Chlorination of Bacterial and Neutrophil Proteins during Phagocytosis and Killing of Staphylococcus aureus*

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Myeloperoxidase is proposed to play a central role in bacterial killing by generating hypochlorous acid within neutrophil phagosomes. However, it has yet to be demonstrated that these inflammatory cells target hypochlorous acid against bacteria inside phagosomes. In this investigation, we treated Staphylococcus aureus with varying concentrations of reagent hypochlorous acid and found that even at sublethal doses, it converted some tyrosine residues in their proteins to 3-chlorotyrosine and 3,5-dichlorotyrosine. To determine whether or not ingested bacteria were exposed to hypochlorous acid in neutrophil phagosomes, we labeled their proteins with $^{13}$C$_9$-tyrosine and used gas chromatography with mass spectrometry to identify the corresponding chlorinated isotopes after the bacteria had been phagocytosed. Chlorinated tyrosines were detected in bacterial proteins 5 min after phagocytosis and reached levels of approximately 2.5/1000 mol of tyrosine after 60 min. Inhibitor studies revealed that chlorination was dependent on myeloperoxidase. Chlorinated neutrophil proteins were also detected and accounted for 94% of total chlorinated tyrosine residues formed during phagocytosis. We conclude that hypochlorous acid is a major intracellular product of the respiratory burst. Although some react with the bacteria, most react with neutrophil components.

Since Metchnikoff (1) first observed phagocytosis of bacteria in white blood cells, numerous investigations have been undertaken to determine the mechanisms responsible for bacterial killing. It is now well established that neutrophils use oxidative and non-oxidative mechanisms to kill bacteria (2, 3). However, our understanding of the precise ways in which these host defenses work is still in its infancy. This is especially true for how neutrophils use oxygen to fight infection. When they phagocytose bacteria, neutrophils rapidly consume oxygen, which is reduced to superoxide by an NADPH oxidase. Superoxide is not toxic to bacteria but spawns numerous other reactive oxygen species that have been implicated in bacterial killing (4). Chief among these is hypochlorous acid, which is formed from hydrogen peroxide and chloride in a reaction catalyzed by myeloperoxidase (5).

Results from several studies indicate that myeloperoxidase is required for the majority of oxygen-dependent killing of certain bacteria such as Staphylococcus aureus (4, 6, 7). It is released into phagosomes along with superoxide and hydrogen peroxide (8). However, myeloperoxidase is a complex enzyme with several different activities (5), and it can not be assumed that its predominant function within neutrophil phagosomes is to produce hypochlorous acid. Early studies showing incorporation of chlorine-36 (9) and iodination of bacterial and neutrophil proteins (10) indicated that hypohalous acids are produced when neutrophils undergo phagocytosis. More recently, it was demonstrated that free tyrosine enclosed inside red blood cell ghosts was chlorinated when these vesicles were phagocytosed by neutrophils (11). Also, fluorescein conjugated to polyacrylamide microspheres was extensively chlorinated when the beads were ingested by neutrophils (12). Approximately 10% of oxygen consumed by the neutrophils was accounted for as chlorofluorescein, which was suggested to be sufficient to kill bacteria (12).

It has yet to be shown that hypochlorous acid is produced in phagosomes when neutrophils engulf bacteria, and whether it is responsible for killing them. Therefore, we sought evidence that hypochlorous acid reacts with ingested bacteria by showing that bacterial molecules are modified by a reaction that is specific to hypochlorous acid. The modification we selected was the conversion of tyrosine residues in proteins to 3-chlorotyrosine and 3,5-dichlorotyrosine (11, 13–16). Although these chlorinated molecules are minor products of the reaction of hypochlorous acid with proteins, they are stable and specific for this oxidant and as such are appropriate markers for verifying its production (17). In this study, we show that tyrosine residues in bacterial proteins are chlorinated after bacteria have been phagocytosed by neutrophils. This result demonstrates that hypochlorous acid is produced in phagosomes and reacts with bacteria. We also show that the vast majority of chlorination occurs on neutrophil rather than bacterial proteins.

MATERIALS AND METHODS

The concentration of the hypochlorous acid stock solution (Reckitt and Coleman, Auckland, New Zealand) was determined using either 5-thio-2-nitrobenzoic acid as described previously (18) or its absorbance maximum at 292 nm ($\varepsilon_{292} = 350 \text{ M}^{-1} \cdot \text{cm}^{-1}$) (19). L-Tyrosine-[U-$^{13}$C$_9$] and L-tyrosine-RING-[13C$_6$] were obtained from Cambridge Isotope Laboratories (Andover, MA). 3,5-Dichlorotyrosine was kindly prepared by Dr. Christina Chai (Department of Chemistry, Australian National University, Canberra, Australia). Chlorinated tyrosine [13C$_n$] internal standards were prepared as described previously (16). S. aureus ATCC 27217 (502a) was purchased from the New Zealand Communicable Disease Center (Porirua, New Zealand). Columbia sheep blood agar plates were from Port Richard (New Zealand), and nutrient broth no. 1 was from Oxoid Ltd. (Basingstoke, UK). All other chemicals were purchased from the Sigma or BDH (Poole, UK).

Hypochlorous Acid Treatment of S. aureus—S. aureus was grown overnight in nutrient broth at 37 °C with agitation. In experiments...
Neutrophil and Bacterial Chlorination

RESULTS

Chlorination and Killing of Bacteria Treated with Reagent Hypochlorous Acid—We initially measured chlorination of tyrosine residues in bacterial proteins after treatment with various doses of hypochlorous acid with the aim of recovering bacteria from neutrophil phagosomes and predicting their level of exposure to hypochlorous acid. S. aureus were treated with hypochlorous acid at doses ranging from 1 to 30 nmol for 20 min at 37 °C. This resulted in the conversion of tyrosine residues to 3-chlorotyrosine and 3,5-dichlorotyrosine (Fig. 1). Bacterial viability was completely lost at a dose of approximately 25 nmol of hypochlorous acid/108 bacteria (Fig. 2). The corresponding level of chlorination was approximately 6 mol of 3-chlorotyrosines/1000 mol of tyrosine. At all doses of hypochlorous acid, yields of 3,5-dichlorotyrosine were at least 4-fold less than those of 3-chlorotyrosine. These results demonstrate that chlorinated tyrosine residues are formed in bacterial proteins at and below bactericidal doses of hypochlorous acid.

Table I

| Amino acid                  | M+ | M+–HF | M+–CF3–O |
|-----------------------------|----|-------|----------|
| l-[13C6]tyrosine (l-[13C6]Tyr) | 415| 395   |          |
| l-[12C]tyrosine (l-[12C]Tyr) | 449| 455   | 386      |
| l-[12C]tyrosine (l-[12C]Tyr) | 424| 401   | 361      |

Fig. 1. GC/MS detection of tyrosine and chlorinated tyrosines in hypochlorous acid-treated bacteria. Bacteria (1 × 108) were treated with 30 nmol of hypochlorous acid for 20 min at 37 °C. Proteins were acid-hydrolyzed, and the resulting amino acids were solid phase-extracted. l-[12C]Tyrosine (m/z 212), 3-chloro-[12C]Tyrosine (m/z 245), and 3,5-dichloro-[12C]Tyrosine (m/z 289) were derivatized, and the n-propyl trifluoroacetic anhydride derivatives were separated by GC/MS and co-eluted with authentic standards. The negative ion chemical ionization GC/MS chromatogram of the separation is shown (the ion current scale changes from 0—150 to 0—12 at the axis break). The trace is representative of at least 10 experiments.

Fig. 2. The relationship between chlorination and viability in S. aureus treated with hypochlorous acid. Bacteria (1 × 108) were treated with hypochlorous acid at doses ranging from 1 to 30 nmol for 20 min at 37 °C. Aliquots of each sample were diluted and plated to assess viability. The bacteria remaining in the sample were acid-hydrolyzed and assayed for chlorinated tyrosines as in Fig. 1. Closed circles represent 3-chlorotyrosine, and open triangles represent viability. All the data from four independent experiments are presented.
The above experiments were performed at pH 7.4. However, the pH inside phagosomes changes during phagocytosis. Initially, the pH is 7.4, but rises to almost 8 in the first 5 min and then declines to 6 throughout the next hour (24, 25). When S. aureus (1 × 10⁸) were treated with 10 nmol of hypochlorous acid at pH 6, 7.4, and 8, the degree of 3-chlorotyrosine formation was 1.1, 1.8, and 2.9/1000 tyrosines, respectively. These values are means of duplicate experiments in which the ranges differed by <10%. At all pH values, the levels of 3-chlorotyrosine were approximately 4-fold higher than those of 3,5-dichlorotyrosine (data not shown).

Chlorination of Bacteria Ingested by Neutrophils—When opsonized bacteria were added to neutrophils at a ratio of 20:1, they were phagocytosed with a half-life of 9 min. Once ingested, the half-life for killing was 3 min. Twenty minutes after adding bacteria to neutrophils, 80% had been ingested, and 70% were killed. The ingested bacteria were isolated, and their proteins were subsequently analyzed by GC/MS for chlorinated tyrosines. We were able to distinguish between bacterial and neutrophil proteins because the neutrophil proteins contained tyrosine. We were able to distinguish between bacterial and neutrophil proteins because the neutrophil proteins contained tyrosine.

The mass spectrum of 3-chloro-[13C₆]tyrosine is shown in Fig. 3A. The inset shows the structure of the derivative and its expected fragmentation pattern. The above experiments were performed at pH 7.4. However, the pH inside phagosomes changes during phagocytosis. Initially, the pH is 7.4, but rises to almost 8 in the first 5 min and then declines to 6 throughout the next hour (24, 25). When S. aureus (1 × 10⁸) were treated with hypochlorous acid at pH 6, 7.4, and 8, the degree of 3-chlorotyrosine formation was 1.1, 1.8, and 2.9/1000 tyrosines, respectively. These values are means of duplicate experiments in which the ranges differed by <10%. At all pH values, the levels of 3-chlorotyrosine were approximately 4-fold higher than those of 3,5-dichlorotyrosine (data not shown).

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also formed but to a lesser extent, reaching a maximum of approximately 0.8/1000 mol of tyrosine. Its mass spectrum gave the characteristic 9:6:1 ratio expected for a doubly chlorinated molecule (data not shown).

As shown in Fig. 5, chlorination was decreased by diphenyleneiodonium, which inhibits the neutrophil NADPH oxidase, and by the heme poison azide. Chlorination was substantially less in bacteria that had been ingested by neutrophils from an individual who was deficient in myeloperoxidase. These results confirm that chlorination of bacterial proteins resulted from the generation of hypochlorous acid by myeloperoxidase. Tau- 

tine and catalase did not inhibit chlorination. These latter results imply that chlorination of bacteria occurred in a compartment that was not accessible to extracellular scavengers of hypochlorous acid and hydrogen peroxide.

Chlorination of Neutrophil Proteins during Phagocytosis of Bacteria—Several studies have revealed that neutrophils are susceptible to their own oxidants (26, 27). To ascertain whether hypochlorous acid also reacts with neutrophil components, neutrophil proteins were examined for evidence of chlorination. We first determined how readily reagent hypochlorous acid chlorinated tyrosine residues in neutrophil proteins. As shown in Fig. 6, the formation of 3-chlorotyrosine was relatively inefficient at low doses of hypochlorous acid but increased exponen- 
tially as the dose was increased. Presumably, at low doses the hypochlorous acid would preferentially react with thiols, methionine residues, and ascorbate in neutrophils (28, 29). At higher doses, more would be available to chlorinate tyrosyl residues in proteins. This effect was not evident when bacteria were treated with hypochlorous acid (Fig. 2), even though the lowest doses of oxidant/tyrosine residue were similar (3 nmol of hypochlorous acid/40 pmol of Tyr for bacteria and 50 nmol of hypochlorous acid/480 pmol of Tyr for neutrophils). It is not entirely clear why a scavenging effect was not observed with bacteria. However, it may simply reflect the sensitivity of our assay, because 6.5-fold less 3-chlorotyrosine was formed in bacteria (40 fmol) than in neutrophils (265 fmol) at the lowest doses of hypochlorous acid.

Next, we examined the ability of neutrophils to chlorinate their own proteins. After neutrophils had undergone phagocytosis of S. aureus, they were lysed, the bacteria were pelleted, and the remaining soluble protein was assayed for its content of 3-chlorotyrosine and 3,5-dichlorotyrosine. There was minimal [13C6]tyrosine contamination in this fraction, confirming that it contained predominantly neutrophil proteins (data not shown). Both 3-chlorotyrosine and 3,5-dichlorotyrosine were detected in neutrophil proteins (Fig. 7). The level of chlorination rose steadily over 60 min with 4 mol of 3-chlorotyrosine and 2 mol of 3,5-dichlorotyrosine formed/1000 mol of tyrosines (Fig. 7). The inhibitor profile was similar to that seen with bacterial chlorination and indicated that chlorination of neutrophil proteins was dependent on myeloperoxidase (Fig. 8). However, azide dramatically enhanced the chlorination of neutrophil proteins. This effect is most probably attributed to greater inhibition of cytosolic catalase compared with the myeloperoxidase contained in the azurophilic granules (30). Thus, in the presence of azide, there would be more hydrogen peroxide available for conversion to hypochlorous acid.

The extent of chlorination in each cell type was compared to establish the relative amounts of hypochlorous acid that had reacted with bacteria or neutrophils. Twenty minutes after bacteria were added to neutrophils, the ratio of 3-chlorotyrosine to tyrosine was twice that observed with bacterial proteins (Table II). To calculate the absolute amounts of chlorinated tyrosines in bacterial and neutrophil proteins, the total amounts of tyrosine residues in each cell type were measured. As shown in Table II, there were considerably more tyrosine
residues in the neutrophils than the bacteria. The ratios for chlorination were then multiplied by the total amounts of tyrosine to get absolute values for the contents of 3-chlorotyrosine and 3,5-dichlorotyrosine in bacteria and neutrophils. This comparison revealed that 94% of incorporated chlorine was present in neutrophil proteins. That is, the majority of the hypochlorous acid generated during phagocytosis had reacted with the neutrophil.

We calculated the efficiency of chlorination in each cell type at a dose of reagent hypochlorous acid that converted one tyrosine in 1000 to 3-chlorotyrosine. With $1 \times 10^9$ bacteria, 3 nmol of hypochlorous acid gave rise to 40 fmol of 3-chlorotyrosine, i.e. 13 fmol of 3-chlorotyrosine were formed/nmol hypochlorous acid (see Fig. 2). In contrast, the addition of 100 nmol of hypochlorous acid to $5 \times 10^8$ neutrophils produced 482 fmol of 3-chlorotyrosine, i.e. 5 fmol of 3-chlorotyrosine were formed/nmol hypochlorous acid (see Fig. 6). Thus, tyrosine residues in bacteria were chlorinated ~2.5 times more efficiently as those in neutrophils.

**DISCUSSION**

We have shown that hypochlorous acid is produced inside neutrophil phagosomes during bacterial killing and that it reacts with the bacteria. Surprisingly though, we have also demonstrated that the majority of chlorination occurs on neutrophil proteins. Only 6% of the chlorinated tyrosines were present in bacterial proteins. These results indicate that the majority of hypochlorous acid produced during phagocytosis reacts with neutrophil components rather than the ingested bacteria. We were able to reveal these novel findings by labeling bacteria with $[^{13}C]$,chyrosine so that we could distinguish between bacterial and neutrophil proteins. The detection of chlorinated tyrosine residues in proteins by GC/MS analysis gave unequivocal evidence that hypochlorous acid had been produced within neutrophils when they phagocytosed bacteria and was also used to determine the fate of the oxidant. Our conclusions are based on the caveat that the formation of chlorinated tyrosines adequately describes the reactions of hypochlorous acid within neutrophils. It is possible that because these biomarkers are minor products of protein modification by hypochlorous acid (14), they may not accurately reflect what happens with the majority of the oxidant. However, this presumed disadvantage has to be weighed against the real advantage that 3-chlorotyrosine and 3,5-dichlorotyrosine are the only biomarkers of protein modification that are specific to hypochlorous acid (15, 31).

Others (32–34) have demonstrated that hypochlorous acid is produced extracellularly when neutrophils are stimulated with a variety of agonists. It has also been shown to be generated within phagosomes (11, 12). However, our study is the first to show that hypochlorous acid is produced inside phagosomes when neutrophils ingest bacteria. Inhibitor studies confirmed that chlorination of proteins was dependent on myeloperoxidase. A lack of inhibition by catalase and taurine, which scavenges hydrogen peroxide and hypochlorous acid, respectively, is consistent with the proposal that chlorination of bacteria and neutrophils were intracellular events. Neither catalase nor taurine would be expected to enter the cell or the phagosome in sufficient quantities to affect reactions of myeloperoxidase. Our report finally confirms the original proposal of Klebanoff (35) that neutrophils use the myeloperoxidase/hydrogen peroxide/chloride system to generate hypochlorous acid for antimicrobial defense.

We had anticipated the bacteria would be heavily chlorinated inside phagosomes. However, the extent of chlorination after 20 min of phagocytosis was equivalent to that achieved in isolated bacteria that were treated with only 5 nmol of reagent hypochlorous acid/10^6 cells. It is possible that chlorinated proteins were preferentially exported from bacteria (36) and/or had enhanced proteolytic digestion (37). However, the recovery of $[^{13}C]$,tyrosine that was incorporated into bacterial proteins varied considerably among experiments. Yet the extent of chlorination showed little variation. This indicates that the chlorination of the bacterial protein we isolated was representative of the total, and that we had not failed to detect highly chlorinated bacterial proteins. It is possible to underestimate the extent of chlorination, because 3-chlorotyrosine reacts readily with hypochlorous acid to form additional chlorinated products (15, 16). However, we checked for this possibility by measuring the levels of 3,5-dichlorotyrosine that were low and confirmed that the bacteria were minimally chlorinated. Our estimates of the amount of hypochlorous acid that reacted with bacteria inside phagosomes were based on the extent of bacterial chlorination by reagent hypochlorous acid at pH 7.4. The efficiency of this reaction was found to increase over the pH range that occurs during phagocytosis. In the first 20 min of phagocytosis, the phagosome has a slightly alkaline pH (24, 25). Therefore, our estimates of the amount of hypochlorous acid that reacted with the bacteria would be expected to be slightly higher than the actual values. Even so, they do indicate that the bacteria had been subjected to low but microbicidal doses of hypochlorous acid.

It is conceivable that because the presence of other antimicrobial agents, bacteria are likely to be more susceptible to killing by hypochlorous acid generated inside phagosomes than to reagent hypochlorous acid added to them in isolation. For example, elastase is known to greatly enhance the bactericidal activity of the myeloperoxidase-glucose oxidase system (38). Consequently, we cannot make a firm conclusion on the precise contribution hypochlorous acid made to the killing of the bacteria.

Previously, it has been shown that when neutrophils ingest *S. aureus* at a ratio of 20 bacteria/phagocyte, they consume approximately 1.8 nmol of oxygen/10^6 cells/min (39). If this rate was sustained, we would expect that the maximum amount of hypochlorous acid, which could be produced in our experiments, with $5 \times 10^6$ cells over 20 min would be 180 nmol.

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**TABLE II**

Comparison of chlorination in bacterial and neutrophil proteins

|                  | $1 \times 10^9$ Bacteria | $5 \times 10^8$ Neutrophils |
|------------------|--------------------------|-----------------------------|
| 3-chlorotyrosine  | 1.2 ± 0.2 (n = 12)        | 2.0 ± 0.2 (n = 12)          |
| 3,5-dichlorotyrosine | 0.6 ± 0.1 (n = 9)      | 0.6 ± 0.1 (n = 10)          |
| Total Tyr (pmol)  | 39.9 ± 5.3 (n = 10)      | 482 ± 91 (n = 9)            |
| Total 3-chlorotyrosine (fmol) | 48               | 964                         |
| Total 3,5-dichlorotyrosine (fmol) | 24             | 289                         |
| Total chlorination (fmol) (chlorotyrosine + 2x dichlorotyrosine) | 96             | 1542                        |
| % Total chlorination | 6                | 94                          |

*a* The value given is for the 80% bacteria that was ingested after 20 min.
Adding 130 nmol of reagent hypochlorous acid to neutrophils gave a similar degree of chlorination of their proteins as that observed when the cells underwent phagocytosis (compare Fig. 6 with Fig. 7). Based on these values, we can estimate that approximately 70% of the oxygen consumed by neutrophils was used to make intracellular hypochlorous acid. Thus, hypochlorous acid should be considered as a major product of the respiratory burst when neutrophils ingest and kill bacteria. Hurst and co-workers (12) previously estimated that a minimum of 10% hydrogen peroxide is converted to hypochlorous acid. This value was based on the extent to which fluorescein conjugated to polyacrylamide microspheres was chlorinated. The value is likely to be a lower limit, because they did not consider chlorination of neutrophil components in their study.

Our finding that the majority of the chlorinated tyrosines formed during phagocytosis is in neutrophil proteins demonstrates that most of the hypochlorous acid produced during the respiratory burst reacts with the neutrophils. We checked the efficiency of chlorination in each cell type and found that 3-chlorotyrosine was formed approximately twice as readily in bacterial proteins than in neutrophil proteins. Thus, based on the assumption that reagent hypochlorous acid reacts similarly to hypochlorous acid generated inside neutrophils, we conclude that the greater degree of chlorination of neutrophil proteins during phagocytosis cannot be attributed to them being more efficiently chlorinated than bacteria. Previously, it was found that neutrophil components including methionine residues (26) and thiols (27, 40) undergo extensive oxidation when the cells are stimulated. In these studies it was inferred that hypochlorous acid was responsible for most of the oxidant measured. However, specific biomarkers were not used to categorically prove its involvement. Analogous results to those presented here have been reported for iodination of proteins following phagocytosis of bacteria (10, 41). Most of the radioactive iodine was associated with neutrophil proteins, not bacterial proteins, which supports our proposal that most of the hypochlorous acid reacts with neutrophil components. A simple explanation for this result is that because hypochlorous acid can readily pass through cell membranes (42) and diffuse considerable distances compared with the size of a bacterium (43), it will react mainly with the neutrophil, which provides the biggest sink for this reactive oxidant. The extensive oxidation of neutrophil components by hypochlorous acid does raise the possibility that modification of some of them may be play a beneficial role in microbial killing. In future studies, it will be important to identify which neutrophil molecules are oxidized during phagocytosis and to determine how this affects their antimicrobial activity.

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