Role of Myeloperoxidase in the Killing of *Staphylococcus aureus* by Human Neutrophils: Studies with the Myeloperoxidase Inhibitor Salicylhydroxamic Acid

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We have used salicylhydroxamic acid (SHAM) to inhibit intraphagosomal myeloperoxidase activity in order to evaluate the role of this enzyme in the killing of *Staphylococcus aureus* by human neutrophils. 50 μM -SHAM reduced the luminol-dependent chemiluminescence response stimulated during phagocytosis of unopsonized latex beads and opsonized *S. aureus* by over 80% and 60%, respectively. When opsonized *S. aureus* were incubated with neutrophils, 45% were killed within 15 min incubation and 60% by 1 h. However, in neutrophil suspensions incubated with 50 μM -SHAM, only 13% were killed by 15 min whilst 71% still remained viable after 1 h. This inhibitor had no effect upon the number of bacteria phagocytosed or upon degranulation. In a cell-free system, 2.5 μM - H_2O_2 alone killed 55% of the bacteria, whereas in the presence of myeloperoxidase (i.e. 10 mU myeloperoxidase and 2.5 μM - H_2O_2) virtually all of the bacteria were killed: the addition of 50 μM -SHAM abolished this myeloperoxidase-enhanced killing but did not affect the H_2O_2 -dependent killing. We therefore conclude that in normal neutrophils whilst H_2O_2 is required for killing of this pathogen, both myeloperoxidase-dependent and -independent pathways exist.

INTRODUCTION

In order to carry out their important role in host defence, polymorphonuclear leucocytes (neutrophils) possess a battery of cytotoxic enzymes and associated pathways which can be utilized for microbial killing. These may be classified as those whose activity is either dependent or independent of the requirement for molecular O_2 . The latter group includes the activities of proteases, defensins, permeability-inducing proteins and lysozyme (Elsbach & Weiss, 1983; Spitznagel, 1984; Ganz *et al.*, 1985). O_2 -dependent processes result in the generation of a series of reactive oxygen metabolites (e.g. O_2^- , H_2O_2 , $\cdot\text{OH}$, HOCl , and $^1\text{O}_2$) during a respiratory burst of non-mitochondrial O_2 uptake (Babior, 1978, 1984). The possession of such a diverse array of bactericidal systems with different biochemical modes of action is necessary to ensure the maximal killing potential against a wide range of pathogens which may themselves show resistance to any one particular system. Indeed, experimental evidence has confirmed this selective toxicity: bacterial-permeability-inducing peptide preferentially kills Gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium* (Elsbach & Weiss, 1983), defensins are toxic to *Cryptococcus neoformans* and *Pseudomonas aeruginosa* (Ganz *et al.*, 1985) and the efficient killing of *Staphylococcus aureus* requires the activation of O_2 -dependent processes (Edwards *et al.*, 1987, 1988*a, b*).

The generation of a full complement of reactive oxidants by neutrophils requires the activities of two enzyme systems. Firstly, a plasma-membrane-bound NADPH oxidase is activated to

Abbreviation: SHAM, salicylhydroxamic acid.

produce O_2^- and H_2O_2 during the respiratory burst (Rossi, 1986) and, secondly, myeloperoxidase (an enzyme normally located within azurophilic granules and discharged to the site of oxidant generation by the process of degranulation; Klebanoff & Clark, 1978), may react with H_2O_2 and possibly O_2^- to produce HOCl and related compounds (Harrison & Schultz, 1976). Since its discovery, myeloperoxidase has been shown to possess peroxidase, catalase, superoxide dismutase, oxidase and DNA-binding activities (Winterbourne *et al.*, 1985; Cuperus *et al.*, 1986; Edwards & Lloyd, 1987; Murao *et al.*, 1988) and, *in vitro*, the myeloperoxidase- H_2O_2 -halide system has potent antibacterial, antifungal, antiviral and antitumour properties (Klebanoff & Clark, 1978). However, the role of this enzyme in bacterial killing has recently been questioned because of the finding that neutrophils from apparently healthy individuals may be deficient in this enzyme (Lehrer & Cline, 1969; Salmon *et al.*, 1970; Klebanoff & Pincus, 1971; Nauseef, 1988). The degree of this deficiency varies but it must be borne in mind that in normal neutrophils this enzyme may constitute 5% of the total protein (Klebanoff & Clark, 1978). Myeloperoxidase-deficient neutrophils have impaired ability to kill *Candida* and *S. aureus in vitro* (Kitahara *et al.*, 1981; Parry *et al.*, 1981; Lanza *et al.*, 1987) and there is a close correlation between a deficiency in this enzyme and the occurrence of preleukaemia and neoplasms (Breton-Gorius *et al.*, 1975; Lanza *et al.*, 1987; Pui *et al.*, 1987; Nauseef, 1988).

Since it has been proposed that in myeloperoxidase-deficient neutrophils other bactericidal systems are elaborated (Klebanoff & Pincus, 1971; Kitahara *et al.*, 1981), and also that a specific inhibitor does not exist (Edwards & Swan, 1986), definition of the role of this enzyme in microbial and tumour cell killing by normal neutrophils has not as yet been possible. The most commonly used myeloperoxidase inhibitor in neutrophil studies is sodium azide, but since this is a non-specific haem inhibitor and also quenches $\cdot OH$ and 1O_2 (Halliwell & Gutteridge, 1985), its broad toxicity renders it useless for cytotoxicity studies. We have recently shown that salicylhydroxamic acid (SHAM), previously used as an inhibitor of the alternative cyanide-insensitive oxidase of plant mitochondria (Schonbaum *et al.*, 1971) and some other redox enzymes (Rich *et al.*, 1978), is a potent inhibitor of the myeloperoxidase system (Davies & Edwards, 1989). The aim of the present study was to use this inhibitor to establish the role of myeloperoxidase in the killing of *S. aureus* by normal human neutrophils.

METHODS

Isolation and preparation of neutrophils. Polymorphonuclear leucocytes (neutrophils) were isolated from heparinized venous blood from healthy volunteers utilizing either a dextran/Ficoll sedimentation procedure (Edwards & Swan, 1986) or Mono-Poly Resolving Medium (Flow Laboratories) as described previously (Edwards *et al.*, 1987). After purification, cells were suspended in a buffer containing (mM): NaCl, 120; KCl, 4.8; KH_2PO_4 , 1.2; $CaCl_2$, 1.3; $MgSO_4$, 1.2; HEPES, 25 (pH 7.4); and 0.1% bovine serum albumin. Neutrophils (>98% purity) were counted using a Fuchs-Rosenthal haemocytometer slide and used within 4 h of preparation.

Bactericidal assays. *Staphylococcus aureus* (Oxford) was grown overnight on nutrient agar plates and then opsonized with 10% (v/v) pooled normal human serum by the method of Turner *et al.* (1986) as described in detail by Edwards *et al.* (1987). After opsonization, unbound proteins were removed by centrifugation and bacteria were suspended in sterile buffer at known concentrations. Opsonized bacteria (3×10^7 ml⁻¹) were incubated with neutrophil suspensions (10^6 ml⁻¹) with gentle agitation in the presence or absence of SHAM at the stated concentration, and at suitable time intervals samples were removed for the estimation of viable bacteria as described previously (Edwards *et al.*, 1987). Alternatively, opsonized bacteria were incubated in a cell-free myeloperoxidase/ H_2O_2 system (Klebanoff, 1968) containing 2.5 μM - H_2O_2 and 120 mM-Cl⁻ in the presence or absence of 10 mU purified myeloperoxidase and 50 μM -SHAM. Viable bacteria were then estimated after 30 min incubation.

Analytical methods. Chemiluminescence was measured using an LKB Wallac 1250 luminometer in neutrophil suspensions containing 10^6 cells ml⁻¹ and 10 μM -luminol: suspensions were stimulated by the addition of 4×10^9 latex beads ml⁻¹ (1.01 μm diameter) or opsonized bacteria (3×10^7 ml⁻¹) in the presence or absence of SHAM at the stated concentration. Human myeloperoxidase was purified from buffy coats as described by Pember *et al.* (1983) and had an A_{430}/A_{280} ratio of 0.68.

Electron microscopy. Neutrophils incubated with opsonized *S. aureus* in the presence or absence of 50 μM -SHAM for 20 min at 37 °C were fixed in cold 0.15 M-cacodylate-buffered glutaraldehyde, pH 7.4 (2.5%, v/v) and post-

fixed with osmium tetroxide (1%, v/v). The mixtures were embedded in Araldite, thin-sectioned and stained with Reynold's lead citrate and uranyl acetate (1%, w/v). Thin sections were examined using a Philips 301 electron microscope.

Statistical analysis. Data were compared using Student's *t*-test.

Chemicals. Luminol, HEPES and SHAM were from Sigma. All other chemicals were of the highest purity available.

RESULTS AND DISCUSSION

Effect of SHAM on chemiluminescence during phagocytosis

In order to establish that SHAM permeated neutrophils and inhibited myeloperoxidase activity within phagolysosomes, neutrophils were incubated with various concentrations of inhibitor and the chemiluminescence stimulated upon phagocytosis was measured. As shown in Fig. 1, 50 μ M-SHAM inhibited the chemiluminescence stimulated during phagocytosis of opsonized *S. aureus* by over 60% and that measured during phagocytosis of unopsonized latex beads by over 80%. Since SHAM did not inhibit uptake of particles (see later) and as it has no inhibitory effect on oxidant generation (Davies & Edwards, 1989), these data show that this compound effectively inhibited the intraphagosomal activity of myeloperoxidase after both receptor-dependent and -independent uptake of particles.

Effect of SHAM on the killing of S. aureus by neutrophils

Since 50 μ M-SHAM resulted in considerable inhibition of intraphagosomal myeloperoxidase activity (Fig. 1), we determined whether this inhibition resulted in impaired bactericidal activity towards *S. aureus*. Neutrophil suspensions were incubated with opsonized *S. aureus* in the presence or absence of 50 μ M-SHAM. At suitable time intervals after the addition of luminol and bacteria, portions were removed for estimation of viable bacteria (after lysis of neutrophils) and measurement of chemiluminescence (Fig. 2). In control suspensions the bacteria were rapidly killed during phagocytosis; in suspensions treated with 50 μ M-SHAM, killing was clearly reduced. For example, after 15 min incubation with the inhibitor, 87% (± 10 ; mean $\pm$ SD, $n = 7$) remained viable, compared to 56% (± 9 , $n = 9$) in the control, and by 60 min 71% were viable (± 11 , $n = 10$) compared to 43% (± 8 , $n = 10$) in control suspensions. Control experiments showed that this concentration of inhibitor alone had no effect on the viability of the bacteria.

Luminol-dependent chemiluminescence traces obtained from these experiments are shown in Fig. 3. In control suspensions the maximum rate of increase in chemiluminescence occurred within the first 15–20 min (corresponding to the time of greatest killing) and then slowly declined over the remainder of the experiment after reaching a maximum value of about 70 mV. In identical suspensions treated with 50 μ M-SHAM, the maximum rate of chemiluminescence was 15 mV at 20 min and after this time the rate slowly declined.

Samples were fixed with glutaraldehyde 30 min after phagocytosis of *S. aureus* and electron micrographs analysed in order to assess whether SHAM had any effect upon the rate of particle uptake or degranulation. Enumeration of intracellular, phagocytosed bacteria in these micrographs revealed no significant difference in the rate of particle uptake in control (7.6 ± 4.9 bacteria per neutrophil; mean $\pm$ SD, $n = 101$ neutrophils counted) or SHAM-treated suspensions (7.6 ± 4.2 bacteria per neutrophil, $n = 102$ neutrophils counted). Furthermore, these experiments showed that migration of granules towards the phagosome and degranulation were not affected by treatment with this compound (Fig. 4). Thus, we attribute the reduced killing (Fig. 2) to inhibition of myeloperoxidase and not to any effect of SHAM on phagocytosis or degranulation.

Effect of SHAM on the killing of S. aureus by a cell-free myeloperoxidase/H₂O₂ system

We have previously shown that SHAM has no effect on the kinetics of oxidant generation during the respiratory burst (Davies & Edwards, 1989). However, in order to exclude the possibility that this compound affected H₂O₂-dependent (but myeloperoxidase-independent)

Fig. 1

Fig. 2

Fig. 1. Effect of SHAM on chemiluminescence during phagocytosis. Suspensions of neutrophils (10^6 , total volume 1 ml) were incubated at 37°C with $10\ \mu\text{M}$ -luminol in the presence or absence of SHAM at the stated concentration. Phagocytosis was then stimulated by the addition of either 3×10^7 opsonized *S. aureus* (●) or 4×10^9 unopsonized latex beads (○) and the chemiluminescence response measured.

Fig. 2. Effect of SHAM on the killing of *S. aureus* by neutrophils. Suspensions of neutrophils (10^6 , total volume 1 ml) were incubated at 37°C in the presence (●) or absence (■) of $50\ \mu\text{M}$ -SHAM. At time zero, 3×10^7 opsonized *S. aureus* were added to the suspensions and portions removed at intervals for estimation of viable bacteria. Results presented are means of at least seven experiments, with error bars representing standard deviations.

Fig. 3. Luminol-dependent chemiluminescence during killing of *S. aureus*. Suspensions of neutrophils (10^6 , total volume 1 ml) containing $10\ \mu\text{M}$ -luminol were incubated at 37°C in the presence (○) or absence (●) of $50\ \mu\text{M}$ -SHAM. At time zero, 3×10^7 opsonized *S. aureus* were added and the chemiluminescence response measured at time intervals. Results presented are typical of at least seven experiments.

killing of *S. aureus*, the killing of opsonized bacteria in a cell-free myeloperoxidase/ H_2O_2 system (see Methods) was examined. When opsonized bacteria were incubated with $2.5\ \mu\text{M}$ - H_2O_2 alone, $55\% (\pm 20; \text{mean} \pm \text{SEM}, n = 8)$ of the bacteria were killed within 30 min incubation. In suspensions containing $10\ \text{mU}$ purified myeloperoxidase plus $2.5\ \mu\text{M}$ - H_2O_2 , considerably more bacteria were killed ($95\% \pm 19, n = 11$), i.e. the purified enzyme enhanced H_2O_2 -dependent killing of this organism. However, in suspensions containing $10\ \text{mU}$ purified myeloperoxidase, $2.5\ \mu\text{M}$ - H_2O_2 plus $50\ \mu\text{M}$ -SHAM, the level of killing ($44\% \pm 7, n = 9$) was similar to that observed by H_2O_2 alone, i.e. the inhibitor abolished the myeloperoxidase-enhanced killing.

The results of these experiments are consistent with previous reports regarding the mechanisms of killing of *S. aureus* by human neutrophils and show several important features.

Fig. 4. Electron micrograph of ingested *S. aureus*. Neutrophils were incubated as described in the legend to Fig. 2. After 20 min incubation with opsonized *S. aureus*, suspensions were fixed with glutaraldehyde as described in Methods. After embedding, sectioning and staining at least 100 neutrophils from control or SHAM-treated suspensions were examined and the numbers of ingested bacteria counted. The figure shows a section of a neutrophil that had been incubated with 50 μ M-SHAM before the addition of bacteria. Bar, 1 μ m.

Firstly, H_2O_2 (in the absence of myeloperoxidase) is capable of killing this pathogen. Secondly, the H_2O_2 -dependent killing of this organism is considerably enhanced by the presence of myeloperoxidase. Thirdly, oxygen-independent granule enzymes do not play a role in this killing process since neutrophils from patients with chronic granulomatous disease cannot kill this pathogen (Edwards *et al.*, 1987): the only biochemical defect in these patients is their inability to generate reactive oxidants (Tauber *et al.*, 1983). Thus, we confirm that the efficient killing of *S. aureus* by human neutrophils requires the activation of oxygen-dependent processes (Edwards *et al.*, 1987, 1988a, b) but now show that both myeloperoxidase-dependent and -independent pathways exist.

SHAM can act as a peroxidase inhibitor and (at a concentration of 50 μ M) scavenge the myeloperoxidase product HOCl (Davies & Edwards, 1989). Therefore, its ability to reduce bacterial killing may reside in its ability to either inhibit the enzymic activity of myeloperoxidase or else scavenge one of its major products, or a combination of these two effects. The use of SHAM as an inhibitor of myeloperoxidase has, for the first time, enabled the role of this enzyme in *S. aureus* killing by normal neutrophils to be evaluated. The data presented here confirm the observations of experiments with myeloperoxidase-deficient neutrophils which could not kill this pathogen as efficiently as controls (Kitahara *et al.*, 1981; Lanza *et al.*, 1987). Thus, by virtue of its non-toxicity towards the target cell, SHAM is a powerful analytical tool which can now be used to delineate the function of myeloperoxidase in other inflammatory events.

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REFERENCES

- BABIOR, B. M. (1978). Oxygen-dependent killing by phagocytes. *New England Journal of Medicine* **298**, 659–668.
- BABIOR, B. M. (1984). The respiratory burst of phagocytes. *Journal of Clinical Investigation* **73**, 599–601.
- BRETTON-GORIUS, J., HOUSSAY, D. & DREYFUS, B. (1975). Partial myeloperoxidase deficiency in a case of preleukaemia. I. Studies of fine structure and peroxidase synthesis of promyelocytes. *British Journal of Haematology* **30**, 273–278.
- CUPERUS, R. A., MUIJSERS, A. O. & WEVER, R. (1986). The superoxide dismutase activity of myeloperoxidase: formation of compound III. *Biochimica et biophysica acta* **871**, 78–84.
- DAVIES, B. & EDWARDS, S. W. (1989). Inhibition of myeloperoxidase by salicylhydroxamic acid. *Biochemical Journal* **258**, 801–806.
- EDWARDS, S. W. & LLOYD, D. (1987). CO-reacting haemoproteins of neutrophils: evidence for cytochrome *b*-245 and myeloperoxidase as potential oxidases during the respiratory burst. *Bioscience Reports* **7**, 193–199.
- EDWARDS, S. W. & SWAN, T. F. (1986). Regulation of superoxide generation by myeloperoxidase during the respiratory burst of human neutrophils. *Biochemical Journal* **237**, 601–604.
- EDWARDS, S. W., SAY, J. E. & HART, C. A. (1987). Oxygen-dependent killing of *Staphylococcus aureus* by human neutrophils. *Journal of General Microbiology* **133**, 3591–3597.
- EDWARDS, S. W., SAY, J. E. & HUGHES, V. (1988*a*). Gamma interferon enhances the killing of *Staphylococcus aureus* by human neutrophils. *Journal of General Microbiology* **134**, 37–42.
- EDWARDS, S. W., HART, C. A., DAVIES, J. M., PATTISON, J., HUGHES, V. & SILLS, J. A. (1988*b*). Impaired neutrophil killing in a patient with defective degranulation of myeloperoxidase. *Journal of Clinical and Laboratory Immunology* **25**, 201–206.
- ELSBACH, P. & WEISS, J. (1983). A reevaluation of the role of the O₂-dependent and O₂-independent microbial systems of phagocytes. *Reviews of Infectious Diseases* **5**, 843–853.
- GANZ, T., SELSTED, M. E., SZKLAREK, D., HARWIG, S. S. L., DAHER, K., BAINTON, D. F. & LEHRER, R. I. (1985). Defensins: natural peptide antibiotics of human neutrophils. *Journal of Clinical Investigation* **76**, 1427–1435.
- HALLIWELL, B. & GUTTERIDGE, J. M. C. (1985). *Free Radicals in Biology and Medicine*. Oxford: Clarendon Press.
- HARRISON, J. E. & SCHULTZ, J. (1976). Studies on the chlorinating activity of myeloperoxidase. *Journal of Biological Chemistry* **251**, 1371–1374.
- KITAHARA, M., EYRE, H. J., SIMONIAN, Y., ATKIN, C. L. & HASSTEDT, S. J. (1981). Hereditary myeloperoxidase deficiency. *Blood* **57**, 888–893.
- KLEBANOFF, S. J. (1968). Myeloperoxidase-halide-hydrogen peroxide antibacterial system. *Journal of Bacteriology* **95**, 2131–2138.
- KLEBANOFF, S. J. & CLARK, R. A. (1978). *The Neutrophil: Function and Clinical Disorders*. Amsterdam: North-Holland.
- KLEBANOFF, S. J. & PINCUS, S. H. (1971). Hydrogen peroxide utilization in myeloperoxidase-deficient leukocytes: a possible microbicidal control mechanism. *Journal of Clinical Investigation* **50**, 2226–2229.
- LANZA, F., FIETTA, A., SPISANI, S., CASTOLDI, G. L. & TRANIELLO, S. (1987). Does a relationship exist between neutrophil myeloperoxidase deficiency and the occurrence of neoplasms? *Journal of Clinical and Laboratory Immunology* **22**, 175–180.
- LEHRER, R. I. & CLINE, M. J. (1969). Leukocyte myeloperoxidase deficiency and disseminated candidiasis: the role of myeloperoxidase in resistance to *Candida* infections. *Journal of Clinical Investigation* **48**, 1478–1488.
- MURAO, S. I., STEVENS, S. J., ITO, A. & HUBERMAN, E. (1988). Myeloperoxidase: a myeloid cell nuclear antigen with DNA binding properties. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 1232–1236.
- NAUSEEF, W. M. (1988). Myeloperoxidase deficiency. In *Phagocytic Defects 1: Abnormalities Outside of the Respiratory Burst*, pp. 135–158. Edited by J. T. Curnutte. Philadelphia: W. B. Saunders.
- PARRY, M. F., ROOT, R. K., METCALF, J. A., DELANEY, K. K., KAPLOW, L. S. & RICHAR, W. J. (1981). Myeloperoxidase deficiency. Prevalence and clinical significance. *Annals of Internal Medicine* **95**, 293–301.
- PEMBER, S. O., SHAPIRA, R. & KINKADE, J. M., JR (1983). Multiple forms of myeloperoxidase from human neutrophilic granulocytes: evidence for differences in compartmentalization, enzymatic activity and sub-unit structure. *Archives of Biochemistry and Biophysics* **221**, 391–403.
- PUI, C.-H., BEHM, F. G., KALWINSKY, D. K., MURPHY, S. B., BUTLER, D. L., DAHL, G. V. & MIRRO, J. (1987). Clinical significance of low levels of myeloperoxidase positivity in childhood acute nonlymphoblastic leukemia. *Blood* **70**, 51–54.
- RICH, P. R., WIEGAND, N. K., BLUM, H., MOORE, A. L. & BONNER, W. D., JR (1978). Studies on the mechanism of inhibition of redox enzymes by substituted hydroxamic acids. *Biochimica et biophysica acta* **525**, 325–337.
- ROSSI, F. (1986). The O₂ forming NADPH oxidase of the phagocytes: nature, mechanisms of activation and function. *Biochimica et biophysica acta* **853**, 65–89.
- SALMON, S. E., CLINE, M. J., SCHULTZ, J. & LEHRER, R. I. (1970). Myeloperoxidase deficiency. Immunological study of a genetic leukocyte defect. *New England Journal of Medicine* **282**, 250–253.
- SCHONBAUM, G. R., BONNER, W. D., JR, STOREY, B. T. & BAHR, J. T. (1971). Specific inhibition of the cyanide-insensitive respiratory pathway in plant mitochondria by hydroxamic acids. *Plant Physiology* **47**, 124–128.
- SPITZNAGEL, J. K. (1984). Nonoxidative antimicrobial

- systems in leucocytes. In *Regulation of Leukocyte Function* (Contemporary Topics in Immunology vol. 14), pp. 234–283. Edited by R. Snyderman. New York: Plenum.
- TAUBER, A. I., BORREGAARD, N., SIMONS, E. & WRIGHT, J. (1983). Chronic granulomatous disease: a syndrome of phagocyte oxidase deficiencies. *Medicine* **62**, 286–309.
- TURNER, M. W., GRANT, C., SEYMOUR, N. D., HARVEY, B. & LEVINSKY, R. J. (1986). Evaluation of C3b/C3bi opsonization and chemiluminescence with selected yeasts and bacteria using sera of different opsonic potential. *Immunology* **58**, 111–115.
- WINTERBOURNE, C. C., GARCIA, R. C. & SEGAL, A. W. (1985). Production of the superoxide adduct of myeloperoxidase (compound III) by stimulated human neutrophils and its reactivity with hydrogen peroxide and chloride. *Biochemical Journal* **228**, 583–592.