Oxidative Response of Human Neutrophils, Monocytes, and Alveolar Macrophages Induced by Unopsonized Surface-Adherent *Staphylococcus aureus*

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In contrast to results with bacterial suspensions, phagocytosis of unopsonized bacteria readily occurs when bacteria are adhered to glass or plastic surfaces. However, in contrast to neutrophils, alveolar macrophages produced much less DNA denaturation as measured by acridine orange metachromasia of phagocytized *Staphylococcus aureus*. We have studied the phagocytosis of unopsonized surface-adherent *S. aureus* and the subsequent production of reactive oxygen species by peripheral blood neutrophils, monocytes, and alveolar macrophages. Phagocyte-free systems were then used to show the relationship of the reactive oxygen species produced by neutrophils and alveolar macrophages and the denaturation of unopsonized *S. aureus* DNA with acridine orange. Peripheral blood neutrophils, monocytes, and alveolar macrophages from normal human volunteers were added to vials with adherent *S. aureus* without opsonin. Bacterial uptake and luminol- and lucigenin-dependent chemiluminescence were measured. Neutrophils developed much greater luminol-dependent chemiluminescence than monocytes or alveolar macrophages. Compared with neutrophils and monocytes, alveolar macrophages developed significantly greater concentrations of superoxide, as measured by lucigenin-dependent chemiluminescence and ferricytochrome *c* reduction. These findings suggested that products of the myeloperoxidase-hydrogen peroxide-halide pathway were generated when peripheral blood neutrophils were stimulated and that alveolar macrophages primarily produced superoxide. When these reactive oxygen species were generated in phagocyte-free systems containing *S. aureus*, products of the myeloperoxidase-hydrogen peroxide-halide pathway produced denaturation of *S. aureus* DNA, whereas superoxide did not. Thus, differences in reactive oxygen species produced during phagocytosis may be related to the different capacities of neutrophils and alveolar macrophages to denature unopsonized adherent *S. aureus* DNA.

A model for phagocytosis of bacteria has been developed in our laboratory that utilizes bacteria adhered to a surface as a stimulus (14). Compared with suspension assays, this model may more closely simulate the behavior of phagocytes exposed to bacteria on epithelial surfaces. This model with adherent bacteria has shown significant differences in response of phagocytic cells when compared with phagocyte systems with bacteria in suspension. For example, when bacteria are adhered to a surface, efficient uptake by phagocytes is possible without the presence of serum opsonins. Peripheral blood neutrophils monocytes and alveolar macrophages exhibited >65% uptake of unopsonized bacteria. When phagocytized by neutrophils, 45% of the phagocytized *Staphylococcus aureus* showed denatured DNA. In contrast, only 6% of *S. aureus* had denatured DNA when phagocytized by alveolar macrophages (17).

The purpose of the present study was to determine whether unopsonized, adherent *S. aureus* DNA denaturation might be related to a difference in reactive oxygen species formed during phagocytosis. Two chemiluminogenic probes were used to study the oxidative metabolic response of neutrophils, monocytes, and alveolar macrophages during engulfment of glass-adherent staphylococci. 5-Amino-2,3-dihydrophthalazine-1,4-dione (luminol) was used since this probe measures primarily products of the myeloperoxidase (MPO)-hydrogen peroxide-halide pathway of the respiratory

burst during phagocytosis (3-5, 9, 11, 12, 23). Dimethyl biacridium nitrate lucigenin) was the second probe used since the chemiluminescence with this probe reflects superoxide production (2-4, 20; P. Stevens and D. Hong, Fed. Proc. 41:273, 1982). To show that this difference in reactive oxygen species between neutrophils and alveolar macrophages could in turn produce the observed differences in *S. aureus* DNA denaturation, phagocyte-free systems were used to generate these oxygen species. Superoxide in concentrations observed in the phagocytizing alveolar macrophages produced no metachromasia of *S. aureus* exposed to acridine orange. In contrast, addition of MPO to H₂O₂ in the presence of Cl⁻ and the addition of OCl⁻ alone at concentrations observed in the phagocytizing neutrophil produced metachromasia of acridine orange-exposed *S. aureus*.

MATERIALS AND METHODS

Cell preparation. Alveolar macrophages were obtained from healthy human donors by subsegmental lavage of the right middle lobe with sterile saline (13, 14). Lavage fluid was passed through a wire mesh to remove mucus, centrifuged for 10 min at 160 × *g*, and suspended in Hanks balanced salt solution (HBSS) containing 0.1% gelatin (GHBSS). The cells were then counted on a hemacytometer, and viability of >95% was assured by trypan blue exclusion. The mean (range) cell differentials were: alveolar macrophages, 77% (44 to 97%); neutrophils, 3% (0 to 11%); and lymphocytes, 20% (0 to 56%).

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Neutrophils were isolated from heparinized venous blood by isopycnic centrifugation and hypotonic lysis of erythrocytes (8, 21). Monocytes were isolated as previously described (1) by adherence to preconditioned plastic flasks. The purity of neutrophil and monocyte suspensions and viability by trypan blue exclusion both exceeded 95%. Final cell suspensions of all three phagocyte types contained 5×10^4 cells per ml of GHBSS for the chemiluminescence assay and 5×10^6 cells per ml of GHBSS for phagocytosis and ferricytochrome *c* reduction.

Bacterial adherence. *S. aureus* Cowan I colonies from a blood agar plate were grown for 18 h at 37°C in 10 ml of Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) containing 20 μ l of [3 H]adenine (specific activity, 33 Ci/mmol; Research Products International Corp., Mt. Prospect, Ill.) when required. The bacteria were then centrifuged, washed twice with phosphate-buffered saline (PBS), pH 7.4, and suspended to a concentration of 2×10^9 CFU/ml of PBS.

For phagocytosis, 1 ml of radiolabeled bacteria (2×10^8 CFU/ml of PBS) was added to each well of a 24-well tissue culture dish (Costar, Cambridge, Mass.) and incubated at 37°C for 2 h. The supernatant containing nonadherent bacteria was removed, and wells were washed with 2 ml of PBS. Of bacteria added to the well, 12% were adherent as determined by counting the initial inoculum of radiolabeled bacteria and the nonadherent bacterial population.

For the chemiluminescence assay the bacteria were further diluted to 7×10^8 CFU/ml of PBS, and 1 ml was added to glass scintillation vials (Research Products International) and allowed to adhere for 2 h at 37°C in a stationary position. About 1.4×10^7 bacteria became adherent during this time as determined by radiolabel assay (16). The supernatant was then decanted, and the vials were used for the chemiluminescence assay.

For the ferricytochrome *c* assay, 200 μ l of the bacterial suspension of 2.5×10^9 CFU/ml of PBS was added to each well of a 96-well, flat-bottomed microtiter plate (Costar) and incubated for 2 h in a stationary position at 37°C. After incubation the supernatant was decanted, leaving approximately 10^8 adherent bacteria.

Phagocytosis. Bacterial uptake from a plastic surface was determined as described by Lee et al. (16). Briefly, 0.5 ml of a phagocyte suspension (2.5×10^6 cells) was added to each well of a 24-well tissue culture dish (Costar) containing adherent, radiolabeled bacteria. The plates were incubated at 37°C in a stationary position for the specified times. Phagocytic cells and bacteria were then removed by trypsin and EDTA (0.5% trypsin and 200 mM EDTA; GIBCO Laboratories, Grand Island, N.Y.) in normal saline. Greater than 80% of the plastic surface-adherent bacteria are removed by the addition of trypsin to the wells as determined by measuring the radioactivity in each of the various suspensions added to and removed from the wells. Non-cell-associated bacteria were separated by differential centrifugation. Phagocytosis was expressed as a percentage of the total radioactivity recovered by using the following formula: percent uptake = $(A)/(A + B) \times 100$, where *A* represents phagocyte-associated radioactivity and *B* represents non-phagocyte-associated radioactivity.

Chemiluminescence. After the bacteria were adhered to the glass, a standard technique of chemiluminescence measurement was followed (16, 19). Briefly, 4.5 ml of HBSS with 0.1% gelatin to prevent nonspecific phagocyte adherence to the glass (25; Stevens and Hong, Fed. Proc., 1982) and 20 μ l of stock solution of chemiluminogenic probes were added to each vial. The concentration of stock solution was 275 μ M

for luminol and 6.88 mM for lucigenin. Final concentrations were 1 μ M for luminol and 25 μ M for lucigenin, which gave counts within the range of the scintillation counter. The vials were dark adapted and counted at room temperature in a Beckman LS 150 set in the out-of-coincidence mode to obtain background values. One milliliter of cell suspension (5×10^4 phagocytes per ml of GHBSS) was then added to the mixture. The vials were counted for 0.2 min at 5-min intervals for 75 min. In some experiments, the peroxidase inhibitor sodium azide (Fisher Scientific Co., Fairlawn, N.J.) at 10 μ M final concentration or superoxide dismutase (SOD; Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 100 μ g/ml was added, and the volume of GHBSS was decreased to maintain a final volume of 5.5 ml. Results reported are the values of the test solutions (phagocytes plus bacteria) minus solutions with cells but without adherent bacteria.

Superoxide microassay. To assay the kinetics of superoxide generation and release into the supernatant a microtiter plate assay of SOD-inhibitable ferricytochrome *c* reduction was used (22). SOD (0.02 ml; 1 mg/ml) was added to the appropriate wells of the 96-well flat-bottom microtiter plates containing adherent bacteria and enough GHBSS to obtain a volume of 0.05 ml. Cytochrome *c* (Sigma: 4.2 mg/ml) and cells (5×10^6 /ml) were mixed in 1:2 proportions, and 0.15 ml of this mixture was added to the wells for a final volume of 0.2 ml. Plates were incubated at 37°C for the specified time intervals. Immediately after incubation, plates were read on a Flow Titertek Multiskan MCC/340, an 8-channel vertical light path photometer, at a reading wavelength of 550 nm. The plates were blanked on a column of wells containing ferricytochrome *c*, cells, and SOD. Variability in turbidity, thickness of plastic, and depth of liquid in each well was compensated for by measuring absorption of light at 630 nm. Values reported are nanomoles of ferricytochrome *c* reduced per 10^6 cells as calculated from the absorbance of stimulated cells minus the absorbance of stimulated cells plus SOD, by using 2.11×10^4 M $^{-1}$ cm $^{-1}$ as the extinction coefficient for ferricytochrome *c* (18).

Cell-free systems to produce reactive oxygen products. To generate $\cdot O_2^-$, 0.05 ml of xanthine (Sigma) at 10^{-3} M was combined with 0.05 ml of xanthine oxidase (Sigma) at 0.04 U/ml in 0.35 ml of GHBSS. The concentration of $\cdot O_2^-$ was determined by SOD-inhibitable cytochrome *c* reduction at 30 and 60 min.

To study H_2O_2 , aqueous H_2O_2 (Mallinkrodt, Inc., St. Louis, Mo.) was diluted to concentrations of 10^{-6} to 10^{-4} M in GHBSS.

To generate HOCl, MPO (a generous gift from Professor Beulah Holmes Gray) at 120 and 1,200 U/ml (final concentrations) was added to the various concentrations of H_2O_2 in HBSS. One unit of MPO was defined as the amount of MPO sufficient to produce an increased absorbance at 450 nm of 0.001 absorbance units per min at room temperature in a Titertek Multiscan plate reader (model mcc 340; Flow Laboratories, Inc., McLean, Va.) with *o*-dianisidine as the substrate. Neutrophils contain 120 to 240 U of MPO per 10^6 cells. NaOCl (EM Science) alone at 10^{-4} to 10^{-6} M (final concentration) in GHBSS was also used.

S. aureus (0.5 ml; 2×10^7 CFU/ml of GHBSS) was allowed to adhere to cover slips at 37°C for 2 h. After 60 min of exposure to one of the above solutions, the slides were stained for 30 s with 1:10,000 acridine orange (Sigma) in saline. Slides were read in a Zeiss epifluorescence microscope with a 490-nm excitation wavelength. The percentages of green, orange, and red *S. aureus* were calculated.

FIG. 1. Chemiluminescence with surface-adherent unopsonized *S. aureus*. Results represent the mean peak chemiluminescence $\pm$ 1 standard error of the mean for 14 neutrophil (PMN), 11 alveolar macrophage (AM), and 7 monocyte (MN) samples. Shown are significant differences for neutrophil versus macrophage ($P < 0.001$) luminol-enhanced chemiluminescence and for macrophage versus neutrophil ($P < 0.001$) lucigenin-dependent chemiluminescence.

RESULTS

The chemiluminescence responses of the three phagocyte populations to surface-adherent unopsonized *S. aureus* were compared in the presence of luminol and lucigenin. In the luminol-dependent chemiluminescence assay, neutrophils produced significantly greater chemiluminescence at all time points beyond 5 min than either monocytes or alveolar macrophages. The light flux showed a unimodal peak for each cell type. A comparison of these peaks is shown in Fig. 1. In contrast, in the presence of lucigenin, alveolar macrophages exhibited significantly greater peak chemiluminescence than neutrophils or monocytes. Again, the light flux showed a unimodal peak for each cell type. The luminol-dependent chemiluminescent response of neutrophils and monocytes was inhibited 78 and 87%, respectively, by sodium azide, an inhibitor of heme-containing enzymes such as myeloperoxidase (Table 1). The small amount of luminol-dependent chemiluminescence of alveolar macrophages was only 6% inhibited by sodium azide. SOD, an enzyme that decreases the concentration of superoxide, markedly decreased the lucigenin-dependent chemiluminescent response of alveolar macrophages (91%) and moderately decreased neutrophil (68%) and monocyte (81%) lucigenin-dependent chemiluminescence (Table 1).

A detailed kinetic comparison of the phagocytosis of

surface-adherent, unopsonized *S. aureus*, the rates of luminol and lucigenin chemiluminescence, and the superoxide released is presented in Fig. 2 for neutrophils and Fig. 3 for alveolar macrophages. Nearly 50% of available radiolabeled, unopsonized, surface-adherent *S. aureus* was neutrophil associated by 5 min (Fig. 2A). Increasing lucigenin and luminol chemiluminescence was also observed after 5 min (Fig. 2B), as was increased superoxide release by ferricytochrome *c* reduction at 10 min (Fig. 2C). The uptake by alveolar macrophages of radiolabeled, unopsonized, adherent *S. aureus* reached 40% of available bacteria by 5 min (Fig. 3A). A significant increase in lucigenin chemiluminescence was observed at this time, but a significant increase in luminol chemiluminescence was not detected (Fig. 3B). Superoxide release by ferricytochrome *c* reduction showed a significant increase by 10 min (Fig. 3C).

Lucigenin chemiluminescence and ferricytochrome *c* reduction were significantly greater at all time points with alveolar macrophages than with neutrophils. On the other hand, luminol chemiluminescence of alveolar macrophages was significantly lower from 15 through 60 min than that of neutrophils.

The ratio of ferricytochrome *c* reduced by neutrophils to that by alveolar macrophages was similar to the ration of lucigenin-dependent chemiluminescence by neutrophils to that by alveolar macrophages during phagocytosis of surface-adherent *S. aureus*.

In a previous study from this laboratory, neutrophils and alveolar macrophages showed efficient and similar uptake of unopsonized, adherent *S. aureus* (17). Of those *S. aureus* phagocytosed by neutrophils, 45% showed evidence of DNA denaturation by metachromasia to orange red after exposure to acridine orange. In contrast only 6% of those *S. aureus* phagocytized by alveolar macrophages showed metachromasia.

To demonstrate that the differences in the reactive oxygen species produced by neutrophils and alveolar macrophages under these conditions could account for these observed differences in acridine orange metachromasia of *S. aureus* phagocytized by these two cell types (17), we employed several cell-free systems to generate relevant reactive oxy-

TABLE 1. Chemiluminescence inhibition with sodium azide and SOD^a

Probe	Addition	% Inhibition of peak chemiluminescence		
		Neutrophils	Monocytes	Macrophages
Luminol	Sodium azide	78 $\pm$ 22.2	87 $\pm$ 3.8	6 $\pm$ 0.04
	SOD	27 $\pm$ 3.2	18 $\pm$ 10.7	98 $\pm$ 2.9
Lucigenin	Sodium azide	-52 $\pm$ 10.7	-1 $\pm$ 15.5	8 $\pm$ 2.9
	SOD	68 $\pm$ 2.2	81 $\pm$ 7.5	91 $\pm$ 1.1

^a Values represent the means ($\pm$ 1 standard error of the mean) of the percent peak chemiluminescence of vials with sodium azide or SOD divided by peak chemiluminescence of vials run simultaneously without sodium azide or SOD (sample size, 3).

FIG. 2. Time course of unopsonized surface-adherent uptake of *S. aureus* and generation of reactive oxygen species by polymorphonuclear leukocytes. (A) Cell-associated bacteria were evident by 5 min. (B) This was followed by increases in lucigenin and by luminol chemiluminescence (graphed as integrals versus time). (C) Increasing superoxide concentrations in the medium surrounding cells can be detected by 10 min and continue to increase through 60 min after exposure to surface-adherent unopsonized *S. aureus* as measured by cytochrome *c* reduction.

gen species. Using xanthine-xanthine oxidase to produce 7.6 nmol of $\cdot O_2^-$ per 2×10^6 adherent *S. aureus* cells failed to produce acridine orange metachromasia. H_2O_2 concentrations from 10^{-5} to 10^{-4} M also showed no *S. aureus* with orange-red metachromasia. However, the addition of myeloperoxidase to H_2O_2 in a solution containing Cl^- to produce HOCl led to an orange-red metachromasia of the acridine orange-loaded *S. aureus*. Finally, increasing amounts of NaOCl alone produced increasing orange-red metachromasia at concentrations from 10^{-6} to 10^{-4} M (Table 2).

DISCUSSION

Luminol and lucigenin were used to measure chemiluminescence since they have been found to greatly enhance the sensitivity of such measurements and to detect different products of oxygen metabolism (2-4, 6; Stevens and Hong, Fed. Proc., 1982). Luminol undergoes oxidative dioxygenation and interacts more efficiently with the H_2O_2 -HOCl reactants of the myeloperoxidase system, although reaction with $OH\cdot$ - O_2 may be possible (4). The pattern of azide-inhibitable chemiluminescence with luminol was neutrophils

> monocytes > alveolar macrophages. This is consistent with the known pattern of relative peroxidase activities in these cell types (7).

This pattern and these amounts of luminol chemiluminescence are well known in assays using opsonized bacteria to stimulate neutrophils or monocytes. However, most unopsonized bacteria in suspension (including *S. aureus* Cowan II) induce much less luminol chemiluminescence in neutrophils and monocytes than observed here. This is most likely due to the relatively small numbers of unopsonized bacteria in suspension that are phagocyte associated (13). In contrast, when unopsonized bacteria are adherent to a surface, they promptly associate with neutrophils or monocytes in amounts comparable to those seen in suspension systems with opsonized bacteria. As our results show, the kinetics of the luminol chemiluminescence response in neutrophils closely followed the kinetics of association of the radiolabeled staphylococcus to this cell.

In contrast to luminol, lucigenin undergoes reductive dioxygenation and interacts preferentially with $\cdot O_2^-$ (3). Lucigenin chemiluminescence and the superoxide concen-

FIG. 3. Time course of uptake of unopsonized surface-adherent *S. aureus* and generation of reactive oxygen species by alveolar macrophages. (A) Significant cell-associated bacteria are detected by 5 min. (B) Increasing lucigenin chemiluminescence can be detected at this time. However, unlike in the polymorphonuclear leukocyte, little increase in luminol chemiluminescence is detected. (C) Increasing superoxide concentrations in the medium surrounding alveolar macrophages were also evident by 5 min after exposure to surface-adherent, unopsonized *S. aureus*.

TABLE 2. Percentage of adherent *S. aureus* cells with acridine orange metachromasia in phagocyte-free systems

Prepn	% Green (no metachromasia)	% Orange or red
7.7 nmol of $\cdot O_2^-$ /~ 2×10^6 <i>S. aureus</i>	100	0
[H ₂ O ₂]/~ 2×10^6 <i>S. aureus</i>		
10^{-6} M	100	0
10^{-5} M	100	0
10^{-4} M	100	0
10^{-4} M H ₂ O ₂ -120 U of MPO/ ~ 2×10^6 <i>S. aureus</i>	80	20
10^{-4} M H ₂ O ₂ -1,200 U of MPO/ ~ 2×10^6 <i>S. aureus</i>	60	40
[OCl ⁻]/~ 2×10^6 <i>S. aureus</i>		
10^{-6} M	11	89
10^{-5} M	5	97
10^{-4} M	0	100

tration detected by the ferricytochrome *c* assay were significantly greater in alveolar macrophages than in neutrophils or monocytes during phagocytosis of unopsonized bacteria on a surface. The kinetics of superoxide generation and release detected by the ferricytochrome *c* assay correlated with the lucigenin chemiluminescence kinetics in both neutrophils and alveolar macrophages. Moreover, the kinetics of the lucigenin chemiluminescence and ferricytochrome *c* reduction followed the kinetics of bacterial association to these phagocytes. Certainly the known paucity of peroxidase activity in alveolar macrophages (10) might account for the persistence of higher concentrations of superoxide compared with that in neutrophils, which are richer in peroxidases (e.g., MPO). These peroxidases increase forward reactions away from $\cdot O_2^-$, e.g., $\cdot O_2^- + 2H^+ \rightarrow 2H_2O_2$ and $H_2O_2 + Cl^- \xrightarrow{MPO} HOCl + H_2O$, thereby decreasing the $\cdot O_2^-$ concentration by mass action. This is also consistent with the observed increase in the lucigenin chemiluminescence in neutrophils when sodium azide, an inhibitor of MPO, was present. Thus, neutrophils and alveolar macrophages not only take up unopsonized adherent *S. aureus* efficiently but also generate reactive oxygen species in amounts similar to those generated during phagocytosis of opsonized *S. aureus* in suspension.

Interestingly, our results show at least a twofold greater amount of SOD-inhibitable cytochrome *c* reduction from alveolar macrophages during the first 15 min of phagocytosis of unopsonized bacteria on a surface than has been reported for those cells when phagocytosing opsonized zymosan particles in suspension (24). Whether this increase is due to factors related to phagocytosis while the alveolar macrophage is adherent to a surface or to the difference in the particles requires further investigation.

The differences we observed in major reactive oxygen species produced by alveolar macrophages and neutrophils correlate with the results of a previous study in which the ability of alveolar macrophages and neutrophils to denature staphylococcal DNA in a surface-adherent system was assayed by using acridine orange (17). Whereas similar numbers of cell-associated staphylococci were found in alveolar macrophages and neutrophils at 60 min, neutrophil bacterial killing was found to be seven times more effective than killing by alveolar macrophages with the acridine orange system. Alveolar macrophages, which had less MPO-

hydrogen peroxide-halide activity, showed less intercalation of acridine into the DNA of the phagocytized unopsonized *S. aureus*, whereas neutrophils produced greater intercalation into their phagocytized unopsonized *S. aureus* DNA, producing the characteristic red metachromasia of the dye.

To determine whether the generation of different reactive oxygen species could explain these observed differences in acridine orange metachromasia, cell-free systems were used to generate different relevant oxygen species. We found that the generation of $\cdot O_2^-$ by xanthine in the presence of xanthine oxidase failed to produce any orange-red metachromasia in the *S. aureus* exposed to acridine orange. The amount of $\cdot O_2^-$ generated was similar to that detected with SOD-inhibitable cytochrome *c* reduction in our systems containing phagocytes. Similarly, the addition of H₂O₂ at concentrations expected in our system containing phagocytes (15) did not produce metachromasia in *S. aureus* exposed to acridine orange. However, the use of H₂O₂ and increasing amounts of MPO did indeed produce increasing proportions of *S. aureus*, showing orange-red metachromasia, as did NaOCl alone.

The reactive oxygen species produced by alveolar macrophages between host and peripheral blood neutrophils and monocytes during phagocytosis of surface-adherent bacteria may also relate to epithelial cell damage. Current studies are examining this hypothesis.

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