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Received 10 January 2003
Accepted 28 March 2003

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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During phagocytosis, neutrophils undergo a burst of respiration in which oxygen is reduced to superoxide (O2·−), which dismutates to form H2O2. Myeloperoxidase (MPO) is discharged from the cytoplasmic granules into the phagosome following particle ingestion. It is thought to utilize H2O2 to oxidize halides, which then react with and kill ingested microbes. Recent studies have provided new information as to the concentration of O2·− and proteins, and the pH, within the vacuole. This study was conducted to examine the antimicrobial effect of O2·−, H2O2 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 of125I, 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 O2·−, the product of the univalent reduction of oxygen (Babior et al., 1973). O2·− has minimal antibacterial activity (Rosen & Klebanoff, 1979; Hurst & Barrette, 1989) and dismutates to produce H2O2. H2O2 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 O2·−, 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 O2·−, H2O2, and products of chloride oxidation (HOCl), under these conditions.125I 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 × 10^6) were incubated at 37 °C in 1 ml PBS (140 mM NaCl, 10 mM KCl, 10 mM NaH2PO4, 5 mM glucose, pH 7·3) in a rapidly stirred chamber. IgG opsonized S. aureus (NCTC 12981) or E. coli (ATCC 11775) (1 × 10^6 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 (±S.D.) 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 10 and 100 mM H2O2 in PBS at pH 5.5, 6.5 and 7.5 without MPO were added and diluted 1/10 in ice-cold LB broth (Difco). Serial tenfold dilutions were then made, and plated in triplicate on LB agar plates. Surviving bacteria were enumerated by Segal et al. (1981) omitting lysostaphin. Results were calculated as the mean (±S.D.) 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.

In vitro killing of S. aureus and E. coli by H2O2, HOCl and superoxide (KO2). From an overnight culture, bacteria (2 × 10^8 c.f.u. ml^-1) 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 H2O2 (1-0, 10 or 100 mM) or HOCl (1 or 5 mM) 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 H2O2 and up to 1 mM HOCl in the presence of a mixture of azurophil and specific demembranated granules (25 mg ml^-1). Membranes were removed by Percoll granule disruption as described by Vila 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^-1. Bacteria (2 × 10^8 c.f.u. ml^-1) were added to the granule protein prior to the addition of H2O2 or HOCl. Killing of bacteria by O2 was performed similarly to that described for H2O2 and HOCl. As a source of O2, KO2 was employed and added as a powder to the reactions. Since concentrations of KO2 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 KO2, which resulted in a rise in the pH to approximately 7-5.

Iodination studies. Iodination studies were performed as described by Klebanoff & Clark (1976). Neutrrophils (1 × 10^6) were resuspended in 1 ml PBS supplemented with 40 mM KI and 100 μC (3700 Bq) 125I. 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 IgGopsonized 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 thiourea, 50 mM HEPES, pH 8·0) and the cell mixture was placed in a magnetically stirring oxygenated chamber at 37 °C for 10 min. The cell mixture was taken into 1 ml cold PBS containing 10 % trichloroacetic acid (TCA). This experiment was also carried out with IgGopsonized E. coli, added at a ratio of 100:1.

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 (clips 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, 
Msfit 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 O₂, H₂O₂ and HOCl

The bactericidal effect of increasing concentrations of O₂⁻ was investigated at pH 7·5 after 6 min. Fig. 1(b) shows that 
O₂⁻ itself is relatively non-toxic.

Fig. 2(a–f) shows the killing of S. aureus and E. coli exposed to 
increasing concentrations of H₂O₂ at pH values of 7·5, 6·5 or 
5·5. The bactericidal effect of H₂O₂ was both dose- and pH-
dependent. As the pH was elevated to 7·5, a concentration of 
100 mM H₂O₂ was required to reduce the survival of S. 
aureus by 50 %. The effect of 100 mM H₂O₂ 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/H₂O₂/Cl⁻ 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⁻¹) and reactions were started by 
the addition of 0·1, 1, 10 or 100 mM H₂O₂. The bactericidal 
effect proved to be dependent upon the concentration of 
H₂O₂ and the pH. In the presence of 0·1 (result not shown) 
and up to 1 mM H₂O₂ no bacterial killing was observed 
despite the low pH of 5·5 and the presence of Cl⁻ (Fig. 4a–c).

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With the use of 10 mM H₂O₂ there was a marked bactericidal effect at pH 5.5 and 6.5. Finally, the addition of 100 mM H₂O₂ 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 H₂O₂-dependent (Table 1), with HOCl production increasing with decreasing pH and increasing H₂O₂ concentration. Most of the HOCl was produced within the first few seconds after the addition of H₂O₂.

MPO inhibited killing of bacteria by high concentrations of H₂O₂ at physiological pH. A pH of 7.5 and 100 mM H₂O₂ 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 H₂O₂ 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

![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 × 10⁷ c.f.u. ml⁻¹) and 1 or 5 µM HOCl. Inhibition of killing of S. aureus (e, g) (2 × 10⁷ c.f.u. ml⁻¹) 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 × 10⁷ c.f.u. ml⁻¹) were exposed to 100 µM (●), 250 µM (○), 500 µM (△) or 1 mM (△) HOCl in the presence of granule protein (25 mg ml⁻¹). Each line is representative of the mean (±SE) of three experiments.](http://jmm.sgmjournals.org)
enzymes of membrane proteins (OMP-A, OMP-NMPC) and other
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3 (2·5
3 (1

Fig. 4. E. P. Reeves and others
effect of 100 mM H2O2 (itself had no effect on bacterial viability (h
concentration of 1 (a), 10 (b) or 100 (c) mM was added. (d) MPO
(5 mg ml
SE) of three experiments.

Table 1. Quantification of hypochlorite production
Hypochlorite (mM) produced by purified MPO (5 mg ml
) in PBS at
pH values of 5·5, 6·5 and 7·5 and in the presence of 1, 10 and 100 mM
H2O2 at 37°C. Measurements were made at 0·3, 2·0 and 16·0 min. Mean
values (±SE) of three independent experiments. ND, None detected.

<table>
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<tr>
<th>H2O2 (mM)</th>
<th>Minutes</th>
<th>pH 7·5</th>
<th>pH 6·5</th>
<th>pH 5·5</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0·3</td>
<td>0·09 ± 0·16</td>
<td>0·35 ± 0·12</td>
<td>0·19 ± 0·11</td>
</tr>
<tr>
<td></td>
<td>2·0</td>
<td>0·02 ± 0·16</td>
<td>0·23 ± 0·12</td>
<td>0·24 ± 0·19</td>
</tr>
<tr>
<td></td>
<td>16·0</td>
<td>ND</td>
<td>0·28 ± 0·12</td>
<td>0·22 ± 0·08</td>
</tr>
<tr>
<td>10</td>
<td>0·3</td>
<td>1·14 ± 0·22</td>
<td>2·02 ± 0·79</td>
<td>4·84 ± 0·09</td>
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<tr>
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<td>2·0</td>
<td>0·99 ± 0·10</td>
<td>1·86 ± 0·57</td>
<td>4·44 ± 0·09</td>
</tr>
<tr>
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<td>16·0</td>
<td>1·30 ± 0·03</td>
<td>1·57 ± 0·16</td>
<td>3·49 ± 0·09</td>
</tr>
<tr>
<td>100</td>
<td>0·3</td>
<td>1·29 ± 0·40</td>
<td>14·80 ± 1·13</td>
<td>13·26 ± 1·58</td>
</tr>
<tr>
<td></td>
<td>2·0</td>
<td>0·18 ± 0·37</td>
<td>8·64 ± 0·59</td>
<td>7·61 ± 1·32</td>
</tr>
<tr>
<td></td>
<td>16·0</td>
<td>0·82 ± 0·26</td>
<td>6·80 ± 0·55</td>
<td>4·16 ± 0·37</td>
</tr>
</tbody>
</table>

Fig. 4. Bacterial killing by the MPO/H2O2/Cl
 system. S. aureus
(1 × 10
 c.f.u. ml
) in 0·01 M phosphate buffer at pH 7·5 (○), 6·5
(C) or 5·5 (□) was mixed with MPO (5 mg ml
). H2O2 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 H2O2 (○) was prevented in the presence of MPO
(5 mg ml
) at pH 7·5. Each line is representative of the mean
(±SE) of three experiments.

(2·5 × 10
 c.f.u. ml
) 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 H2O2
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
 by H2O2 plays a primary role in
this killing. However, initial experiments to demonstrate the
toxicity of the MPO/H2O2/Cl
 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
physiological concentrations of granule proteins (which
rather than the 100-fold higher concentration present within
the phagocytic 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 H2O2 and up to 5 μM HOCI
demonstrated bactericidal activity, which decreased signifi-
cantly with increasing pH, an effect related to the higher
activity of HOCl than OCl
. (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 concentra-
tion (25 mg ml
) than that present within the phagocytic
vacuole strongly suggest that the enormous amount of
protein will consume the available HOCl immediately
vacuole strongly suggest that the enormous amount of
protein will consume the available HOCl immediately
vacuole (Segal et al., 1997) would be totally ineffective against
bacteria within the confines of the vacuole.

Furthermore, the target proteins of iodination reactions are

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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 H₂O₂ 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

**Fig. 5.** 2D gel electrophoresis and autoradiographs of neutrophils before and after phagocytosis of *S. aureus*. Neutrophils (1 × 10⁷) in 1 ml PBS (pH 7·3) containing 100 μCi (3700 kBq) from in (a–d) were mixed in a rapidly stirring oxygenated chamber at 37 °C without (a, c) or with (b, d) IgG opsonized *S. aureus* (1 × 10⁸ 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.

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

<table>
<thead>
<tr>
<th>Neutrophilic</th>
<th>Other human</th>
<th>Bacterial</th>
</tr>
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<tbody>
<tr>
<td>Granule-associated</td>
<td>14. GDP dissociation inhibitor</td>
<td><em>S. aureus</em></td>
</tr>
<tr>
<td>1. Lactoferrin</td>
<td>15. Glucose-6P-dehydrogenase</td>
<td>27. Ornithine transcarbamoylase</td>
</tr>
<tr>
<td>2. Gelatinase-associated lipocalin</td>
<td>16. Glutathione-S-transferase P</td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td>3. Cathepsin G</td>
<td>17. Esterase D</td>
<td></td>
</tr>
<tr>
<td>4. Lysosome</td>
<td>18. Phosphoglycerate mutase 1</td>
<td>Outer-membrane protein A</td>
</tr>
<tr>
<td>7. Elastase fragment</td>
<td>20. Haemoglobin alpha 2</td>
<td>OMP W precursor</td>
</tr>
<tr>
<td>9. Prion</td>
<td>22. Fibrin</td>
<td>Asparaginase</td>
</tr>
<tr>
<td>10. Profilin</td>
<td>23. Fibrinogen</td>
<td>Hydroperoxide reductase</td>
</tr>
<tr>
<td>11. Annexin III</td>
<td>24. 25. IgG</td>
<td>Fructose-bisphosphate aldolase</td>
</tr>
<tr>
<td>13. Actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*These proteins are not shown on the gel.

which the concentration of \(H_2O_2\) is high and where the cata
tase activity of MPO can be constantly regenerated
through the reduction by \(O_2^-\) (Kettle & Winterbourn, 2001). This theory is supported by the observation that
HOCl decreased markedly the activity of proteolytic enzymes
(Schiller et al., 2001). 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 oxida-
tive destruction.

**ACKNOWLEDGEMENTS**

We are grateful to The Wellcome Trust, the Chronic Granulomatous
Disease Research Trust and the Austrian Science Fund (grant no.
J-1845-MED) for providing financial support.

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