

## Hydrogen Peroxide Production and Killing of *Staphylococcus aureus* by Human Polymorphonuclear Leukocytes

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The effects of bacterial neuraminidase on production of hydrogen peroxide ( $H_2O_2$ ) and killing of *Staphylococcus aureus* by human polymorphonuclear leukocytes (PMN) were studied. The concentration of  $H_2O_2$  was measured by the disappearance of scopoletin fluorescence in the presence of horseradish peroxidase. The results indi-

cated that desialylation of human PMN inhibited the stimulation of  $H_2O_2$  production during phagocytosis. It also markedly impaired the killing of *S. aureus*. Impaired killing of *S. aureus* by desialylated PMN was due to impaired intracellular killing rather than defective phagocytosis.

**D**URING PHAGOCYTOSIS, hexose monophosphate shunt (HMPS) activity and hydrogen peroxide ( $H_2O_2$ ) and superoxide ( $O_2^-$ ) production are markedly stimulated.<sup>1-3</sup> These phagocytosis-associated oxidative metabolic changes are essential for the intracellular microbicidal ability of polymorphonuclear leukocytes (PMN).<sup>4</sup> However, the relative roles of these oxidative metabolic activities in the intracellular killing of ingested microorganisms are still unclear.<sup>4</sup> We have previously demonstrated that inhibition of surface sulfhydryl groups of human PMN has no effect on the ingestion of particles and phagocytosis-associated stimulation of  $O_2^-$  production, but does inhibit  $H_2O_2$  production and HMPS activation during phagocytosis.<sup>5</sup> Under this condition, killing of *Staphylococcus aureus* is normal, suggesting that  $O_2^-$  or its derivatives, such as hydroxyl radical, plays an important role in the killing of *S. aureus*. Recently we have demonstrated that removal of membrane sialic acids from human PMN by bacterial neuraminidase impairs the stimulation of  $O_2^-$  production, while it has no effect on phagocytosis and phagocytosis-associated activation of HMPS.<sup>6</sup> In this study, we report the effect of desialylation on  $H_2O_2$  production and killing of *S. aureus* by human PMN.

### MATERIALS AND METHODS

**Chemicals.** Scopoletin (6-methyl-7-hydroxy-1:2-benzopyrone), *p*-chloromercuribenzenesulfonic acid (PCMBSA), horseradish peroxidase\* (HRP, Type II, E.C.1.11.1.7), and neuraminidase† (from *Clostridium perfringens*, Type V, E.C.3.2.1.18) were obtained from Sigma Chemical Co., St. Louis, Mo.  $H_2O_2$  as a 30.2% solution was obtained from Fisher Scientific Co., Pittsburgh, Pa. Lysostaphin (specific activity 196 units/mg) was obtained from Schwarz-Mann.

\*Horseradish peroxidase: approximately 160 purpurogallin units/mg; 1 unit will form 1 mg purpurogallin in 20 sec at 20°C, pH 6.

†Neuraminidase: 0.5 unit/mg protein; 1 unit liberates 1.0  $\mu$ mole of N-acetyl-neuraminic acid per minute at pH 5.0, 37°C, using N-acetyl-neuramin-lactose as substrate.

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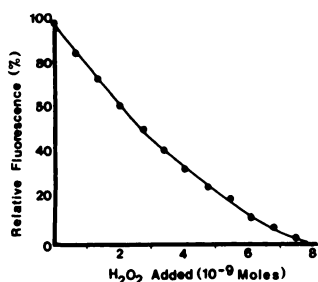


Fig. 1. Relationship between the intensity of fluorescence of scopoletin in the presence of horseradish peroxidase and increasing amounts of  $H_2O_2$  added to the medium.

Orangeburg, N.Y. Ficoll was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and sodium diatrizoate (Hypaque) from Winthrop Laboratories, New York, N.Y. Polystyrene latex (0.79  $\mu$ m in diameter) was obtained from Dow Chemical Co., Indianapolis, Ind.

**Isolation of human PMN.** Isolation of human PMN was performed as described previously.<sup>5</sup> Briefly, venous blood was obtained from normal individuals; leukocytes were isolated by dextran sedimentation of red blood cells, differential centrifugation, and  $NH_4Cl$  lysis of contaminating red cells. The leukocytes then were suspended in 10 ml modified Hanks' balanced salt solution (with 5 mM glucose),<sup>7</sup> and placed on top of a 10-ml Ficoll-Hypaque mixture and centrifuged at 400 g for 40 min at 20°C according to Böyum.<sup>8</sup> The pellet, which consisted of 97%-99% PMN by differential counting, was washed twice with modified Hanks' solution (5 mM glucose); PMN were diluted to a final concentration of  $1 \times 10^7$  PMN/ml.

**Measurement of  $H_2O_2$ .** The concentration of  $H_2O_2$  was determined by the disappearance of the fluorescence of scopoletin in the presence of HRP.<sup>9</sup> The basic assay procedure was adapted to permit direct comparison of unknown and known  $H_2O_2$  solutions. Scopoletin was dissolved in 0.5 M phosphate buffer (pH 7.0) at a concentration of 0.2 mg/ml. HRP was dissolved in 0.05 M phosphate buffer (pH 7.0) at a concentration of 1 mg/ml. Hydrogen peroxide (30.2%) was diluted 1:1000 in distilled water and stored at 4°C as a stock solution in an aluminum foil-covered flask. Before each experiment the  $H_2O_2$  concentration of this solution was determined from its absorption at 230 nm using an extinction coefficient of  $81 M^{-1} cm^{-1}$ .<sup>10</sup>

An aliquot of the stock solution then was diluted to obtain a  $H_2O_2$  standard with a concentration of 35  $\mu$ M. Scopoletin (0.01 ml) and HRP (0.05 ml) were added to 1 ml of distilled  $H_2O$  in a fluorescence cuvette. The fluorescence intensity was measured with an Aminco-Bowman spectrofluorometer (American Instruments Co., Silver Spring, Md.) with the exciting wavelength set at 350 nm and the emission wavelength at 460 nm. The reduction of fluorescence was determined after adding each 0.01 ml of standard solution or samples. In each experiment, the measurement was done first with several 0.01-ml aliquots of standard solution, followed by several aliquots of unknown solution, and then again with several aliquots of standard in the same cuvette. Thus the loss of fluorescence with addition of a standard  $H_2O_2$  solution was determined both before and after addition of the unknown solution in the same cuvette. All fluorescence intensities were first corrected to a constant volume. The concentration of  $H_2O_2$  in the unknown solution relative to that in the standard was determined from the ratio of the loss of fluorescence due to the unknown to the average fluorescence loss due to the standard. A curve of fluorescence loss obtained by adding only the standard solution is shown in Fig. 1. The curve was linear when the relative fluorescence was above 50%. Therefore, in all experiments, the fluorescence intensity was kept above 50%. With the assay performed in this way,  $H_2O_2$  concentrations from 1 to 100  $\mu$ M could be accurately measured.

**Measurement of  $H_2O_2$  production by human PMN.** Human PMN ( $1 \times 10^7$  cells) were preincubated with neuraminidase (0.11 units/ml), PCMBSA (0.1 mM) or modified Hanks' solution for 20 min at 37°C in a shaking water bath. Sodium azide ( $NaN_3$ , 2 mM) was then added to inhibit the breakdown of  $H_2O_2$  by cellular enzymes.<sup>10</sup> Latex particles at a final concentration of 0.17% were added to stimulate phagocytosis and the mixture then was incubated at 37°C for 30 min. At the end of incubation, the PMN were removed by centrifugation at 4°C at 200 g for 10 min. The supernatant was passed through a 0.22- $\mu$ m millipore filter to remove the remaining latex particles. The concentration of  $H_2O_2$  in the clear solution was determined by the disappearance of scopoletin fluorescence in the presence of HRP as described

**Table 1. Effects of Neuraminidase, PCMBSA, and  $\text{NaN}_3$  on the Measurement of  $\text{H}_2\text{O}_2$  by the Disappearance of Scopoletin Fluorescence**

	$\text{H}_2\text{O}_2$ Added ( $\mu\text{M}$ )	$\text{H}_2\text{O}_2$ Measured ( $\mu\text{M}$ ) <sup>*</sup>
Hanks' solution alone	28	30 ± 4
+ $\text{NaN}_3$ (2 mM)	28	27 ± 4
+ PCMBSA (0.1 mM)	28	26 ± 6
+ Neuraminidase (0.11 units/ml)	28	29 ± 2

\*Results are mean ± SEM of three determinations.

above. The effects of neuraminidase, PCMBSA, and  $\text{NaN}_3$  on the assay system were tested. As shown in Table 1, all these substances at the concentrations used had no effect on the determination of  $\text{H}_2\text{O}_2$ .

*Measurement of the killing of S. aureus by human PMN.* The effect of desialylation on the killing of *S. aureus* by human PMN was carried out by preincubating PMN ( $2 \times 10^7$ ) in modified Hanks' solution in sterile 50-ml serum vials with and without 0.11 units/ml neuraminidase in a water bath with shaking for 20 min at 37°C. At the end of preincubation, an inoculum of *S. aureus* (ATCC 25923) and serum was introduced to a final concentration of 20% serum and a bacteria-to-PMN ratio of 10:1. Control experiments were performed in the absence of PMN with and without neuraminidase. The cell and bacterial mixtures were further incubated for 2 hr at 37°C. At the end of incubation, 0.1 ml of the incubation mixture was removed and 9.9 ml of sterile distilled water was added to lyse the PMN. Serial dilutions were made and the number of bacteria was determined by the pour plate technique as described previously.<sup>5</sup> In the absence of PMN, 0.11 units/ml neuraminidase had no effect on bacterial survival or growth over the 2-hr experimental period. The percentage of bacterial killing was calculated from the number of bacteria surviving in the presence and absence of PMN.

In order to differentiate whether the impaired killing of *S. aureus* by the desialylated PMN was due to defective phagocytosis or impaired intracellular killing, the extracellular *S. aureus* were eliminated by lysostaphin according to Tan et al.<sup>11</sup> The experiments were carried out as described above. At the end of a 2-hr incubation and after 0.1 ml of the incubation mixture was removed for the determination of the percentage of bacterial killing, 0.8 ml lysostaphin (20 units/ml) was added to the bacterial and cell mixtures. The mixtures were further incubated for 20 min at 37°C in the water bath with shaking. The cells and bacteria then were collected by centrifugation at 2500 g for 10 min at 4°C. The pellet was suspended in 1 ml of 0.25% trypsin and incubated for 15 min at 37°C to inactivate the residual lysostaphin. The cells and bacteria were collected again by centrifugation. Five milliliters of sterile distilled water was added to lyse the PMN. Serial dilutions were made and the number of bacteria was determined by the pour plate technique.

*Statistical significance.* The statistical significance of each experiment was obtained based on the pair difference.<sup>12</sup>

## RESULTS

*Effect of neuraminidase on  $\text{H}_2\text{O}_2$  production by human PMN.* In a previous study<sup>6</sup> we have shown that preincubation of human PMN with 0.11 units/ml neuraminidase for 20 min at 37°C removes 40%–60% of the total neuraminidase-accessible sialic acids from the plasma membrane. These desialylated PMN ingest particles normally, but the stimulation of  $\text{O}_2^-$  production that usually accompanies phagocytosis is markedly inhibited.<sup>6</sup> Superoxide is an unstable intermediate. In the presence of hydrogen donor,  $\text{O}_2^-$  undergoes dismutation, either enzymatically or spontaneously, producing  $\text{H}_2\text{O}_2$  and oxygen.<sup>3,4</sup> Therefore the effect of neuraminidase on  $\text{H}_2\text{O}_2$  production by human PMN has been studied. Hydrogen peroxide has been quantified by the disappearance of fluorescence of reduced scopoletin.<sup>9</sup> Scopoletin is a compound that fluoresces

**Table 2. Effects of Neuraminidase and PCMBSA on the H<sub>2</sub>O<sub>2</sub> Production by Human PMN\***

	Neuraminidase (0.11 units/ml)	PCMBSA (0.1 mM)
Resting cells		
Control	2.13 ± 0.93	2.13 ± 0.93
Experiment	1.30 ± 1.01	1.00 ± 0.68
p value†	> 0.4	> 0.3
Phagocytosing cells		
Control	297 ± 101	297 ± 101
Experiment	42 ± 12	29 ± 21
p value†	< 0.05	< 0.02

\*Results expressed as mean ± SEM (10<sup>-10</sup> moles H<sub>2</sub>O<sub>2</sub>/hr/10<sup>6</sup> PMN) based on seven determinations for each control and each experiment.

†p values obtained based on paired differences.

at 460 nm with an intensity directly proportional to its concentration when activated by light of 350 nm wavelength. During its oxidation by HRP, fluorescence is lost with a stoichiometry directly proportional to the H<sub>2</sub>O<sub>2</sub> concentration in the medium.<sup>9,13</sup> As is shown in Table 2, desialylation markedly inhibits (86%) the stimulation of H<sub>2</sub>O<sub>2</sub> production during phagocytosis, while it has no significant effect on the resting cells. We have previously demonstrated that inhibition of surface -SH groups by PCMBSA inhibits the stimulation of H<sub>2</sub>O<sub>2</sub> production by human PMN during phagocytosis.<sup>6</sup> In that study, H<sub>2</sub>O<sub>2</sub> production was measured by two relatively insensitive techniques, i.e., oxidation of <sup>14</sup>C-formate and iodination of ingested bacteria. In this study, H<sub>2</sub>O<sub>2</sub> production has been determined by a highly sensitive technique. The results confirm our previous observations (Table 2).

*Effect of desialylation on the killing of S. aureus by human PMN.* The myeloperoxidase (MPO) mediated antimicrobial system has been proposed as the major pathway by which ingested organisms are killed by human PMN.<sup>4</sup> Hydrogen peroxide, but not O<sub>2</sub><sup>-</sup>, is essential for this system.<sup>14</sup> On the other hand, O<sub>2</sub><sup>-</sup> has been implicated in the antimicrobial mechanisms in human PMN.<sup>3,4</sup> Since desialylation prevents the stimulation of O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> production during phagocytosis, its effect on the killing of *S. aureus* by human PMN has been studied. As is shown in Table 3, desialylation markedly inhibits the killing of *S. aureus*. The reduced killing of *S. aureus* by desialylated PMN could be due to either reduced phagocytosis or impaired intracellular killing. In order to distinguish these two possibilities, lysostaphin was used to eliminate the extracellular *S. aureus* after the bacteria had been incubated with PMN for

**Table 3. Effect of Neuraminidase on the Killing of S. aureus by Human PMN\***

	<i>S. aureus</i> Killed (%)
Control	91 ± 2.2
Neuraminidase (0.11 units/ml)	17 ± 5.9
p value	< 0.001

\*Leukocytes were preincubated with and without neuraminidase for 20 min. Then *S. aureus* (ATCC 25923) were added to a final concentration of bacteria-to-PMN ratio of 10:1. At the end of incubation (2 hr), surviving bacteria were counted by the pour plate technique. The results are expressed as mean ± SEM of the percentage killed based on seven experiments.

**Table 4. Use of Lysostaphin to Distinguish Between Impaired Intracellular Killing and Defective Phagocytosis\***

	<i>S. aureus</i>	
	Colony Count ( $\times 10^7$ )	(%)
Before lysostaphin		
No PMN	9.37	100
PMN alone	0.28	3
PMN + neuraminidase (0.11 units/ml)	8.36	89
After lysostaphin		
No PMN	0.02	0.2
PMN alone	0.21	2.2
PMN + neuraminidase (0.11 units/ml)	8.18	87

\*Leukocytes were preincubated with and without neuraminidase for 20 min. Then *S. aureus* (ATCC 25923) were added and incubated for 2 hr at 37°C. At the end of incubation, 0.1 ml was removed to determine the surviving bacteria (before lysostaphin). Lysostaphin (0.8 ml, 2 units/0.1 ml) was added to the remaining bacterial and cell mixtures to eliminate the extracellular *S. aureus*. The intracellular surviving *S. aureus* were then determined (after lysostaphin). The results are the actual colony counts obtained.

2 hr. Lysostaphin is a muralytic enzyme. It does not enter neutrophils,<sup>11</sup> and it specifically and rapidly eliminates extracellular *S. aureus*.<sup>11,15</sup> Preliminary experiments showed that neuraminidase had no effect on the killing of *S. aureus* by lysostaphin. Table 4 shows the results of a typical experiment. In this experiment, untreated PMN killed 97% of *S. aureus*, whereas desialylated PMN only killed 11%. Lysostaphin eliminated more than 99% of *S. aureus* when PMN were not present. In contrast, 87% of the 89% *S. aureus* in the desialylated PMN vial survived lysostaphin treatment, suggesting that almost all the *S. aureus* were inside the cells. Thus, the reduced killing of *S. aureus* by desialylated PMN was due to impairment of intracellular killing. Furthermore, this experiment confirmed our previous observation<sup>6</sup> that desialylation had no effect on the ingestion of particles.

#### DISCUSSION

Ingestion of particles by PMN is accompanied by a burst of oxidative metabolic changes, such as increased oxygen consumption, stimulation of the HMPS activity, and  $H_2O_2$  and  $O_2^-$  production.<sup>1-3</sup> HMPS activity and  $H_2O_2$  production in resting leukocytes also can be stimulated by a variety of surface-active agents,<sup>16-18</sup> endotoxin,<sup>16</sup> phospholipase C,<sup>18</sup> concanavalin A,<sup>19</sup> or leukocyte antibodies in the absence of complement.<sup>20</sup> Recently Goldstein and co-workers<sup>21</sup> have provided evidence that complement and immunoglobulins can stimulate  $O_2^-$  production by human leukocytes independent of phagocytosis. Thus a mere perturbation of plasma membrane is sufficient for the stimulation of PMN oxidative metabolism. We have studied the role of surface functional groups (e.g., -SH groups, sialic acids) on the stimulation of oxidative metabolism by human PMN during phagocytosis.<sup>5,6</sup> PCMBSA was used to inhibit surface -SH groups, and neuraminidase was used to delete membrane sialic acids. Neither PCMBSA nor neuraminidase significantly penetrate the plasma membrane.<sup>5,22,23</sup> Thus, intracellular metabolism is not affected by these substances.

**Table 5. Effects of PCMBSA and Neuraminidase on the Phagocytosis-associated Stimulation of Oxidative Metabolism and Killing of *S. aureus* by Human PMN\***

	PCMBSA-treated PMN (0.1 mM)	Desialylated PMN (Neuraminidase 0.11 units/ml)
Phagocytosis (latex particles)	107 (5)	101 (8)
1- <sup>14</sup> C-glucose oxidation	27 (10)	100 (6)
H <sub>2</sub> O <sub>2</sub> production	10 (7)	14 (7)
Superoxide production	85 (3)	11 (7)
Killing of <i>S. aureus</i>	94 (3)	17 (7)

\*The results are calculated from previous<sup>5,6</sup> and the present work and expressed as the percentage of the control. The numbers in parentheses indicate the number of experiments. Hydrogen peroxide was measured by the disappearance of the scopoletin fluorescence in the presence of HRP. Superoxide was measured by the reduction of ferricytochrome C, which could be inhibited by O<sub>2</sub><sup>-</sup> dismutase.<sup>6</sup>

This conclusion is substantiated by the observation that PCMBSA treatment and desialylation have no effect on the oxidative metabolism in resting human PMN.<sup>6,24</sup> As is summarized in Table 5, both PCMBSA treatment and desialylation have no effect on the phagocytosis of particles. PCMBSA also has no effect on the stimulation of O<sub>2</sub><sup>-</sup> production, while it inhibits the activation of HMPS and H<sub>2</sub>O<sub>2</sub> production during phagocytosis. In contrast, desialylation inhibits the production of O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub>, but not the stimulation of the HMPS activity associated with phagocytosis. Although H<sub>2</sub>O<sub>2</sub> can stimulate HMPS activity in PMN,<sup>5,25</sup> our observations are best explained by the hypothesis that (1) O<sub>2</sub><sup>-</sup> production and HMPS activation during phagocytosis are controlled by separate membrane mechanisms requiring intact surface sialic acids and -SH groups, respectively, and (2) the HMPS plays a primary role in producing NADPH for H<sub>2</sub>O<sub>2</sub> production rather than H<sub>2</sub>O<sub>2</sub> catabolism.

The mechanisms of bacterial killing by PMN have been the subject of intensive investigation. The MPO-mediated antimicrobial system proposed by Klebanoff<sup>26</sup> is one of the best studied. In this system, MPO, H<sub>2</sub>O<sub>2</sub>, and an oxidizable cofactor such as a halide anion form a potent antimicrobial system.<sup>4,26</sup> The assumption that it contributes significantly to the microbicidal activity of the intact leukocytes is based on the following observations:<sup>4</sup> (1) the components of the MPO-mediated antimicrobial system are present in the leukocyte and their formation or release occurs at a time appropriate to the microbicidal act; (2) these components are capable of reaction in the phagocytic vacuole; (3) leukocytes deficient in H<sub>2</sub>O<sub>2</sub> formation have decreased microbicidal activity; and (4) PMN deficient in MPO have decreased microbicidal activity.

Our results suggest that O<sub>2</sub><sup>-</sup> plays a more important role than H<sub>2</sub>O<sub>2</sub> in the killing of *S. aureus*. In PCMBSA-treated cells, O<sub>2</sub><sup>-</sup> production is normal but H<sub>2</sub>O<sub>2</sub> production is markedly inhibited. These PCMBSA-treated cells have a normal capacity to kill *S. aureus*.<sup>5</sup> In contrast, desialylated PMN have markedly reduced O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> production. They also are severely impaired in killing *S. aureus*. This reduced killing of *S. aureus* has been further shown to be due to impaired intracellular killing rather than impaired phagocytosis. The normal killing of *S. aureus* by PCMBSA-treated cells cannot be attributed to the small amount of H<sub>2</sub>O<sub>2</sub> produced, since desialylated PMN generate at least an equal amount of H<sub>2</sub>O<sub>2</sub> as PCMBSA-treated cells. Studies in patients with qualitative dysfunction of neutrophils tend to support our hypothesis. Leuko-

cytes from patients with chronic granulomatous disease (CGD) have impaired  $\text{H}_2\text{O}_2$  as well as  $\text{O}_2^-$  production with markedly impaired intracellular killing of catalase-positive bacteria. These patients suffer from severe infections with granuloma formation and usually die in early life.<sup>27,28</sup> In contrast, PMN from patients with severe MPO deficiency have mild to moderate impairment of bacterial killing, and these patients usually have milder clinical courses.<sup>29,30</sup> Leukocytes from patients with glucose-6-phosphate dehydrogenase (G-6-PD) deficiency who have less than 5% leukocyte G-6-PD activity have impaired  $\text{H}_2\text{O}_2$  production during phagocytosis and mild to moderate intracellular bacterial defect.<sup>31,32</sup> Again, they have relatively milder clinical courses. Thus, it is tempting to suggest that the difference in the severity of infections in patients with CGD and those with MPO deficiency and G-6-PD deficiency is due to impaired  $\text{O}_2^-$  production in CGD PMN. Further studies will be necessary to evaluate our thesis.

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