sub>2</sub>O<sub>2</sub> there was a marked bactericidal effect at pH 5.5 and 6.5. Finally, the addition of 100 mM H<sub>2</sub>O<sub>2</sub> resulted in total killing of bacteria at low pH.

Quantification of HOCl production by MPO under exactly the same experimental conditions showed the *in vitro* activity of MPO to be pH- and H<sub>2</sub>O<sub>2</sub>-dependent (Table 1), with HOCl production increasing with decreasing pH and increasing H<sub>2</sub>O<sub>2</sub> concentration. Most of the HOCl was produced within the first few seconds after the addition of H<sub>2</sub>O<sub>2</sub>.

MPO inhibited killing of bacteria by high concentrations of H<sub>2</sub>O<sub>2</sub> at physiological pH. A pH of 7.5 and 100 mM H<sub>2</sub>O<sub>2</sub> resulted in a reduction of the bacterial colony count by 60% (Figs 2a and 4d). This was reduced to 10% or less in the presence of MPO (Fig. 4c, d). The lack of killing at pH 7.5 was coupled with low levels of HOCl production (Table 1). Maximally 1 mM HOCl was produced at pH 7.5, a concentration incapable of bacterial killing in the presence of granule protein (Fig. 3e–h).

### Identification of iodinated proteins

Proteins that were iodinated when neutrophils phagocytosed opsonized bacteria were cut from 2D PAGE gels, digested and identified by MALDI-TOF MS.

A variety of iodinated proteins were present in phagocytosing cells when compared with resting cells (Fig. 5). At least 40 spots, varying from very high molecular masses to about 8 kDa, became apparent (Fig. 5c, d). The identity of the iodinated spots is shown in Table 2. They mainly belonged to the contents of the azurophilic and specific granules (mainly lactoferrin, lysozyme, gelatinase-associated lipocalin and lysozyme). Other neutrophilic intracellular cytoskeletal proteins (profilin, annexin and actin) and plasma proteins including fibrinogen and fibrin were also identified. In contrast, the main bacterial-associated protein was the opsonizing IgG. Most of the iodinated proteins appear to be located within the phagocytic vacuole, or in the case of the cytoskeletal proteins, just surrounding it. Proteins like haemoglobin and fibrinogen would either be iodinated after their uptake with engulfed particles, or iodinated by MPO and H<sub>2</sub>O<sub>2</sub> secreted into the extracellular space.

Iodination of granular and cytosolic proteins, as well as extracellular human proteins, was much more obvious than that of bacterial proteins. Only if a great excess of bacteria ( $2.5 \times 10^9$  c.f.u. ml<sup>-1</sup>) was used was the iodination of outer-membrane proteins (OMP-A, OMP-NMPC) and other enzymes of *E. coli* observed (Table 2). At such high numbers of bacteria to neutrophils (100 : 1), frustrated phagocytosis takes place (Henson, 1971) with degranulation and H<sub>2</sub>O<sub>2</sub> release to the outside of the cell, and under these conditions iodination of these organisms is probably occurring in the extracellular medium.

There is no doubt that the generation of ROS is essential for efficient killing of bacteria (Klebanoff, 1967a, 1968) and fungi (Lehrer, 1969) by neutrophils. The question is how these ROS accomplish this. The current view is that HOCl formed by oxidation of Cl<sup>-</sup> by H<sub>2</sub>O<sub>2</sub>

plays a primary role in this killing. However, initial experiments to demonstrate the toxicity of the MPO/H<sub>2</sub>O<sub>2</sub>/Cl<sup>-</sup> system were performed with very low concentrations of enzyme. MPO was used in the range of 50 µg (Thomas, 1979; Kettle & Winterbourn, 1988) rather than the 100-fold higher concentration present within the vacuole (Reeves *et al.*, 2002). Most importantly, the pH was 5.0-5.5 (Klebanoff, 1967b, 1968, 1970) or less (Belding & Klebanoff, 1970), rather than the 7.6-8.0 that pertains in the vacuole (Segal *et al.*, 1981; Cech & Lehrer, 1984; Jiang *et al.*, 1997). In this study, 100 mM H<sub>2</sub>O<sub>2</sub> and up to 5 µM HOCl demonstrated bactericidal activity, which decreased significantly with increasing pH, an effect related to the higher activity of HOCl than OCl<sup>-</sup> (Dychdala, 2001).

Markedly more pronounced than the influence of pH are physiological concentrations of granule proteins (which include about 20% MPO) or pure MPO. Oxidants like HOCl are known to react with thio groups, thioethers, and aliphatic or aromatic groups (Test *et al.*, 1984). Most of these reactions lead to an immediate loss in oxidative capacity resulting in the loss of microbicidal properties. *in vitro* experiments employing a lower granule protein concentration (25 mg ml<sup>-1</sup>) than that present within the phagocytic vacuole strongly suggest that the enormous amount of protein will consume the available HOCl immediately *in vivo*. Thus estimates of approximately 28 µM HOCl production (Jiang *et al.*, 1997) would be totally ineffective against bacteria within the confines of the vacuole.

Furthermore, the target proteins of iodination reactions are largely those of the engulfing neutrophil rather than the microbial prey. This was demonstrated by results of this, and a previous study (Segal *et al.*, 1983). Regarding chlorination, Chapman *et al.* (2002) established that 94% of the total chlorinated tyrosine residues formed during phagocytosis were those of neutrophil proteins.

How do these new data fit in with the current dogma on the role of ROS in microbial killing? Confirmation of the involvement of MPO in the killing process was made through the use of MPO knockout mice (Aratani *et al.*, 1999), in which killing of *Candida albicans* was defective. However, deficiency of MPO is a common condition in humans and does not lead to obvious susceptibility to bacterial infection (Forehand *et al.*, 1995). Therefore an alternative system must dominate to compensate for this deficiency. A more recent study using elastase- and cathepsin-G-deficient mice showed that killing of *C. albicans* was grossly defective despite perfectly normal iodination (Reeves *et al.*, 2002), implicating granule proteases and questioning the conventional theory of MPO action.

Doubt has also been cast on another aspect of oxidative killing. It was thought that patients with CGD were more susceptible to catalase-positive microbes because the catalase-negative organisms generated H<sub>2</sub>O<sub>2</sub> as substrate for MPO-mediated halogenation (Mandell & Hook, 1969), thereby providing the substrate for their own destruction. However, catalase-deficient *S. aureus* (Messina *et al.*, 2002) and *Aspergillus nidulans* (Chang *et al.*, 1998) were shown to be at least as virulent as the catalase-positive variety in a mouse model of CGD.

An alternative role for MPO has been suggested in which it protects the microbicidal enzymes against oxidative damage (Reeves *et al.*, 2002) by ROS. In addition to its peroxidase activity, MPO can also act as a catalase. This latter role may dominate under the alkaline conditions in the vacuole, in which the concentration of H<sub>2</sub>O<sub>2</sub> is high and where the catalase activity of MPO can be constantly regenerated through the reduction by O<sub>2</sub><sup>-</sup> (Kettle & Winterbourn, 2001). This theory is supported by the observation that HOCl decreased markedly the activity of proteolytic enzymes (Schiller *et al.*, 2000). Similarly, mechanisms of oxidant denaturation of degradative enzymes could explain the predisposition to atherosclerosis seen in MPO-deficient mice (Brennan *et al.*, 2001). In conclusion, results

obtained support the novel concept that the function of the neutrophil oxidative pathway is to provide optimal conditions for bacterial killing by proteases rather than their direct oxidative destruction.

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## Abbreviations

<b>CGD</b>	chronic granulomatous disease
$O_2^-$	superoxide
<b>MALDI-TOF</b>	matrix-assisted laser desorption ionization time-of-flight
<b>MPO</b>	myeloperoxidase
<b>ROS</b>	reduced oxygen species.

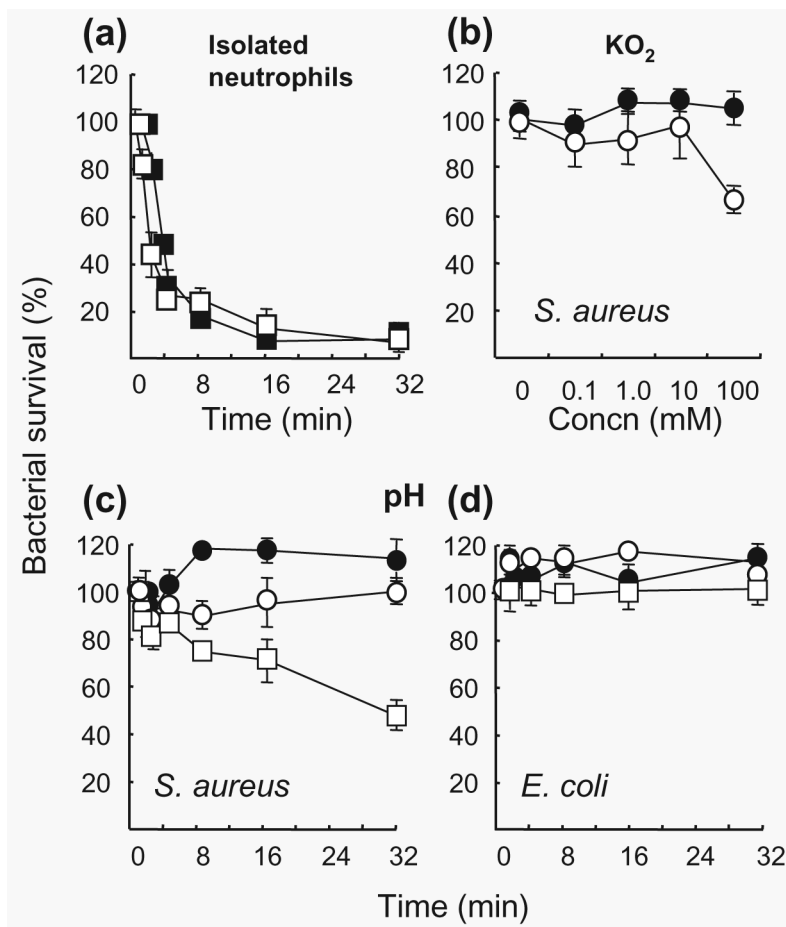
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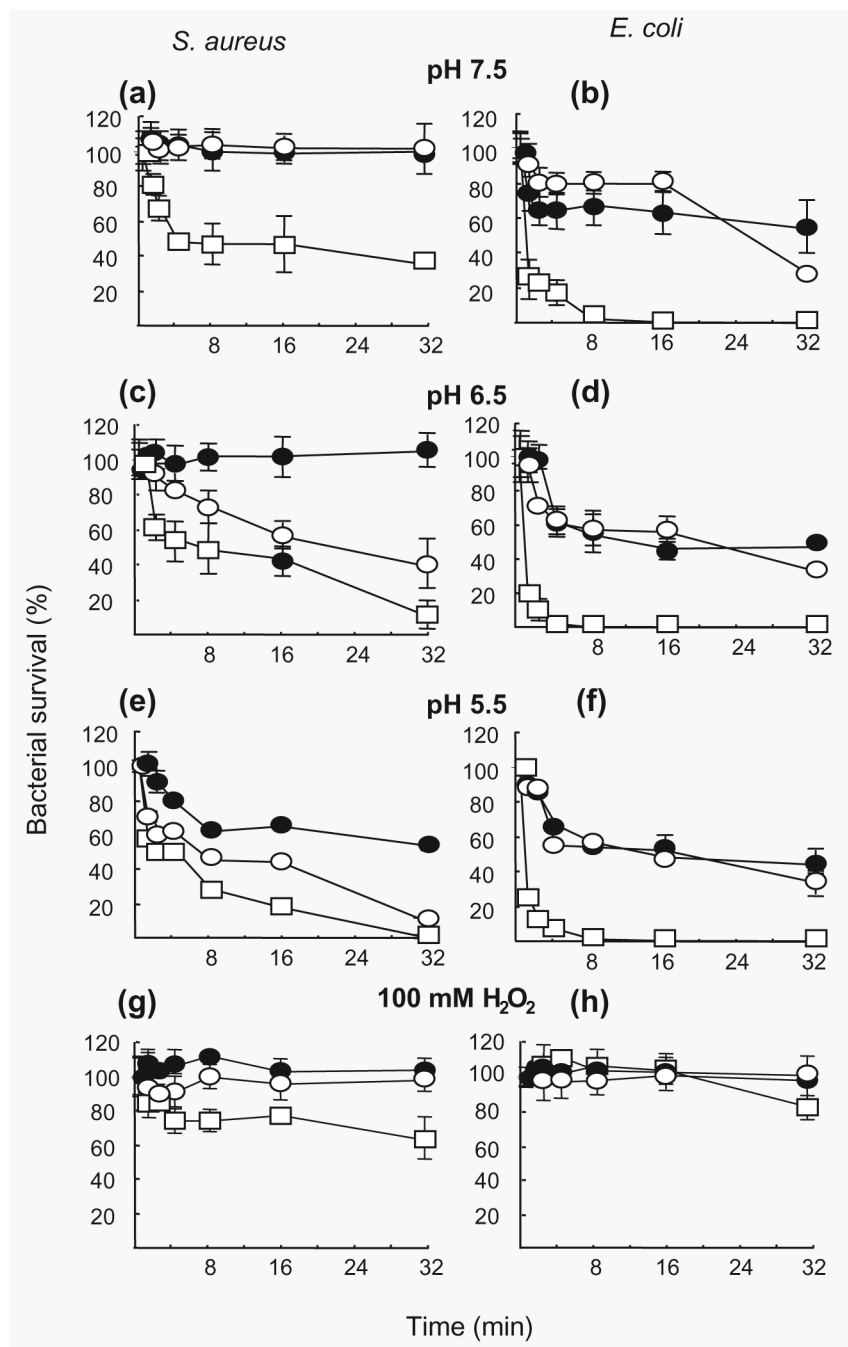
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**Fig. 1.**

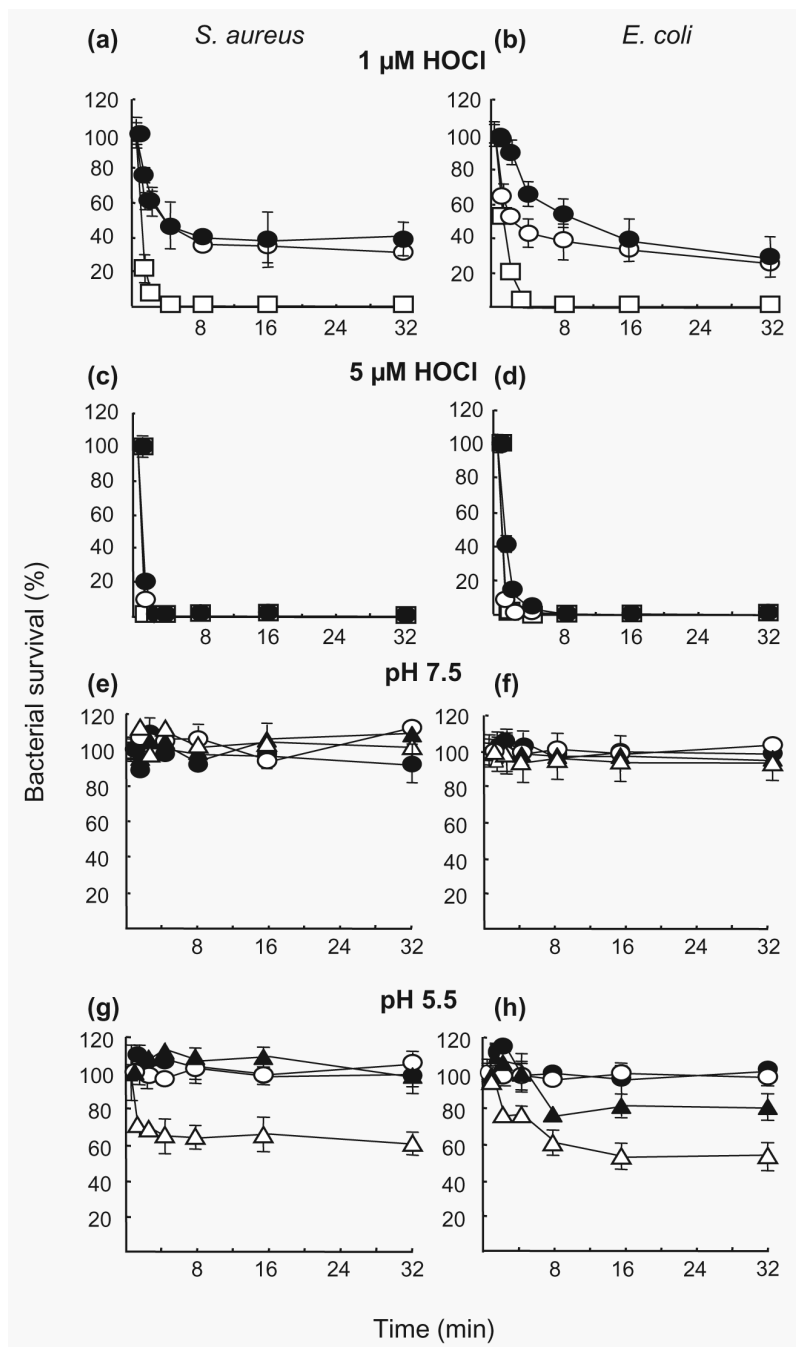
Effect of pH on bacterial viability, destruction by stimulated neutrophils and bactericidal effects of  $O_2^-$ . (a) IgG opsonized *S. aureus* (■) or *E. coli* (□) ( $1 \times 10^8$  c.f.u.  $ml^{-1}$ ) was mixed at a ratio of one target organism to five neutrophils in 1 ml PBS (pH 7.3) for the indicated periods of time and bacterial viability was determined. The mean ( $\pm$ SE) of three experiments is shown. No significant difference was observed between killing of *S. aureus* and *E. coli*.

(b) Bactericidal effect of  $O_2^-$  was determined by suspending *S. aureus* ( $1 \times 10^7$  c.f.u.) in 0.01 M phosphate buffer (pH 7.5) (●) or buffer containing different concentrations of  $KO_2$  (○).

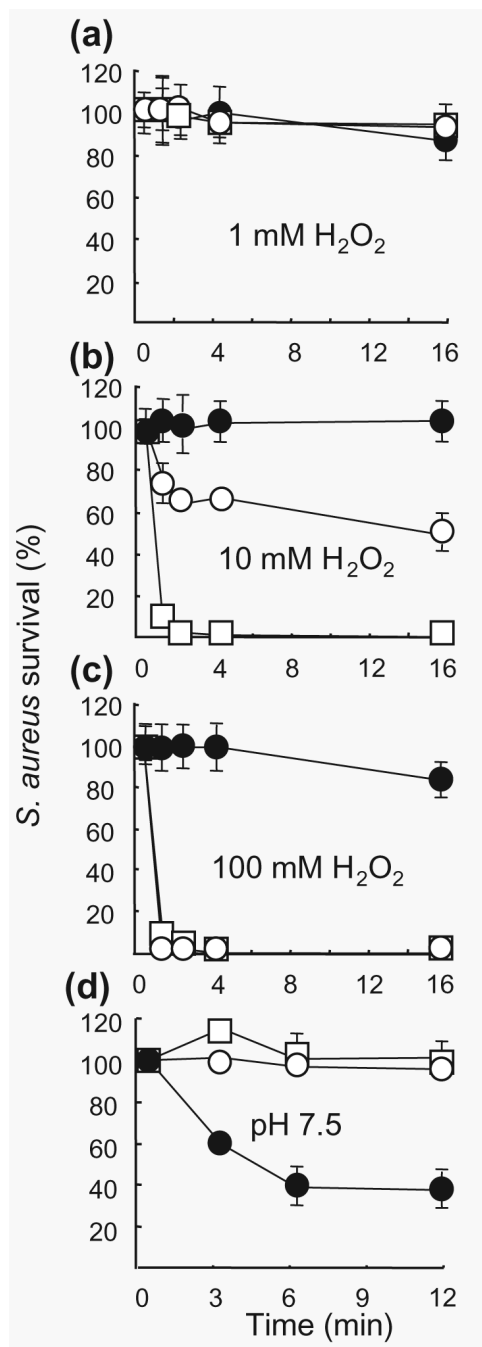
Each point is the mean of triplicate experiments ( $\pm$ SE). (c, d) To determine the effect of pH on bacterial viability, *S. aureus* or *E. coli* ( $1 \times 10^7$  c.f.u.  $ml^{-1}$ ) was incubated at 37 °C in 0.01 M phosphate buffer, pH 5.5 (□), 6.5 (○) or 7.5 (●). Reduction in survival of *S. aureus* at pH 5.5 compared to 6.5 was found to be significant,  $P < 0.033$ .



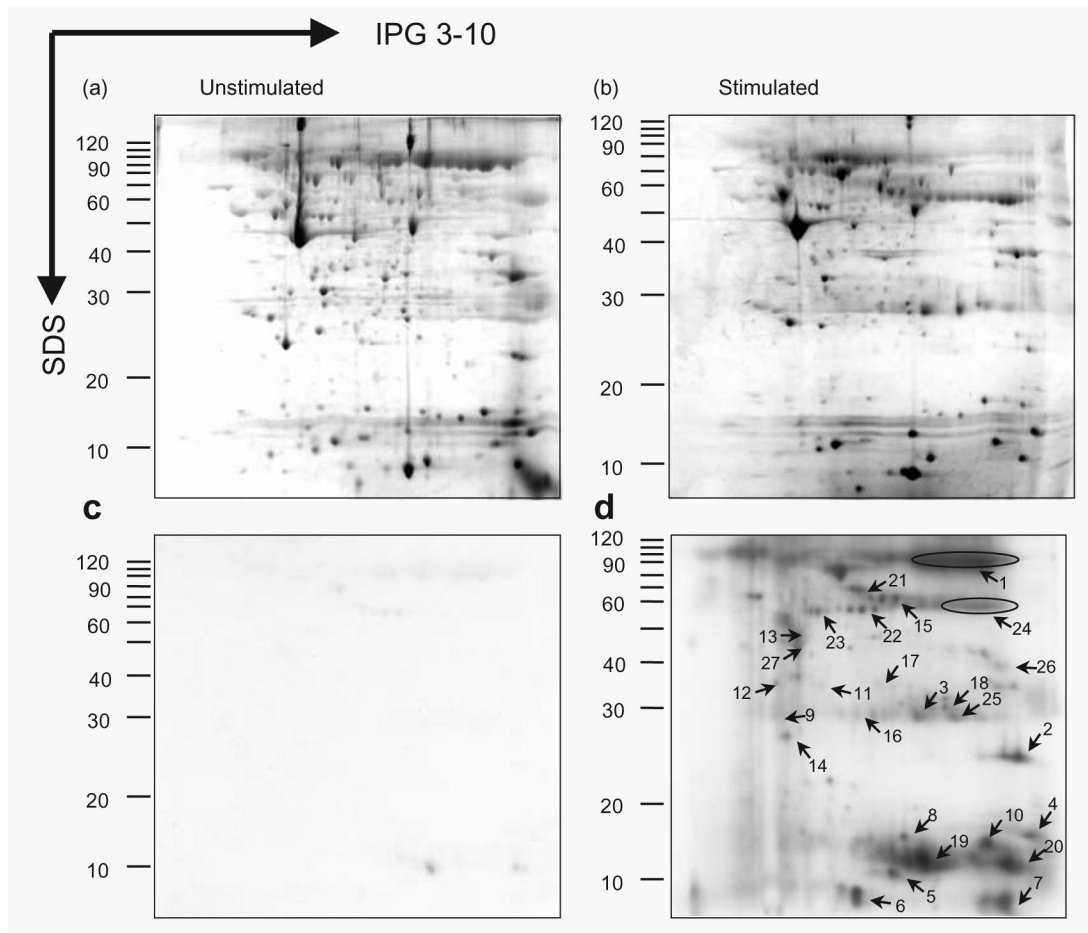
**Fig. 2.** Bactericidal activity of H<sub>2</sub>O<sub>2</sub> in the presence and absence of granule protein. The reaction mixture contained *S. aureus* (a, c, e) or *E. coli* (b, d, f) ( $2 \times 10^7$  c.f.u. ml<sup>-1</sup>) in 0.01 M phosphate buffer at pH 5.5, 6.5 or 7.5 with 1 (●), 10 (○) or 100 (□) mM H<sub>2</sub>O<sub>2</sub> added at 37 °C. Aliquots were removed at the times indicated. The experiment was repeated with 100 mM H<sub>2</sub>O<sub>2</sub> and granule protein (g, h) (25 mg ml<sup>-1</sup>) at pH 7.5 (●), 6.5 (○) or 5.5 (□). Each value is derived from triplicate plating. The mean values ( $\pm$ SE) from four experiments are shown. Changes in viability greater than 50% were always significant ( $P < 0.05$ ).



**Fig. 3.** Bactericidal activity of HOCl in the presence and absence of granule protein. The reaction mixture 0.01 M phosphate buffer, pH 7.5 (●), 6.5 (○) or 5.5 (□), contained *S. aureus* (a, c) or *E. coli* (b, d) ( $2 \times 10^7$  c.f.u. ml<sup>-1</sup>) and 1 or 5  $\mu\text{M}$  HOCl. Inhibition of killing of *S. aureus* (e, g) ( $2 \times 10^7$  c.f.u. ml<sup>-1</sup>) or *E. coli* (f, h) by HOCl was observed in 0.01 M phosphate buffer, pH 7.5 or 5.5, with added granule protein. Bacteria ( $2 \times 10^7$  c.f.u. ml<sup>-1</sup>) were exposed to 100  $\mu\text{M}$  (●), 250  $\mu\text{M}$  (○), 500  $\mu\text{M}$  (▲) or 1 mM ( $\Delta$ ) HOCl in the presence of granule protein (25 mg ml<sup>-1</sup>). Each line is representative of the mean ( $\pm$ SE) of three experiments.



**Fig. 4.** Bacterial killing by the MPO/H<sub>2</sub>O<sub>2</sub>/Cl<sup>-</sup> system. *S. aureus* ( $1 \times 10^7$  c.f.u. ml<sup>-1</sup>) in 0.01 M phosphate buffer at pH 7.5 (●), 6.5 (○) or 5.5 (□) was mixed with MPO (5 mg ml<sup>-1</sup>). H<sub>2</sub>O<sub>2</sub> at a concentration of 1 (a), 10 (b) or 100 (c) mM was added. (d) MPO itself had no effect on bacterial viability (□), whilst the bactericidal effect of 100 mM H<sub>2</sub>O<sub>2</sub> (●) was prevented in the presence of MPO (5 mg ml<sup>-1</sup>) (○) at pH 7.5. Each line is representative of the mean ( $\pm$ SE) of three experiments.



**Fig. 5.** 2D gel electrophoresis and autoradiographs of neutrophils before and after phagocytosis of *S. aureus*. Neutrophils ( $1 \times 10^7$ ) in 1 ml PBS (pH 7.3) containing  $100 \mu\text{Ci}$  ( $3700 \text{ kBq}$ )  $^{125}\text{I}$  were mixed in a rapidly stirring oxygenated chamber at  $37^\circ\text{C}$  without (a, c) or with (b, d) IgG opsonized *S. aureus* ( $1 \times 10^8$  c.f.u.). After 4 min the suspension was taken into 10% TCA. Coomassie blue 2D stained gels (a, b) and corresponding autoradiographs (c, d) (216 h exposure) are shown. Iodinated proteins (labelled 1–27) were excised from the SDS gel and identified.

**Table 1**  
**Quantification of hypochlorite production**

Hypochlorite (mM) produced by purified MPO (5 mgml<sup>-1</sup>) in PBS at pH values of 5.5, 6.5 and 7.5 and in the presence of 1, 10 and 100 mM H<sub>2</sub>O<sub>2</sub> at 37 °C. Measurements were made at 0.3, 2.0 and 16 min. Mean values (±SE) of three independent experiments. ND, None detected.

H <sub>2</sub> O <sub>2</sub> (mM)	Minutes	pH 7.5	pH 6.5	pH 5.5
1	0.3	0.09 ± 0.16	0.35 ± 0.12	0.19 ± 0.11
	2.0	0.02 ± 0.16	0.23 ± 0.12	0.24 ± 0.19
	16.0	ND	0.28 ± 0.12	0.22 ± 0.08
10	0.3	1.14 ± 0.22	2.02 ± 0.79	4.84 ± 0.09
	2.0	0.99 ± 0.10	1.86 ± 0.57	4.44 ± 0.09
	16.0	1.30 ± 0.05	1.57 ± 0.16	3.49 ± 0.09
100	0.3	1.29 ± 0.40	14.80 ± 1.13	13.26 ± 1.58
	2.0	0.18 ± 0.37	8.64 ± 0.59	7.61 ± 1.32
	16.0	0.82 ± 0.26	6.80 ± 0.55	4.16 ± 0.37

**Table 2**  
**List of proteins that were iodinated following phagocytosis of *S. aureus* or *E. coli* by isolated neutrophils**

Iodinated proteins were trypsin-digested and mass-spectrometric peptide maps were acquired. Proteins were identified by comparing mass fingerprints to the NCBI database.

Neutrophilic	Other human	Bacterial
<b>Granule-associated</b>	14. GDP-dissociation inhibitor	<i>S. aureus</i>
1. Lactoferrin	15. Glucose-6P-dehydrogenase	27. Ornithine transcarbamoylase
2. Gelatinase-associated lipocalin	16. Glutathione-S-transferase P	
3. Cathepsin G	17. Esterase D	<i>E. coli</i> *
4. Lysozyme	18. Phosphoglycerate mutase 1	Outer-membrane protein A
5, 6. Calgranulin A	19. Haemoglobin beta	OMP-NMPC
7. Elastase fragment	20. Haemoglobin alpha 2	OMP W precursor
8. Myeloperoxidase	21. Albumin	Superoxide dismutase
	22. Fibrin	Malate dehydrogenase
<b>Cytoskeletal</b>	23. Fibrinogen	Asparaginase
9. Grancalcin	24, 25. IgG	Hydroperoxide reductase
10. Profilin	26. Fructose-bisphosphate aldolase A	Fructose-bisphosphate aldolase
11. Annexin III		Chaperonin CPN10
12. Annexin V		
13. Actin		

\* These proteins are not shown on the gel.