Biochimica et Biophysica Acta, 971 (1988) 266–274
Elsevier

BBA 12323

The bactericidal effects of the respiratory burst and the myeloperoxidase system isolated in neutrophil cytoplasts

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(Received 7 March 1988)

Key words: Neutrophil; Cytoplast; Myeloperoxidase; Bacterial killing; Respiratory burst

Neutrophil polymorphonuclear leucocytes kill bacteria by oxygen-dependent and oxygen-independent mechanisms. Many potentially toxic mechanisms have been described, but the complexity of the phagosomal environment and the synergy between oxidative and non-oxidative systems hamper the investigation of individual bactericidal mechanisms in whole cells. Neutrophil cytoplasts are greatly depleted of granule proteins and permit the investigation of the bactericidal effects of the respiratory burst in isolation. In this study they have been used to examine the role of the respiratory burst and myeloperoxidase in oxygen-dependent killing of *Staphylococcus aureus*. Cytoplasts generated oxygen radicals at comparable rates to human neutrophils and phagocytosed but did not kill *S. aureus*. The selective reconstitution of the myeloperoxidase-hydrogen peroxide-halide system by coating bacteria with myeloperoxidase conferred on cytoplasts the ability to kill intracellular bacteria. However, extracellular killing by diffusible bactericidal factors was not detected in this system.

Introduction

Myeloperoxidase (donor H_2O_2 oxidoreductase EC 1.11.1.7) comprises approx. 5% of the total cellular protein of neutrophils and monocytes [1] and plays an important role in the antimicrobial activity of these cells [2]. The antimicrobial actions of MPO are considered to depend on a variety of compounds formed by hydrogen peroxide-dependent oxidation of halogens, either to form halide radicals or, more importantly, a variety of other compounds including hypochlorous acid [3–8].

Other reactions of MPO may also be important in the phagosome. MPO can react directly with superoxide radical [9] and also reduces formation of hydroxyl radical [10] and could, thus, indirectly promote bacterial killing by protecting other granule components from oxidative damage [11,12]. Evidence derived from studies of patients with MPO deficiency, the use of inhibitors and the importance of hydrogen peroxide and chloride, have led to the general acceptance of the importance of halide oxidation, but a bactericidal action attributable to the MPO system alone has never been definitively demonstrated within the phagocytic vacuole under physiological conditions.

The reactions catalysed by MPO are critically dependent upon the concentration of hydrogen peroxide [9]. Hydrogen peroxide concentrations would be expected to be much higher intraphagosomally where the peroxide is generated in a con-

Abbreviation: MPO, neutrophil myeloperoxidase (donor H_2O_2 oxidoreductase, EC 1.11.1.7).

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fined space than after diffusion or spillage into the extracellular medium. At high concentrations of hydrogen peroxide, MPO could act as a catalase without an antimicrobial effect, whereas at low concentrations, MPO could act peroxidatively to kill microorganisms. Since MPO might have different mechanisms of action within the phagosome and in the extracellular medium the detection of the diffusible products of MPO peroxidation extracellularly does not necessarily reflect intraphagosomal activity. Such diffusible products may however be important in extracellular functions of MPO, which include the killing of microorganisms and control of inflammation [6–8, 13–15].

More direct evidence of peroxidative MPO activity within the phagosome has been sought by localising halogenation to this compartment [2,5], but the relevance of this reaction to killing is unclear [16–18]. Neutrophils in suspension in vitro halogenate mainly extracellular protein [19]. Intracellular halogenation is predominantly localised to components of the neutrophil itself rather than the ingested microbe [18].

Related questions have been raised concerning the mechanism of action of eosinophil peroxidase, an enzymatically and structurally distinct peroxidase [20–22]. This enzyme was adsorbed onto microorganisms and introduced into macrophage [23–25] and rabbit monocyte [26] phagosomes, which are devoid of peroxidase activity, and shown to kill *Staphylococci* [26] and parasites [23,24]. In one study, extracellular killing of peroxidase-coated organisms was also detected [23] and shown to require extracellular hydrogen peroxide. In these studies granule components were available to act synergistically with the peroxidase-mediated killing mechanisms.

This study was undertaken to examine selectively the antibacterial role of human MPO in the physiological environment within the phagosome and exposed to vacuolar concentrations of reduced oxygen species. To do this, cytoplasts were prepared from neutrophils by centrifugation [27] in order to separate the products of the respiratory burst from the granule constituents. Cytoplasts were shown to be incapable of killing the catalase-positive target organism *Staphylococcus aureus* (Oxford strain). The selective reintroduction of MPO crosslinked to the surface of the organisms, unequivocally demonstrated that this enzyme together with the products of the respiratory burst is capable of accomplishing bacterial killing within the phagocytic vacuole. The model was also used to provide information regarding the physiological hydrogen peroxide concentration within this compartment.

Materials and Methods

Normal human neutrophils and neutrophil cytoplasts

Neutrophils were prepared from buffy coat residues by dextran sedimentation, Ficoll Hypaque centrifugation, and hypotonic lysis. For comparison with cytoplasts in superoxide assays, control cells were incubated in 12.5% (w/v) Ficoll 70 with 20 μM cytochalasin B (Sigma) for 20 min and washed five times in heparin (5 IU/ml) saline. Cytoplasts were prepared by the method of Roos et al. [27] by discontinuous density-gradient centrifugation at 33°C on Ficoll 70 with 20 μM cytochalasin B, and washed five times in heparin saline. Smears of cells and cytoplasts were fixed in methanol and stained with Giemsa stain. Membrane integrity was assessed by Trypan blue exclusion and fluorescein diacetate staining. The cytoplast diameter in isotonic saline was measured by light microscopy (×1250) under oil immersion with a stage micrometer.

Myeloperoxidase purification

Neutrophil granules were prepared from leukaemic blood by homogenisation in 8% (w/v) sucrose with enzyme inhibitors and membranes were removed from the post-nuclear supernatant by discontinuous sucrose density-gradient centrifugation as previously described [28]. MPO was purified from granules according to Mathieson et al. [29] to a final absorbance ratio of 0.73. This MPO was dialysed against water and focused on a pH gradient in Ultrodex (LKB, Bromma, Sweden) containing 1% (w/v) ampholytes (pH 9–11) (Ampholine, LKB) in order to ensure minimum contamination by other microbial compounds. MPO focused at the extreme alkaline end of the gradient was eluted, washed and concentrated by filtration (Centricon 30, Amicon Corp., Lexington, MA). Sodium dodecyl sulphate polyacryl-

amide gel electrophoresis demonstrated two bands at 60 and 12 kDa, consistent with MPO [30].

Preparation of MPO-coated and control bacteria

MPO was radiolabelled with ^{125}I (85.3 kBq/pyrogallol unit MPO) by the iodogen method (Pierce Chemical Company, Rockford, IL) and separated from free isotope by gel filtration on Sephadex G25 (Pharmacia) without loss of activity. Inactivated MPO for control experiments was prepared from radiolabelled MPO by incremental addition of 0.5% (v/v) H_2O_2 in methanol [31], until less than 0.001% activity remained, and then dialysed. Active and inactivated radiolabelled MPO was covalently bound to the surface of opsonised *S. aureus* with sulphosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Pierce) according to Christiaansen et al. [32].

Protein and enzyme assays

Protein was assayed by the BCA assay system (Pierce) except in the presence of Ficoll when the Bradford (Bio-Rad, München, F.R.G.) system was used, after solubilisation in 0.1% aqueous Triton X-100 (Sigma). MPO was assayed by guaiacol (Sigma) oxidation according to Chance and Maehly [33] and the results were converted to pyrogallol units by comparison with horseradish peroxidase type 2 (Sigma) as standard. Lysozyme was assayed according to Klass et al. [34] using egg white lysozyme (Sigma) as standard. β-Glucuronidase was assayed according to Rest et al. [35] using 4-methylumbelliferone (Sigma) as standard.

Superoxide production

Superoxide production was assessed quantitatively by superoxide dismutase-inhibitable cytochrome *c*-reduction as previously described [36], using either phorbol myristate acetate (Sigma) 100 ng/ml or opsonised bacteria at various cell-to-target ratios as stimulus. Cytoplasts and control cells (as above) were tested in the presence of 20 μM cytochalasin B. The number of cytoplasts responding was determined by light microscopy after reduction of 0.05% (w/v) nitroblue tetrazolium (Sigma) in RPMI 1640 medium for 30 min at 37°C using 100 ng/ml phorbol myristate acetate as a stimulus.

Iodination

Iodination was determined by a modification of the method of Klebanoff [4]. Active and inactivated MPO-coated *S. aureus* were added at various target-to-cell ratios to neutrophils (10^8 per ml) or cytoplasts ($5 \cdot 10^8$ per ml) preincubated with 740 kBq/ml ^{125}I (as NaI spec. act. 579 MBq ^{125}I/μg iodine, Amersham International, Amersham, U.K.) in RPMI 1640 medium with 10 mM glucose and 1 mg/ml bovine serum albumin (Sigma) as acceptor protein. Samples were precipitated, washed four times in ice-cold 10% (w/v) trichloroacetic acid (Fisons, Loughborough, U.K.) in 100 mM KI and precipitated ^{125}I was quantified in an LKB 1282 Gamma counter. Control incubations to assess resting rates were carried out at 37°C and background iodination assessed at 4°C was subtracted from all values.

Bacteria

S. aureus (Oxford, minimum bactericidal concentration 31.25 mIU benzyl penicillin/ml) was grown and radiolabelled with [^{3}H]glucose as previously described [37]. Antibiotic-resistant *S. aureus* (clinical isolate, minimum bactericidal concentration 16 IU benzyl penicillin/ml) was ^{14}C-labelled by growth overnight in one quarter strength nutrient broth supplemented with 0.1 IU/ml benzyl penicillin (Crystapen, Glaxo, Greenford, U.K.) and 14.8 GBq/l (U-^{14}C)-labelled protein hydrolysate (Amersham) spec. act. 2.10 GBq/matom carbon. Bacteria were opsonised as previously described [37] and washed and aspirated through a 25 gauge needle to disrupt clumps before use.

Cell-free MPO-H_2O_2-Cl system

S. aureus coated with active or inactivated MPO were suspended at 10^6 colony forming units/ml in various concentrations of H_2O_2 in 0.15 M NaCl and incubated at 37°C for 10 min, cooled, diluted serially in distilled water and viability assessed by a drop counting technique. The H_2O_2 sensitivity of active MPO-coated *S. aureus* surviving incubation with cytoplasts in killing assays was assessed similarly following lysis of a sample in distilled water.

Phagocytosis and killing assay

Phagocytosis and killing by neutrophils (10^8/ml) and cytoplasts ($5 \cdot 10^8$/ml) was assayed in suspension as described previously [37] at a target-to-neutrophil ratio of 1 : 1 and a target-to-cytoplast ratio of 1 : 5. Phagocytosis was assessed by solubilisation of tritium from extracellular bacteria using lysostaphin and total killing was assessed by drop counting. Phagocytosis and killing of extracellular *S. aureus* were assayed similarly using additional unopsonised penicillin-resistant *S. aureus*, double-label counting and drop counting on blood agar plates containing 0.1 IU/ml benzyl penicillin.

Electron microscopy and histochemistry

Samples for electron microscopy were fixed in Karnovsky fixative for 30 min, postfixed in 2% (w/v) OsO_4 for 30 min and dehydration was commenced in 30% (w/v) ethanol. Samples were pelleted in high gelling temperature agarose (ICN Biochemicals Ltd., High Wycombe, U.K.) dehydrated and embedded in LR White resin (London Resin Company, London, U.K.). Ultrathin sections stained with lead citrate/NaOH were examined in a Philips EM 400 transmission electron microscope. Samples for MPO histochemistry were incubated in 0.05% (w/v) diaminobenzidine (Sigma) and 0.01% (v/v) H_2O_2 in 0.1 M Tris buffer (pH 7.6) for 10 min following fixation, and then washed and processed as above.

Statistical analysis

Statistical comparisons were made using the Student *t*-test. Regression lines were derived and slopes were analysed by least-squares regression.

Results

Neutrophils and neutrophil cytoplasts

Cell suspensions for cytoplast preparation contained greater than 95% neutrophils with greater than 98% excluding Trypan blue. The efficiency of enucleation was 30% (±4.1 S.E., $n = 17$). Mean cytoplast diameter in isotonic saline was 5 μm and greater than 98% excluded Trypan blue and 91% concentrated fluorescein.

Residual enzyme content of cytoplasts was similar to that previously reported [27,38,39], with

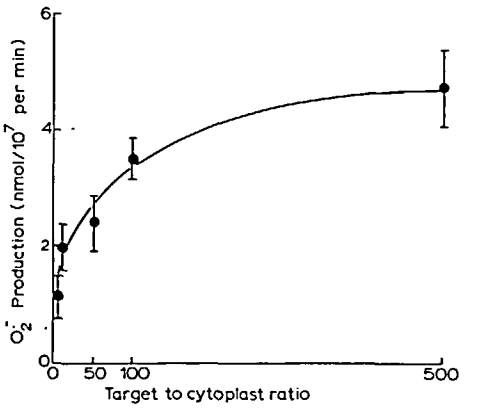

Fig. 1. Maximum rate of superoxide production by cytoplasts ($5 \cdot 10^7$/ml) after addition of *S. aureus* opsonised with mixed human immunoglobulins at various target-to-cell ratios. The mean (±S.E.) of ten experiments is shown.

1.25% (±0.15 S.E.) MPO, 4.6% (±0.53 S.E.) lysozyme and 5.7% (±0.6 S.E., $n = 6$) β-glucuronidase from the neutrophils remaining in the cytoplasts. 78% of the cytoplasts reduced sufficient nitroblue tetrazolium to produce formazan deposits visible by light microscopy after stimulation with phorbol myristate acetate. Maximal rates of superoxide production measured by superoxide dismutase-inhibitable cytochrome *c*-reduction in response to phorbol myristate acetate were 27.5 ± 14.9 S.E. nmol/10^7 cytoplasts per min ($n = 10$) and 99.9 ± 14.9 S.E. nmol/10^7 cells per min ($n = 16$). The superoxide production in response to immunoglobulin opsonised *S. aureus* at various target-to-cell ratios is shown in Fig. 1.

Phagocytosis and killing

The kinetics of phagocytosis and killing of *S. aureus* by normal control cells and cytoplasts are shown in Fig. 2, and the correlation between killing and phagocytosis in cells is shown in Fig. 3. Neutrophils rapidly phagocytosed opsonised *S. aureus*. Cytoplasts phagocytosed to a significant but more limited degree at the lower target-to-cell ratio used and no significant killing of *S. aureus* was seen. Aggregation of cytoplasts prevented continuation of the assay beyond 30 min.

Intracellular killing by the reconstituted MPO-H_2O_2-halide system

MPO was crosslinked to *S. aureus* with an efficiency of 17.2% for the active MPO and 13.2%

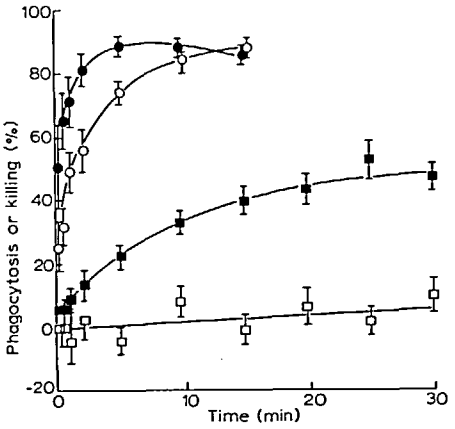

Fig. 2. Phagocytosis (filled symbols) and killing (open symbols) by neutrophils (10^8/ml, target-to:cell ratio, 1:1) (●, ○) and cytoplasts ($5 \cdot 10^8$/ml, target-to-cytoplast ratio, 1:5) (■, □) of immunoglobulin-opsonised *S. aureus*. The mean (±S.E.) of ten experiments is shown.

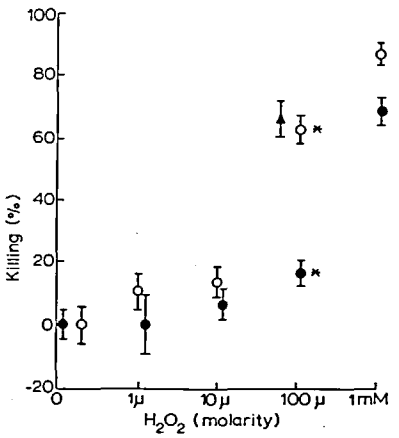

Fig. 4. Killing of *S. aureus* coated with active (○) and inactivated (●) MPO by various concentrations of H_2O_2 in 150 mM NaCl. The killing of *S. aureus* coated with active MPO that survived incubation with cytoplasts is shown for comparison (▲). Mean (±S.E.) of six experiments. * values differ significantly $P < 0.001$.

for the inactivated MPO, corresponding to approx. $5 \cdot 10^3$ molecules of active MPO per bacterium. The enzyme retained its activity as assessed by guaiacol oxidation and bacterial viability was unaffected by the crosslinking procedure (data not shown).

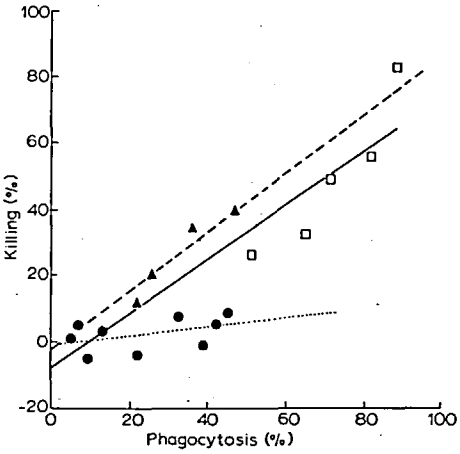

Fig. 3. The relationship between phagocytosis and killing of *S. aureus* by cytoplasts (●·····●, slope 0.136) and intact neutrophils (□———□, slope 0.818) and the effect on killing by cytoplasts of active MPO bound to the bacteria (▲— — —▲, slope 0.892). Regression lines were derived by the least-squares method. The slope represented by ● differs significantly ($P < 0.01$) from those represented by ▲ and □, which do not differ significantly.

S. aureus coated with active but not inactivated MPO were more susceptible to killing by H_2O_2 and NaCl, with the most marked effect occurring at 100 μM H_2O_2 (Fig. 4). At all hydrogen peroxide concentrations, uncoated *S. aureus* was killed to the same extent as those coated with inactivated MPO (data not shown). *S. aureus* coated with active but not with inactivated MPO induced marked iodination (Fig. 5) by cytoplasts.

Cytoplasts phagocytosed *S. aureus* coated with both active and inactive MPO to the same degree as uncoated *S. aureus*, however, only the active MPO-coated *S. aureus* were killed (Fig. 6). Killing was correlated with and did not exceed phagocytosis (Fig. 3). Fig. 4 shows that surviving active MPO-coated *S. aureus* retains the same sensitivity to H_2O_2 and NaCl as the initial inoculum showing that cytoplasts do not selectively kill a subpopulation of bacteria. No significant differences were seen between the phagocytosis and killing of uncoated *S. aureus* bacteria and those coated with inactivated MPO.

Differentiation of intracellular and extracellular killing

When unopsonised plain *S. aureus* bacteria were added into the phagocytosis and killing assays of active MPO-coated *S. aureus* and cyto-

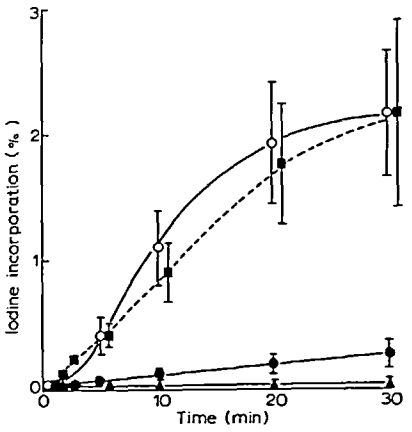

Fig. 5. Iodination by neutrophils (10^8/ml, target-to-cell ratio, 1:1) phagocytosing plain *S. aureus* (■) and cytoplasts ($5 \cdot 10^8$/ml, target-to-cytoplast ratio, 1:1) phagocytosing *S. aureus* coated with active (○) or inactivated MPO (●) in the presence of 1 mg/ml bovine serum albumin. Iodination by cytoplasts in the absence of bacteria is also shown (▲). In all experiments, 1% iodine incorporated was 5.74 (±0.19) fmol iodine fixed. The mean (±S.E.) of six experiments is shown.

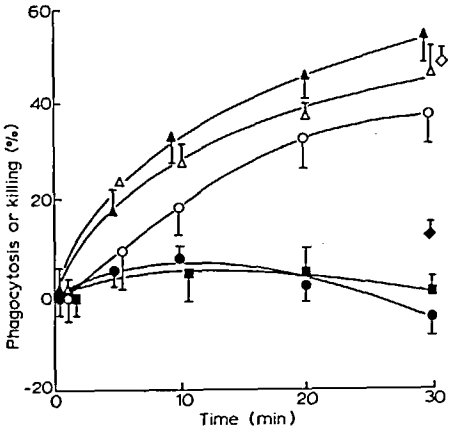

Fig. 6. The effect of coating *S. aureus* with MPO on the killing of intracellular and extracellular *S. aureus* by cytoplasts. Phagocytosis of *S. aureus* coated with active (△) and inactivated (▲) MPO is comparable and a similar proportion of the organisms coated with active enzyme were killed (○), whereas viability was unaffected by coating with inactive enzyme (●). The mean values (±S.E.) from five experiments are shown. Unopsonised *S. aureus* bacteria were not engulfed (◆) to an appreciable extent and were not killed (■) although opsonised *S. aureus* coated with active MPO were phagocytosed from the same reaction mixture (◇). The mean values (±S.E.) from three experiments are shown.

plasts, the MPO coated bacteria were phagocytosed to the same degree as when incubated as the sole target, but the unopsonised *S. aureus* were phagocytosed to a much smaller degree (Fig. 6). No significant killing of these non-phagocytosed targets was seen (Fig. 6).

Electron microscope findings

Cytoplasts were markedly granule-depleted with most cytoplast profiles showing no granules, and a few containing one or two specific granules. Phagocytosis of *S. aureus* was confirmed by electron microscopy and at this low target-to-cell ratio only a small percentage of cytoplasts were involved in phagocytosis, and these cytoplasts often

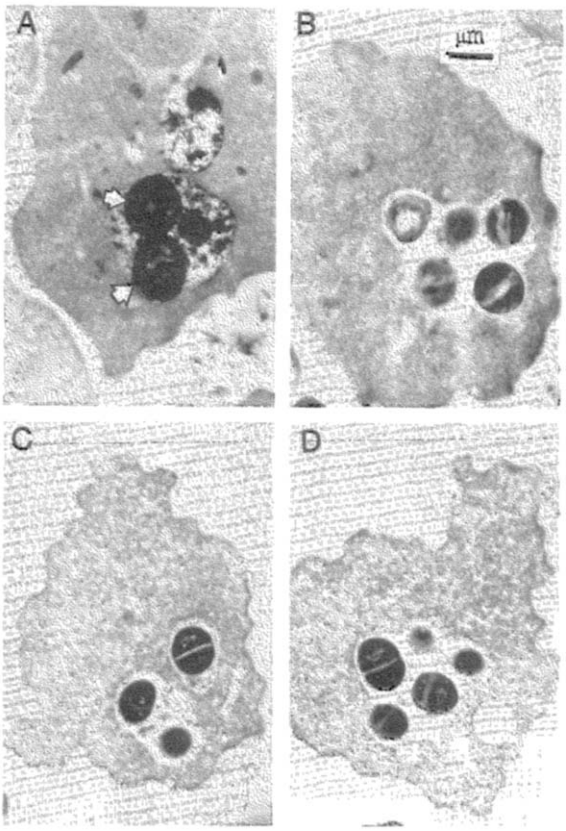

Fig. 7. Electron micrograph of MPO-coated *S. aureus* within phagosomes of cytoplasts stained for MPO by the diaminobenzidine technique. MPO activity is demonstrated by the reaction product (arrowed) seen in the complete incubation (A) but not in controls (B (H_2O_2 omitted), C (diaminobenzidine omitted) or D (MPO inactivated prior to coupling to *S. aureus*).

contained several bacteria. Occasional cytoplasts were seen to contain nuclear lobes or ruptured nuclei, but these were never seen to be phagocytically active. MPO histochemistry performed on samples from phagocytosis assays of active MPO-coated *S. aureus* showed a reaction product around the bacteria within the phagosome, but not around inactivated MPO-coated *S. aureus* or in the experimental controls (Fig. 7). Diaminobenzidine-reactive granules were a very infrequent finding.

Discussion

MPO is known to be important for the antimicrobial activity of neutrophils [2], but although MPO deficiency reduces antimicrobial efficiency in vitro [40–42], the degree of impairment varies considerably between species and target organism, being most marked in the case of fungi [40–42]. Patients with MPO deficiency are not generally predisposed to bacterial infection, and their neutrophils kill bacteria in vitro, although more slowly than normal [3]. Increased phagocytosis [43], a prolonged respiratory burst [44] and non-oxidative killing mechanisms [45] probably compensate, in part, for MPO deficiency. Many different killing systems operate simultaneously within the phagosome and the antibacterial action of human MPO has not been defined within this compartment in the intact cell.

In order to demonstrate that the MPO system alone can contribute to the killing of bacteria within the phagocytic vacuole, cytoplasts were prepared to deplete the cells of myeloperoxidase and other granule contents. Neutrophil cytoplasts can still mount a normal respiratory burst [27,38,39,46–48] and, because they are markedly depleted of granules, can be used to dissociate the effects of the respiratory burst from those of granule components. The intact respiratory burst and lack of granules would be expected to result in a more prolonged phagosomal alkalinisation than in neutrophils [37]. Although neutral pH is not optimal for myeloperoxidase function [4], oxygen-dependent killing of *S. aureus* occurs during the alkaline phase in neutrophil phagosomes [37]. Cytoplasts were observed to phagocytose bacteria [27,48,49] (Fig. 2) and produced superoxide at similar rates to neutrophils [27,46–48,50]. However, they did not kill the catalase-positive bacterium *S. aureus* to any measureable extent (Fig. 2), demonstrating that the products of the respiratory burst are not themselves microbicidal for this microorganism.

Phagocytosis by cytoplasts thus provided a suitable system to examine the effect of the selective reintroduction of MPO. In order to ensure that the MPO was exerting its effect within the vacuole at roughly physiological concentrations, it was directly coupled to the exterior of the bacteria with a heterobifunctional crosslinking agent. *S. aureus* coated with MPO in this manner were rapidly killed after phagocytosis by cytoplasts (Figs. 6 and 3). To ensure that this killing was due to its enzymic effect, control studies were conducted with MPO from which the haem prosthetic group had been removed prior to cross-linking. The apoproteins of MPO were show to be devoid of microbicidal activity.

The concentration of hydrogen peroxide achieved within a single phagocytic vacuole is unknown, but is likely to peak and decay very rapidly rather than reach a steady state [51]. Concentrations of 1 mM killed *S. aureus* in the absence of MPO, and as these organisms survived within cytoplasts it is unlikely that this concentration was achieved in cytoplast vacuoles. MPO coupled to the *S. aureus* did not result in killing at 10 μM hydrogen peroxide, but did so at 100 μM, at which concentration hydrogen peroxide alone was not microbicidal. This suggests that the concentration of hydrogen peroxide achieved in this compartment is in the region of 100 μM.

It has been proposed that neutrophils can kill extracellular bacteria through the MPO-dependent [6,7] and oxygen-dependent [52] generation of diffusible bactericidal factors. This possibility was examined by the addition to the system of unopsonised uncoated *S. aureus,* which could be distinguished from the MPO-coated *S. aureus* on the basis of their antibiotic sensitivity. These uncoated *S. aureus* bacteria were neither phagocytosed from the incubation mixture nor killed (Fig. 6), indicating that this system did not generate MPO-dependent or -independent diffusible factors capable of killing *S. aureus*. Granule components may be required for effective production of these factors [53,54]. It is also unlikely that

sufficient hydrogen peroxide diffused into the medium to kill extracellular MPO-coated bacteria, because of the close relationship between the phagocytosis and killing of the bacteria. This lack of extracellular killing might result from the metabolism or catabolism of hydrogen peroxide by the cytoplasts or MPO, or its destruction by the catalase of the *S. aureus*.

This study has demonstrated that despite the possibility of nonbactericidal reactions between MPO, superoxide radical and hydrogen peroxide, a bactericidal effect does occur in conjunction with the physiological respiratory burst within the phagocytic vacuole. Furthermore, it indicates that the concentration of hydrogen peroxide achieved within the vacuoles probably reaches about 100 μM. Intracellular activity of the MPO system alone does not appear to produce diffusible factors capable of killing extracellular *S. aureus*. This would appear to require the participation of other granule components.

Acknowledgements

This work was supported by the Medical Research Council, The Wellcome Trust and The Special Trustees, University College Hospital, London. We would like to thank Dr. D. Auger, Department of Oral Pathology, The London Hospital Medical College for help with electron microscopy and the National Blood Transfusion Service, South London for providing blood products.

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