Oxygen-dependent Killing of *Staphylococcus aureus* by Human Neutrophils

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Luminol-dependent chemiluminescence was used as a monitor of reactive oxidant generation during phagocytosis of *Staphylococcus aureus* by human neutrophils. Reactive oxidants play a crucial role in the killing of this organism because: (a) *S. aureus* was killed most rapidly when the rate of increase of chemiluminescence was greatest; (b) neutrophils which had been activated to generate reactive oxidants by re-aeration of anaerobic suspensions killed this bacterium more efficiently than control suspensions; and (c) neutrophils from a patient with chronic granulomatous disease could neither generate reactive oxidants nor kill *S. aureus*.

INTRODUCTION

Neutrophilic polymorphonuclear leucocytes (neutrophils) are phagocytic cells of the immune system which play a crucial role in host protection during microbial infections. These cells possess a wide range of cytotoxic enzymes and associated pathways which are utilized for microbial killing during phagocytosis. While many functions of neutrophils, such as chemotaxis and phagocytosis, can occur *in vitro* under anaerobic conditions, their cytotoxic enzymes can be broadly classed as O₂-dependent or O₂-independent (Klebanoff & Clark, 1978). O₂-independent mechanisms include the activities of proteases, hydrolases, lysozyme and cationic proteins (Elsbach & Weiss, 1983; Spitznagel, 1984). O₂-dependent processes involve the generation of a series of reactive oxidants (Karnovsky & Bolis, 1982; Babior, 1984), generated during a respiratory burst of non-mitochondrial O₂ uptake (Sbarra & Karnovsky, 1959): efficient production of a full complement of reactive oxidants requires the activities of an NADPH oxidase and myeloperoxidase (Klebanoff & Clark, 1968; Rossi, 1986).

Much controversy exists in the literature as to the relative importance of O₂-dependent and O₂-independent mechanisms for efficient microbial killing, although clearly, the possession of a wide variety of functionally diverse systems which can operate under varying physiological conditions is beneficial for host protection. On the one hand, the crucial role played by oxidative processes is highlighted in chronic granulomatous disease, in which patients suffer from recurrent, life-threatening infections: the only biochemical defect so far identified resides in their NADPH oxidase and is manifest by the inability of their phagocytes to mount a respiratory burst and hence generate oxidants (Hill, 1984). On the other hand, it has been stated that some microbes are killed *in vitro* under anaerobic conditions almost as efficiently as under aerobic conditions (Vel *et al.*, 1984). This apparent discrepancy is probably at least partly due to the inherent biochemical properties of different types of micro-organisms resulting in differences in susceptibility to the multiplicity of neutrophil products.

The aim of the present study was to evaluate the role played by reactive oxidants in the killing of *Staphylococcus aureus* by human neutrophils. A number of observations prompted this study. Firstly, of a range of pathogens studied, this organism was killed least efficiently under anaerobic conditions (see references in Vel *et al.*, 1984). Secondly, in a group of patients with identifiable defects in neutrophil oxidative metabolism, *S. aureus* was a common pathogen upon presentation (C. A. Hart & S. W. Edwards, unpublished observations). Thirdly, the neutrophils

Fig. 1. Apparatus for the simultaneous measurement of chemiluminescence and O_2 . A thermostatically regulated brass housing was fitted with a Radiometer E5046 O_2 electrode and a photomultiplier tube which was connected to a Thorn-EMI C-10 photon counter. The suspension (total volume 10 ml) was continuously stirred by means of a variable-speed synchronous motor and was 'open' to the gas phase (Degn & Wohlrab, 1971). A cap was fitted with two ports for the inflow and outflow of gases: gas inflow was regulated by a flow meter and digital gas mixers provided O_2/N_2 or O_2/Ar mixtures so that the O_2 tension in the suspension could be varied throughout the physiological range. Additions were made via an injection port in the cap.

from this group of patients had impaired ability to kill *S. aureus in vitro*. Thus, establishing the role played by reactive oxidants in the killing of this pathogen is necessary in order to identify the molecular mechanisms responsible for impaired immune protection in these and other groups of patients.

METHODS

Preparation of neutrophils. Polymorphonuclear leucocytes (neutrophils) were prepared from heparinized venous blood from healthy volunteers (or from a patient with chronic granulomatous disease) either by a combined dextran/Ficoll procedure (Edwards & Swan, 1986) or by centrifugation through M-PRM medium (Flow Laboratories). After centrifugation at 50 *g* for 20–30 min in this latter medium, the polymorphic cell band was removed and diluted with 3 vols of 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); 0.1% bovine serum albumin. This buffer was used for all cell incubations. After sedimentation of the cells by centrifugation at 700 *g* for 5 min, contaminating erythrocytes were lysed by adding 9 ml double-distilled water and shaking vigorously for 25 s: tonicity was then restored by adding 1 ml 9% (w/v) NaCl. The purified cells (>98% neutrophils) were then washed twice and finally suspended in the above buffer to $2-4 \times 10^7$ cells per ml^{-1} ; they were used within 4 h of preparation.

Growth and opsonization of bacteria. *Staphylococcus aureus* (Oxford) was grown overnight on nutrient agar plates at 37 °C. The cells were then scraped from the plates, suspended in sterile buffer and the number of viable cells estimated by measurement of OD_{550} , using suitable calibration curves. Opsonization using pooled serum from healthy donors (stored in portions at –20 °C) was achieved by incubating bacteria (5×10^8 ml^{-1}) with 10% serum (v/v, final concn) for 30 min at 37 °C (Turner *et al.*, 1986), prior to the addition of 3 vols chilled, sterile buffer and whirlmixing for 30 s. After this, the opsonized bacteria were centrifuged at 1000 *g* for 15 min in an MSE Centaur centrifuge, washed three times in buffer and finally resuspended to known concentration.

Bacterial killing. Neutrophils were suspended in buffer to 10^6 cells ml^{-1} in a specially constructed reaction vessel (Fig. 1): the total volume of the suspension was 10 ml and the temperature was maintained at 37 °C. At time zero, pre-opsonized bacteria were added (final concn $3-4 \times 10^7$ bacteria ml^{-1}) and then samples were aseptically removed at frequent time intervals. These samples were diluted 100-fold in sterile double-distilled water, whirlmixed for 5 min to lyse the neutrophils and then diluted in sterile saline. The number of viable bacteria was then estimated by spread-plating suitably diluted samples onto nutrient agar and incubating in air at 37 °C for 16 h.

Chemiluminescence measurements. These were made on 1 ml suspensions of neutrophils using an LKB Wallac 1250 luminometer, or on 10 ml suspensions in a specially designed system employing a Thorn-EMI C-10 photon counter (Fig. 1). This latter apparatus was 'open' to the gas phase (Degn & Wohlrab, 1971), and thus by using gas mixers the O_2 tension in the cell suspensions could be varied throughout the physiological range. In all cases the

final neutrophil concentration was 10^6 cells ml^{-1} , the temperature of operation 37°C and suspensions contained $10\ \mu\text{M}$ -luminol.

Measurement of superoxide generation. This was done by following the rate of reduction of cytochrome *c* (Babior *et al.*, 1973). The assay mixture (total volume 6 ml) contained $75\ \mu\text{M}$ -cytochrome *c* and 2×10^5 cells ml^{-1} . The absorbance increase at 550 nm was measured in an Hitachi (Perkin-Elmer) 557 spectrophotometer operating in the dual-wavelength mode (reference wavelength 540 nm) in an apparatus described by Lloyd *et al.* (1983).

Chemicals. HEPES and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) were from Sigma. All other chemicals were of the highest purity available.

RESULTS

Optimization of phagocytosis of S. aureus and chemiluminescence

Whilst luminol-dependent chemiluminescence is a convenient measure of reactive oxidant generation by neutrophils (Edwards, 1987), the efficiency of detection of these oxidants by photon emission is decreased by the presence of proteins in the assay medium due to non-specific quenching effects. Therefore, since phagocytosis of bacteria requires opsonization by serum proteins, it was necessary to establish the conditions necessary for optimal phagocytosis and detection of oxidants by chemiluminescence. Preliminary experiments showed that while 10% serum was necessary for maximal opsonization and uptake of *S. aureus*, this concentration of serum decreased the chemiluminescence response observed during the phagocytosis of latex beads by 70% (results not shown). Therefore, in order to achieve both maximal phagocytosis of fully opsonized bacteria and maximal detection of chemiluminescence, it was found necessary to pre-opsone the bacteria by incubation with 10% serum for 30 min at 37°C and then to remove excess (unbound) serum proteins prior to the initiation of phagocytosis. Bacteria pre-opsone in this way were rapidly phagocytosed and chemiluminescence was detected after a lag phase of less than 1 min.

Fig. 2

Fig. 3

Fig. 2. Effect of bacterial concentration on neutrophil chemiluminescence. Neutrophils ($10^6\ \text{ml}^{-1}$, total volume 1 ml) were incubated at 37°C in buffer containing $10\ \mu\text{M}$ -luminol. Phagocytosis was initiated by adding different concentrations of pre-opsone *S. aureus* and the maximal chemiluminescence response was measured using an LKB 1250 luminometer. Maximal chemiluminescence (100%) was 20 mV.

Fig. 3. Correlation of killing of *S. aureus* with oxidant generation. Neutrophils ($10^6\ \text{ml}^{-1}$, total volume 1 ml) were isolated either from healthy volunteers (●, ■) or from a patient with X-linked chronic granulomatous disease (○, □) and incubated in buffer at 37°C . At time zero, pre-opsone *S. aureus* ($4 \times 10^7\ \text{ml}^{-1}$) were added and samples were removed at intervals for estimation of viable bacteria (●, ○), or measurement of chemiluminescence with the photon counter (■, □).

Time
Fig. 4

Time
Fig. 5

Fig. 4. Superoxide generation during re-aeration of anaerobic neutrophil suspensions. Neutrophils ($2 \times 10^5 \text{ ml}^{-1}$, total volume 6 ml) were suspended in buffer containing $75 \mu\text{M}$ -cytochrome *c* at 37°C in a reaction vessel fitted within the lightpath of an Hitachi (Perkin-Elmer) spectrophotometer operating in the dual-wavelength mode. With the gas phase comprising air, a steady-state level of reduction of cytochrome *c* (measured at 550 nm , reference wavelength 540 nm) indicated no net production of superoxide (trace B). The gas phase was then switched to Ar and the decreasing O_2 tension in the suspension was measured with an O_2 electrode (trace A) until anaerobiosis was achieved. At the time indicated ($\downarrow$), the gas phase was switched to 10% air.

Fig. 5. Chemiluminescence during re-aeration of anaerobic neutrophil suspensions. Neutrophils (10^6 ml^{-1} , total volume 10 ml) were incubated in buffer containing $10 \mu\text{M}$ -luminol at 37°C using the apparatus described in Fig. 1. With a gas phase of air, a steady-state level of chemiluminescence indicated a constant rate of photon emission (trace B). When the gas phase was switched to O_2 -free N_2 , the O_2 tension in the suspension rapidly decreased until anaerobiosis was attained (trace A). At the time indicated ($\downarrow$), the suspension was re-aerated by switching the gas phase back to air.

Correlation of bacterial killing with chemiluminescence

In order to determine the optimal bacteria : neutrophil ratio for maximal chemiluminescence, neutrophils (10^6) were incubated with different concentrations of pre-opsonized *S. aureus* and the chemiluminescence response was observed. Increasing the bacteria : neutrophil ratio up to 25 : 1 progressively increased the chemiluminescence response, but at higher ratios no further increase in photon emission was observed (Fig. 2). Therefore in all subsequent experiments a *S. aureus* : neutrophil ratio of 30–40 : 1 was used. A suspension of neutrophils ($10^6 \text{ cells ml}^{-1}$) was incubated at 37°C for 5 min and then (time zero) pre-opsonized *S. aureus* ($4 \times 10^7 \text{ ml}^{-1}$) were added. After 15 min incubation, over 65% of the bacteria had been killed (Fig. 3): the rate of killing then declined and by 1 h after the addition of bacteria only about 12% remained viable. In an identical incubation mixture but containing $10 \mu\text{M}$ -luminol, the rate of chemiluminescence increased rapidly during the initial 15 min, coinciding with the phase of rapid bacterial killing (Fig. 3). The rate of chemiluminescence was maximal by 30 min and then slowly declined, as the bacteria were killed at a slower rate. In contrast, when pre-opsonized *S. aureus* were incubated with neutrophils from a patient with X-linked chronic granulomatous disease, no chemiluminescence was detected and all of the bacteria remained viable at the end of the 60 min incubation period (Fig. 3).

Activation of oxidant generation by re-aeration of anaerobic neutrophil suspensions

A suspension of neutrophils was incubated in buffer (in the presence of cytochrome *c*) in a reaction vessel fitted within the lightpath of a dual-wavelength spectrophotometer. The gas

Fig. 6. Rate of killing of *S. aureus*. Suspensions of neutrophils (10^6 ml^{-1} , total volume 10 ml) were incubated at 37°C in the apparatus described in Fig. 1. At time zero, pre-opsonized *S. aureus* ($3 \times 10^7 \text{ ml}^{-1}$) were added to the suspensions and samples were removed at intervals and viable bacteria enumerated. The suspensions contained $210 \mu\text{M-O}_2$. ●, Neutrophils incubated under aerobic conditions throughout; ○, neutrophil suspensions made anaerobic and then re-aerated prior to the introduction of bacteria. The results presented are means of at least seven determinations, with error bars representing standard deviations.

phase comprised air (i.e. $210 \mu\text{M-O}_2$), and the steady-state level of reduction of cytochrome *c* was constant, indicating a negligible rate of superoxide production (Babior *et al.*, 1973). The gas phase was then switched to Ar using a gas mixer and the attainment of anaerobiosis in the suspension was monitored with the oxygen electrode (Fig. 4): no change in the steady-state level of reduction of cytochrome *c* was detected during this period. However, when O_2 was re-introduced into the suspension by switching the gas phase to 10% air, the rate of reduction of cytochrome *c* sharply increased, indicating activation of superoxide production by neutrophils by incubation under these conditions (this cytochrome *c* reduction was inhibited by superoxide dismutase: data not shown). The maximum rate of superoxide generation activated by this mechanism was $1.9 \text{ nmol min}^{-1}$ per 10^6 cells, compared with a rate of $6.5 \text{ nmol min}^{-1}$ per 10^6 cells in identical suspensions stimulated with phorbol myristate acetate ($0.1 \mu\text{g ml}^{-1}$).

In a similar series of experiments using a specially designed apparatus to measure chemiluminescence at defined O_2 tensions (Fig. 1), activation of oxidant generation (as detected by luminol-dependent chemiluminescence) was also observed when anaerobic suspensions of neutrophils were re-aerated (Fig. 5). When the gas phase comprised air, the rate of photon emission by the neutrophil suspension was 2500 c.p.s., but upon anaerobiosis (achieved by switching the gas phase to O_2 -free N_2), this rate of chemiluminescence decreased to 1000 c.p.s., indicating the O_2 -dependence of photon emission in unactivated cells. When the suspension was re-aerated (by switching the gas phase back to air), the rate of chemiluminescence increased rapidly so that when O_2 saturation was attained in the suspension, the rate of chemiluminescence had increased to 9000 c.p.s., i.e. 3.5 times the original aerobic rate.

Killing of S. aureus after re-aeration of anaerobic suspensions of neutrophils

Since re-aeration of anaerobic suspensions of neutrophils resulted in activation of oxidant generation (Figs 4 and 5), the efficiency of killing of *S. aureus* after this activation was determined. Phagocytosis was initiated by the addition of pre-opsonized bacteria either to neutrophil suspensions which were only exposed to air, or to neutrophil suspensions which were first made anaerobic by incubation under O₂-free N₂ and then re-aerated. The neutrophil suspensions which were re-aerated after anaerobic incubation killed *S. aureus* much more efficiently than those in which oxidant generation had not been achieved by such treatment (Fig. 6). For example, after 30 min incubation, the re-aerated neutrophil suspensions had killed 80% (SD $\pm$ 9%, n = 7) of the bacteria, while control suspensions had killed only 45% (SD $\pm$ 9%, n = 14).

DISCUSSION

The possession by neutrophils of a battery of cytotoxic enzymes with functionally diverse modes of action is necessary to provide effective protection against a range of pathogens which may exhibit varying degrees of resistance to any one mechanism. Here, we have presented data demonstrating a close correlation between the killing of *S. aureus* and the generation of reactive oxidants by neutrophils during phagocytosis. This may be summarized as follows. Firstly, the maximal rate of killing occurred when the rate of oxidant generation (as determined by chemiluminescence) was greatest: as the rate of oxidant generation remained constant and then declined, so the rate of killing slowed. Secondly, when oxidant generation was activated in neutrophils by re-aeration of anaerobic suspensions, the rate of killing was enhanced. Thirdly, neutrophils from a patient with chronic granulomatous disease failed to generate reactive oxidants and could not kill *S. aureus*: such neutrophils contain their full complement of lysosomal enzymes and their only biochemical defect resides in their NADPH oxidase, resulting in an inability to generate oxidants (Hill, 1984). Thus, these data taken together strongly support the proposal that the production of oxidants by neutrophils is a necessary event for the efficient killing of *S. aureus*.

Whilst we have shown here that effective killing of *S. aureus* requires the activation of oxidative cytotoxic processes, clearly O₂-independent mechanisms play a crucial role in the killing of other microbial pathogens (Vel *et al.*, 1984). For example, Gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium* are extremely susceptible to the O₂-independent 'bactericidal/permeability-increasing protein' (Elsbach & Weiss, 1983) and neutrophils from patients with chronic granulomatous disease can efficiently kill a variety of types of bacteria *in vitro* in spite of their inability to generate oxidants (Spitznagel, 1984). Comparison of the rates of killing of *Streptococcus pneumoniae* under aerobic or anaerobic conditions revealed that these bacteria were killed more rapidly when O₂ was present throughout the incubation, suggesting that at least for this organism reactive oxidants exert their cytotoxic effects within the first 30 min of phagocytosis (Thore *et al.*, 1985).

We have previously shown that the apparent K_m for O₂ for generation of reactive oxidants is within the range over which local O₂ tensions may limit their rate of production *in vivo* (Edwards *et al.*, 1984). Since reactive oxidants (and hence O₂) are required for killing of *S. aureus*, it is now necessary to determine the O₂-affinity of killing of this organism by neutrophils, so that the efficiency of this process can be predicted in pathological conditions where O₂ tensions may be low. Since we have identified a group of patients whose neutrophils have (a) impaired ability to kill *S. aureus* both *in vitro* and *in vivo*, and (b) defects in oxidative metabolism, we propose that effective host protection by immune phagocytes against *S. aureus* infections requires efficient production of oxidants. We also propose that pathological disorders which directly or indirectly affect the ability of neutrophils to generate these oxidants will render the host susceptible to infections by this organism.

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