Effects of pH on killing of *Staphylococcus aureus* and *Escherichia coli* by constituents of the neutrophil phagolysosome

BARBARA STYRT* and M. S. KLEMPNER†

Department of Medicine, Tufts University School of Medicine and New England Medical Center Hospital, 750 Washington Street, Boston, MA 02111, USA

Summary. Lysosomotropic weak bases impair in-vitro neutrophil functions including intracellular killing of *Staphylococcus aureus* strain 502a. To investigate whether prevention of phagosomal acidification could account for impaired microbicidal activity, a model phagosome was formulated with a freeze-thawed granule extract as a source of lysosomal enzymes and H₂O₂ as a source of toxic oxygen metabolites. The lysosomal extract alone killed *Escherichia coli* strain S15 efficiently at pH 5.5 and 7.0, but had little activity against *S. aureus* 502a. Sublethal concentrations of the two agents, when combined, acted synergically against either organism. Each organism was killed more effectively at pH 5.5 than at pH 7.0 by the lysosome extract–H₂O₂ combination, but the killing of *E. coli* was more rapid than that of *S. aureus* in the same conditions. These findings suggest that impairment of neutrophil anti-staphylococcal activity by weak bases may be mediated by their ability to raise phagosomal pH, and that persistence of *E. coli* in similar conditions does not occur because the latter is killed by lysosomal constituents in a non-pH-dependent fashion.

Introduction

After ingestion of micro-organisms by neutrophils, the pH within the phagocytic vacuole rises transiently (Segal *et al.*, 1981; Cech and Lehrer, 1984) and then falls to acidic levels (Mandell, 1970; Jensen and Bainton, 1973; Bassoe *et al.*, 1983). Interference with neutrophil pH control, by use of weak bases to raise the pH of acidic compartments, impairs measurable neutrophil functions *in vitro* (Klempner and Styrt, 1983; Styrt and Klempner, 1986). We have previously demonstrated that incubation with the lysosomotropic weak base ammonium chloride impairs neutrophil microbicidal activity against *Staphylococcus aureus* strain 502a but not against *Escherichia coli* strain S15 (Styrt and Klempner, 1985). To delineate the reasons for these different effects, we have constructed a cell-free model of the phagosomal environment to assess the importance of pH

variation and weak bases independently in the presence of different elements of the neutrophil's armamentarium.

Materials and methods

Micro-organisms

E. coli S15 (Weiss *et al.*, 1985) was kindly provided by Dr P. Elsbach (New York University). The standard buffer was NaCl 0.9% with 10 mM sodium acetate, pH 5.5. In selected experiments this buffer was adjusted to pH 7.0, or 50 mM NH₄Cl was added and the pH brought back to 5.5. In some instances, where indicated, 10 mM sodium phosphate was substituted for sodium acetate. The bacteria were grown in tryptic soy broth, harvested by centrifugation and suspended in the standard buffer at c. 10⁷ cfu/ml by comparison with McFarland standards.

Cell preparations

Neutrophil polymorphonuclear leukocytes were isolated by Hypaque-Ficoll density gradient centrifugation and dextran sedimentation from heparinised venous blood of healthy adult volunteers (Boyum, 1968). Cells were disrupted by nitrogen cavitation for 20 min at 350 psi and 4°C. A granule fraction was isolated by differential centrifugation as previously described (Klempner *et al.*, 1980).

Received 3 Feb. 1987; revised version accepted 6 Apr. 1987.

* Present address: Department of Medicine, Michigan State University, East Lansing, MI 48823, USA.

† Requests for offprints should be sent to Dr M. S. Klempner, Division of Geographic Medicine and Infectious Diseases, New England Medical Center, 750 Washington Street, Box 41, Boston, MA, USA.

Lysosome extract

The granule fraction was freeze-thawed three times in a dry ice-methanol bath to release enzyme activity. The preparation was then centrifuged for 3 min at 12 000 *g* to sediment any undisrupted granules and membranes. The supernate constituted the lysosomal enzyme preparation. Protein was measured by the method of Bradford (1976), and the extract was stored at -20°C in single-experiment samples.

For optimal recovery of cationic microbicidal proteins, granule extracts were also prepared by a modification of the method of Weiss *et al.* (1978). Briefly, the granule preparation was suspended in 0.2 M sodium acetate, pH 4.0, stirred overnight at 4°C , centrifuged at 12 000 *g* for 5 min, and the supernate was stored at -70°C until used.

Phagosome model

The lysosome extract represented the non-oxygen-dependent enzymic component of neutrophil microbicidal activity. H_2O_2 represented one of the toxic oxygen metabolites produced by the activated neutrophil. Thus when H_2O_2 and lysosome extract were combined, the H_2O_2 -myeloperoxidase-halide system (Hamers and Sips, 1982), was also reconstituted in the standard chloride-containing buffer.

Microbicidal assays

For each experiment, stocks of H_2O_2 and lysosome extract were made up at 100 times the desired final concentration described below. Then 8–10 tubes were set up, each containing 10 μl of H_2O_2 or lysosome extract or both if so specified and buffer such that the total volume was 0.9 ml. When concentrations of reagent were needed that could not be made up in 100-fold concentrated stock (e.g., the highest extract concentrations used), larger volumes of extract were used and the volume of buffer reduced accordingly. The experiment was then started by adding 0.1 ml of the bacterial suspension (previously adjusted to 10^7 cfu/ml). Thus, the final reaction mixture contained approximately 10^6 cfu plus (where indicated) 0.1–300 μg of lysosomal protein and 0.25 nmol–100 μmol of H_2O_2 in a total volume of 1 ml. After bacteria were added, tubes were vortex mixed, and 10 μl were removed immediately from each for measurement of viability by plating serial dilutions on brain heart infusion agar. The suspensions were then incubated for 2 h at 37°C . At designated intervals during this time, further samples were removed for determination of bacterial viability. Except where otherwise indicated, results are expressed as percentage killing relative to control samples incubated without H_2O_2 or lysosome extract. Plates without bacterial growth—reported as 100% killing—therefore represent approximately a 10^3 reduction in viable counts.

Effect of pH variation

The acetate buffer was adjusted to pH 5.5 or 7.0 before reaction mixtures were formulated to examine the effect

of pH modulation on microbicidal activity of the H_2O_2 -lysosome extract system. The pH of the final incubation mixture was also checked to see whether it was significantly altered by addition of H_2O_2 and lysosomal protein.

Effects of NH_4Cl

Acetate buffer was made up with or without 50 mM NH_4Cl and the pH was held constant at 5.5 to assess the influence of NH_4Cl independently of its phagosome-alkalinising effects.

Statistical methods

Results are expressed as mean and SEM of repeated experiments except where otherwise indicated. Student's *t* test was used to measure statistical significance.

Results

Microbicidal activity of lysosomal extract

To measure the susceptibility of *S. aureus* 502a and *E. coli* S15 to the lysosomal extract, the freeze-thawed extract was included in the incubation mixture at total protein concentrations from 0.1 to 300 $\mu\text{g}/\text{ml}$. H_2O_2 was used at concentrations from 0.25 μM to 100 mM. To examine the effects of other variables on this system, concentrations of H_2O_2 and lysosomal extract were selected to cause partial killing, so that either enhancement or inhibition of microbicidal activity could be detected whenever possible.

The antimicrobial activity of the lysosomal extract varied slightly from one preparation to another and diminished if repeated freeze-thawing was continued after the first few cycles. Therefore, each lot of lysosomal protein was standardised for microbicidal activity and frozen in single-use samples to avoid further freeze-thawing. Samples from the same lot were used whenever possible in experiments destined to be compared with one another.

The effect of the lysosomal enzyme preparation on the viability of *E. coli* S15 is shown in fig. 1. Killing occurred rapidly at protein concentrations as low as 1 $\mu\text{g}/\text{ml}$, and more slowly at 0.1 $\mu\text{g}/\text{ml}$. By contrast, no appreciable killing of *S. aureus* 502a occurred at concentrations up to 300 $\mu\text{g}/\text{ml}$ (data not shown), even after incubation for 2 h.

Effect of H_2O_2

H_2O_2 showed less activity against *E. coli* than *S. aureus*; in 2 h, 67.5 (SEM 11.4)% of *S. aureus* were

Fig. 1. Microbicidal activity of lysosomal extract against *E. coli* S15 represented by mean and SEM of three experiments at pH 5.5. No significant differences were seen at pH 7.0.

killed by 0.5 mM H_2O_2 , whereas only 49.8 (SEM 13.1)% of *E. coli* were killed by 1 mM H_2O_2 .

Effect of the H_2O_2 -lysosome extract combination

To examine the influence of other variables on microbicidal activity in the H_2O_2 -lysosome system, it was necessary to use concentrations of these two components with which partial but not complete killing of bacteria could be anticipated. The combination of sublethal concentrations of lysosomal extract (1 µg/ml) and H_2O_2 (500 µM) showed markedly enhanced activity against *S. aureus*. For example, the individual components produced virtually no reduction in colony counts but the combination caused a 92.7 (SEM 5.8)% fall in cfu during the first 3 min of incubation at pH 5.5. In 1 h the combination eliminated bacterial growth, while there was only 11% killing by lysosomal extract alone and 36% by H_2O_2 alone.

The interaction between lysosomal extract 0.1 µg/ml and 1 mM H_2O_2 was also highly effective in killing *E. coli* S15. After 2 h there was 19.4 (SEM 12.2)% killing by lysosomal extract alone and 49.8

(SEM 13.3)% killing by H_2O_2 alone, while no growth was seen in the presence of both agents.

Stability of pH

To ascertain whether the incubation of bacteria with lysosomal extracts, H_2O_2 and NH_4Cl produced any changes in pH during the experiment, pH readings were checked at the end of selected experiments. Mean final pH of suspensions designated as pH 5.5 was 5.55 and that of nominally pH 7 suspensions was 6.93.

Effect of pH and NH_4Cl on bacterial viability

Viable counts were compared after incubation of each micro-organism in the standard buffer at pH 5.5 or adjusted to neutrality, and after incubation with 50 mM NH_4Cl . The baseline survival of *E. coli* was slightly less at pH 7 than at pH 5.5 (table).

Effect of pH changes on killing of *S. aureus* 502a

Fig. 2 shows the results obtained when microbicidal assays against *S. aureus* were performed at pH 5.5 and 7.0. There was no major difference in the effect of either the lysosomal extract alone or H_2O_2 alone. Killing by the combination was severely impaired at 3 min in the more alkaline suspensions and remained somewhat diminished after 2 h. The same relationship with pH was seen when phosphate buffer was used instead of acetate buffer (85.8 SEM 14.2% inhibition of killing after 3 min at pH 7 compared with pH 5.5). Similar results were obtained when the lysosomal extract was prepared with sodium acetate (data not shown).

Table. Effect of pH manipulation and NH_4Cl on bacterial viability

Treatment	Ratio of treated to control cfu at 2 h	
	<i>S. aureus</i>	<i>E. coli</i>
pH 7	1.09 SEM 0.10	0.76 SEM 0.07
NH_4Cl (50 mM)	0.91 SEM 0.08	0.91 SEM 0.10

Each figure represents the ratio (mean and SEM of 2–16 experiments) of viable counts in suspensions incubated in condition indicated (i.e., adjustment of pH to 7.0 or addition of 50 mM NH_4Cl at constant pH 5.5) to same-day, same-experiment controls incubated at pH 5.5 without NH_4Cl . All values are for suspensions without lysosomal extract or H_2O_2 .

Fig. 2. Microbicidal activity of lysosomal extract 1 $\mu\text{g/ml}$ and 0.5 mM H_2O_2 , separately and in combination, against *S. aureus* 502a at pH 5.5 (□) and pH 7.0 (▨), mean and SEM of five experiments.

Effect of NH_4Cl

S. aureus survival was assessed at pH 5.5 in the presence or absence of 50 mM NH_4Cl and the results are shown in fig. 3. Some delay in killing by the combination of agents was again observed, but microbicidal activity reached equivalent maximal levels at 2 h in both conditions. Moreover, when incubation lasted 90 min or longer (four separate experiments), the combination of NH_4Cl and lysosome extract killed 60–90% of organisms, although neither agent alone had significant microbicidal activity.

Effect of pH changes on killing of *E. coli*

When *E. coli* S15 was incubated with different concentrations of lysosomal extract, no significant change in microbicidal activity was caused by raising the pH from 5.5 to 7.0 at any of the time

points or concentrations tested (data not shown). Adding 50 mM NH_4Cl similarly had no major effect.

Fig. 4 shows the result of incubating *E. coli* with lysosome extract 0.1 $\mu\text{g/ml}$ and 1 mM H_2O_2 at pH 5.5 or 7.0. No significant effect could be demonstrated after 3 min but there was so little microbicidal activity that comparisons are difficult to interpret. At 2 h, microbicidal activity at pH 7.0 was less than at pH 5.5.

Comparison of susceptibility of *S. aureus* and *E. coli*

To compare the susceptibility of the two microorganisms in precisely standardised conditions, a series of experiments was performed in which *S. aureus* and *E. coli* were concurrently incubated with lysosomal extract 0.1 $\mu\text{g/ml}$ and 500 μM H_2O_2 at pH 7.0 and 5.5. Results are shown in fig. 5. Microbicidal activity against both bacteria was delayed at pH 7.0; however, at both pH values *E. coli* was killed more rapidly than *S. aureus*.

Fig. 3. Effect of 50 mM NH_4Cl (▨) on microbicidal activity against *S. aureus* of phagosome model maintained at pH 5.5 and incubated for 3 min (mean and SEM of four experiments) or 2 h (mean and range of 2 experiments). Two separate experiments with incubations for 90 min are not shown.

Fig. 4. Microbicidal activity of lysosomal extract 0.1 $\mu\text{g/ml}$ and 1 mM H_2O_2 , separately and in combination, against *E. coli* S15 at pH 5.5 (□) and 7.0 (▨) incubated for 3 min (mean and SEM of four experiments) or 2 h (mean and SEM of three experiments).

Discussion

These studies sought to construct a cell-free model of the neutrophil phagosomal environment and to examine the effect of pH changes on bacterial survival therein. We have demonstrated synergistic bactericidal activity of lysosomal contents and activated oxygen metabolites, analogous to that expected in the intact cell, against both oxidant-susceptible *S. aureus* 502a and granule protein-susceptible *E. coli* S15. In this system, raising the ambient pH from 5.5 to 7.0 impaired killing of both micro-organisms. However, *E. coli* S15 was more effectively killed than *S. aureus* at pH 7.0, and remained susceptible to adequate concentrations of lysosome contents at the elevated pH. These findings suggest that the importance of phagosomal acidification for neutrophil effectiveness in physiological situations varies with different targets.

Although the lowering of intraphagosomal pH after ingestion of bacteria is well documented, the importance of this phenomenon to host defence has not been well defined. No intrinsic neutrophil

abnormality has been associated with failure of acidification, though a lack of the initial alkalisation phase has been reported in chronic granulomatous disease (Segal *et al.*, 1981). However, several organisms which survive within phagocytes have been shown to inhibit phagosomal acidification (Sibley *et al.*, 1985; Weidner and Sibley, 1985; Black *et al.*, 1986), a finding which suggests that an alkaline phagosome may be more hospitable to certain micro-organisms and less conducive to efficient microbicidal activity than an acid one. Thus, for organisms which parasitise phagocytes, ability to inhibit phagolysosomal acidification may be an important factor determining virulence; e.g., virulent strains of *Nocardia asteroides* inhibit phagosomal acidification while less virulent strains do not (Black *et al.*, 1986), and viable but not heat-killed *Toxoplasma gondii* inhibit acidification (Sibley *et al.*, 1985).

Examination of isolated components of the phagosomal environment has elucidated different pH relationships depending on the system examined. Microbicidal events in the phagosome would be expected to include direct attack on the bacterium by granule contents including bactericidal/permeability-increasing protein (BPI) (Weiss

Fig. 5. Microbicidal activity of lysosomal extract 0.1 $\mu\text{g/ml}$ and 0.5 mM H_2O_2 in combination against *S. aureus* and *E. coli* at pH 5.5 (□) and 7.0 (▨); mean and SEM of three experiments.

et al., 1978; Elsbach *et al.*, 1979), defensins (Ganz *et al.*, 1985; Selsted *et al.*, 1985), and acid hydrolases (Elsbach, 1980); and direct effects of toxic oxygen metabolites including superoxide (Babior *et al.*, 1973) and H_2O_2 (Root *et al.*, 1975). Halogenation of bacteria by the myeloperoxidase- H_2O_2 -halide system, requiring participation of granule constituents and products of oxidative metabolism, is presumably another major mechanism of neutrophil microbicidal activity (Klebanoff, 1968). The effects of pH on individual and combined phagosomal contents may be relevant to different clinical situations in which the availability of these components varies.

The microbicidal activity of BPI is reported to be maximal at pH 7.0 (Elsbach *et al.*, 1979); a granule protein with antipseudomonal effects is equally active at pH 5.6 and 7.0 (Hovde and Gray, 1986); and defensins are effective at pH 7.4 (Ganz *et al.*, 1985). The NADPH oxidase which catalyses production of toxic oxygen metabolites also has a pH optimum of 7.3 (Babior, 1983), but a cationic granule protein has maximal microbicidal activity at acid pH (Shafer *et al.*, 1986) and the myeloperoxidase- H_2O_2 -halide system is most active at pH 5.5 (Mandell, 1974). Thus, the net effect of pH on intraphagosomal killing of bacteria is likely to reflect a complex interplay of different pH-dependent processes whose summation may be difficult to predict.

We compared microbicidal activity in the model phagosome at pH 5.5 (chosen to represent the normally acidified phagosome) and at pH 7.0 (to approximate levels that might be reached after alkalisation of intracellular acidic compartments by incubation with a lysosomotropic weak base). There was no difference in bactericidal activity of either lysosomal extract or H_2O_2 alone against either *S. aureus* or *E. coli* at either pH value. In particular, *E. coli* was killed rapidly by the lysosomal extract alone at either pH value.

When sublethal concentrations of the two agents were combined, activity against both organisms was enhanced at pH 5.5 relative to pH 7. This is compatible with the findings of Riley and Robertson (1984) who reported that activity of a granule- H_2O_2 combination against *Brucella abortus* was greatest at pH 5.5-6, but contrasts with the efficacy of a myeloperoxidase H_2O_2 -halide system against *Bacillus cereus* at pH 6.8 (Tenovuo *et al.*, 1985). However, when we compared *S. aureus* and *E. coli* at equivalent reagent concentrations in identical incubation conditions, *E. coli* was killed more rapidly and more completely than *S. aureus*, especially at pH 7.

These studies grew out of our previous work demonstrating impaired intracellular killing of *S. aureus* but not of *E. coli* after incubation of neutrophils with NH_4Cl to alkalise acidic intracellular compartments (Styrt and Klempner, 1985). The effects of NH_4Cl in the whole cell may include collapse of pH gradients, impairment of phagosome-lysosome fusion, and concentration of NH_4Cl itself in the phagosomal milieu. To ascertain whether NH_4Cl might have an effect on intraphagosomal killing not associated with impairment of phagosome-lysosome fusion (Gordon *et al.*, 1980) or elevation of intraphagosomal pH, we added NH_4Cl to the model phagosome while holding the pH constant at 5.5. NH_4Cl did not alter the activity of lysosomal extract against *E. coli* but had paradoxical effects on killing of *S. aureus*. The antistaphylococcal activity of the lysosome extract- H_2O_2 combination was delayed but the effect of extract alone was actually enhanced at long incubation times. The explanation for these effects is not immediately apparent but could involve a slow interaction between NH_4Cl and phagosome constituents to form toxic chloramines (Thomas *et al.*, 1983; Weiss *et al.*, 1983). The initial delay might then reflect preferential interaction of oxygen metabolites with NH_4Cl rather than with bacteria, with long-lived chloramines contributing to late microbicidal effects.

Taken as a whole, our results indicate that a model of the neutrophil phagosome provides maximal activity against disparate organisms at acid pH. In particular, the impaired killing of *S. aureus* 502a in intact neutrophils treated with NH_4Cl may be due in large part to phagosomal alkalisation, although a delay in killing due to NH_4Cl itself may also contribute. Although killing of *E. coli* was also enhanced at acid pH, this pH effect may be less important in the intact cell (and by extrapolation in clinical settings) because such small amounts of lysosomal protein are required for effective killing that dispatch of organisms occurs at pH 7, and greater activity at pH 5.5 confers no practical benefit. The importance of phagosome acidification to neutrophil-mediated host defense against bacteria therefore may depend as much on the characteristic susceptibilities of the target organism as on the pH optima of the various components of the microbicidal system.

We thank R. Noring and F. Craig for expert technical assistance, and Dr P. Elsbach (New York University) for providing *E. coli* S15. This work was supported in part by Grants #AI18028 and AI16732 from the National Institutes of Health. Parts of these studies have been presented at the 1986 National

Meeting of the American Federation for Clinical Research (abstract: *Clinical Research* 34: 534A).

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