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Activity of Polymorphonuclear Leukocytes in the Presence of Sulfide

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Polymorphonuclear leukocytes (PMN) isolated from human blood were exposed to various levels of hydrogen sulfide. The effect on respiratory burst, myeloperoxidase activity, and capacity to phagocytose and kill bacteria were studied. A 1-h exposure of the PMN to 1 mM sulfide did not decrease their myeloperoxidase activity or their capacity to initiate a respiratory burst. Actually the products of the respiratory burst rapidly oxidized sulfide. The phagocytosis and killing of bacteria in the presence of 1 mM sulfide was only decreased to a minor extent. Myeloperoxidase in cell extract was, however, almost completely inhibited by 1 μM sulfide. These results indicate that hydrogen sulfide does not easily permeate PMN. PMN may be able to function in infected sites with high sulfide levels such as in the gingival pockets of periodontal disease. In the oxygenated areas of these sites the PMN may actually help in the detoxification of sulfide.

In polymicrobial infections such as periodontal disease, sulfide levels up to 1 mmol/liter may be reached in the infected sites (16, 21, 28). These levels of sulfide are much higher than levels that may damage epithelial cells (21) or increase the permeability of the oral mucosa (23). In one of the few studies on the effect of sulfide on antimicrobial host defense, it was found that rats inhaling hydrogen sulfide vapor had a reduced capacity to kill staphylococci deposited in the lungs during a bacterial aerosol challenge (29).

In the present study human polymorphonuclear leukocytes (PMN) were exposed to sulfide, and the effects on their capacity to kill bacteria and on their respiratory burst and myeloperoxidase activity were elucidated.

MATERIALS AND METHODS

Materials. Macrodex (6% dextran in saline) and Percoll were obtained from Pharmacia, Uppsala, Sweden; Lymphoprep (Ficoll and sodium metrizoate) was from Nycomed AS, Oslo, Norway; zymosan A (from *Saccharomyces cerevisiae*), phorbol-12-myristate-13-acetate (PMA), monochlorodimedon (MCDM; 1,1-dimethyl-4-iodo-chloro-3,5-cyclohexanedione), and cetyltrimethylammonium bromide were from Sigma Chemicals Co., St. Louis, Mo.; cytochrome *c* (from horse heart) and superoxide dismutase were from Boehringer Mannheim Gmbh, Federal Republic of Germany; sodium sulfide was from E. Merck AG, Darmstadt, Federal Republic of Germany; myeloperoxidase (>98% pure) was from Calbiochem, La Jolla, Calif.; and dimethyl sulfoxide was from Pierce Chemical Co., Rockford, Ill. Tissue culture medium RPMI 1640 with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) was from GIBCO Ltd., Paisley, Scotland. Normal human serum was obtained from 12 healthy donors, pooled, divided into working samples, and frozen at −70°C within 2 h after collection; samples were thawed immediately before use. The buffered salt solution contained 140 mM NaCl, 3 mM KCl, 6.7 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 (pH 7.2).

Bacterial strains. *Streptococcus agalactiae* M732 was used to evaluate the phagocytosis and killing by PMN. This type III group B streptococcus was originally isolated from an infant with meningitis and was kindly provided by Carol Baker, Houston, Tex. The strain has been subdivided into low-density (LD) and high-density (HD) variants by Stellan Håkansson, Umeå, Sweden, by density gradient centrifugation in Percoll. The LD variant has a larger polysaccharide capsule than the HD variant (12–14). When these bacteria were used in the phagocytosis and killing experiments, 10 ml of T-Y medium (15) was inoculated, and the bacteria were incubated for 10 to 12 h. The bacterial cells were washed once in the buffered salt solution, suspended in this solution to an optical density of 2.0 at 520 nm, and diluted 1:20 in RPMI 1640–HEPES to a density of 5×10^7 to 1.5×10^8 bacterial cells per ml.

Isolation of PMN. PMN were isolated from 50 ml of human blood by the method of Tiku and collaborators (32). The blood was drawn from healthy donors into evacuated tubes with heparin or citrate-phosphate-dextrose-adenine solution (Venoject; Terumo Europe N.V., Leuven, Belgium). Four parts of blood were mixed with one part of Macrodex, and the blood cells were allowed to separate for 1.5 h at room temperature. The leukocyte-containing fraction was centrifuged at 275 × *g* for 10 min. The cells of the pellet were suspended in 6 ml of the buffered salt solution and 2 ml of the supernatant fluid after that had been centrifuged at 20,000 × *g* for 2 min (plasma). The suspension of cells was layered over Lymphoprep and centrifuged at 450 × *g* for 30 min at room temperature. The cell pellet, which contained the PMN, was suspended in 2 ml of plasma. The remaining erythrocytes in the cell suspension were lysed by adding 6 ml of 0.87% NH_4Cl. The PMN were washed three times and suspended in the buffered salt solution (2×10^7 cells per ml) or in RPMI 1640–HEPES solution (5×10^7 cells per ml).

Assay of respiratory burst. The respiratory burst of PMN was measured as the superoxide dismutase-inhibitable reduction of cytochrome *c*. The absorbance of cytochrome *c* was recorded in a dual-wavelength spectrophotometer in a stirred cuvette with a thermostat (37°C). A modification of the method by Clark and collaborators (9) was used. The reaction mixture (1 ml) contained 1×10^5 to 5×10^5 cells, 0.1% glucose, 1 mM cytochrome *c*, 69 mM NaCl, 13.5 mM KCl, 60 μM $CaCl_2$, and 100 μM $MgCl_2$ in 10 mM sodium phosphate buffer (pH 7.4). The respiratory burst was activated by adding 10 μl of 2 μM PMA solution containing 1% dimethyl sulfoxide. The difference in change of absorbance

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between reaction mixtures with and without superoxide dismutase (25 μg/ml) was used to calculate the rate of superoxide anion-dependent cytochrome *c* reduction. This calculation was based on a specific absorbance ($A_{550-540}$) of cytochrome *c* of 19.1×10^3 liters per mol per cm (26).

Cell extract. A cell extract was obtained by treating the PMN with 0.2% cetyltrimethylammonium bromide for 15 min, followed by centrifugation at 20,000 × *g* for 5 min.

Assay of myeloperoxidase activity. A method based on conversion of MCDM to dichlorodimedon was used to assay the myeloperoxidase activity (11). The reaction mixture (1 ml) contained 50 μM MCDM, 100 mM KCl, and cell extract in 0.05 M sodium citrate-phosphate buffer (pH 6.0). The reaction was initiated by adding 10 μl of 5 mM H_2O_2 to the mixture. The difference in absorbance between MCDM and dichlorodimedon was used to calculate the peroxidase activity. This difference in A_{278} was 12.2×10^3 liters per mol per cm (11).

A catalase-free myeloperoxidase preparation was obtained by ion-exchange chromatography (20). One part of cell extract was mixed with one part of 20 mM sodium acetate buffer (pH 4.75) and centrifuged at 20,000 × *g* for 5 min. The supernatant fluid was loaded onto a cation-exchange column (Mono-S; Pharmacia) equilibrated with 20 mM sodium acetate buffer (pH 4.75) at 4°C and washed with 0.2 M NaCl in the same buffer, and myeloperoxidase was eluted with 2 M NaCl in this buffer.

To simulate the activity of myeloperoxidase, a syringe pump (model 352; Orion Research, Cambridge, Mass.) was used in some experiments to deliver hypochlorite into the reaction mixture.

Assay of catalase activity. Catalase was assayed by following the oxygen release from 0.33 mM hydrogen peroxide polarographically at 37°C with an oxygen monitor (model 53; Yellow Springs Instruments Co., Yellow Springs, Ohio). The standard reaction mixture (3 ml) contained 60 mM potassium phosphate buffer (pH 7.0), 50 mM NaCl, and cell extract. The reaction was initiated by adding hydrogen peroxide.

Treatment of PMN with sulfide. Sodium sulfide was added to a 1-ml suspension of PMN (10^7 cells per ml) in the buffered salt solution containing 0.1% glucose in a 12-ml tube with a stop cork. The tube was placed horizontally for 1 h (37°C) in a cradle with a rocking movement (8 rpm) and a maximal inclination of 15°. In some experiments serum-opsonized zymosan (4 mg/ml) was added at the same time as sulfide to the PMN suspension. Zymosan had been opsonized by treating one part of a zymosan suspension (80 mg/ml) in buffered salt solution with one part of serum at 37°C for 30 min. After the 1-h experiment the PMN suspension was centrifuged at 450 × *g* for 5 min. Myeloperoxidase activity and sulfide level (30) were determined in the supernatant fluid. The PMN of the pellet were washed three times and suspended in buffered salt solution (10^7 cells per ml). The respiratory burst of the cells and the myeloperoxidase and the catalase activities in the cell extract were determined.

Oxidation of sulfide by activated PMN suspensions. A 1-ml suspension of leukocytes (10^7 cells per ml) in buffered salt solution containing 0.1% glucose was incubated at 37°C. A 100-μl sample was taken and added to a stirred cuvette with a thermostat (37°C) containing 0.9 ml of buffered salt solution with 0.1% glucose and 100 μM sulfide. The decrease in absorbance of sulfide was followed at dual wavelengths ($A_{230-250}$) for 15 min. The change in sulfide level was calculated from an $A_{230-250}$ of 3.71×10^3 liters per mol per cm. Thereafter another 100-μl sample was taken from the leukocyte suspension and treated in the same way, but the respiratory burst of the PMN was activated by the addition of 10 μl of 2 μM PMA containing 1% dimethyl sulfoxide. After 1-h incubation of the original PMN suspension, this latter procedure was repeated.

To another PMN suspension (10^7 cells per ml) sulfide was added to give an initial concentration of 1 mmol/liter. This leukocyte suspension was tested in the same way as the sulfide-free leukocyte suspension. During the 1-h incubation the sulfide level decreased, before the sulfide oxidation in the stirred thermostated cuvette was determined, this reaction mixture had to be supplemented with sulfide to reach a concentration of 100 μmol/liter.

Phagocytosis and killing of bacteria. Bacterial cells (5×10^6) and PMN (5×10^6) were suspended in 1.7-ml Eppendorf tubes in RPMI 1640–HEPES medium containing 5% serum. To some of the tubes, 5 μl of 100 mM sodium sulfide was added to reach a final volume of 500 μl. The addition of sulfide increased the pH in these tubes from 7.2 to 7.35. To get the same pH in all tubes, 1.5 μl of 0.1 M NaOH was added to the other tubes. The tubes were incubated at 37°C for 1 h under end-over-end rotation (60 rpm). Two samples were withdrawn from each tube at the start and at the end of the experiment (18).

One of the two samples from each occasion was used to evaluate the killing of the bacteria. The total number of viable bacteria was determined by diluting and culturing samples on duplicate blood agar plates after PMN had been lysed and the streptococcal chains had been broken up by sonication for 8 s (150 W, 145- by 35-mm tip with an amplitude of 18 μm; MSE Ultrasonic Disintegrator, MSE Scientific Instruments, Crawley, Sussex, United Kingdom).

To estimate the number of bacteria associated with the PMN, the other sample was diluted and centrifuged in siliconized glass tubes at 110 × *g* for 10 min at 4°C. This separated bacteria that were phagocytosed or sticking to PMN into a pellet, whereas the other bacteria stayed in the supernatant fluid. After the supernatant fluid was decanted from the pellet, the cells of the pellet were suspended in the buffered salt solution. Both the supernatant fluid and the cell suspension were sonicated, and samples were cultured on duplicate blood agar plates. The number of viable bacteria was expressed as a percentage of the initial inoculum.

RESULTS

Respiratory burst. When the PMN were stored for 1 h in a solution with an initial sulfide level of 1 mmol/liter, the respiratory burst induced by PMA was only slightly lower (3.6 ± 1.4 nmol of cytochrome *c* reduced by 10^6 cells per min) than that in cells stored in a sulfide-free solution (4.7 ± 1.6 nmol). At a sulfide level of 2 mmol/liter, the activity of the cells was decreased by about 80% (0.9 ± 0.7 nmol).

The sulfide levels in the PMN suspensions decreased significantly during 1 h. The decrease was much higher in suspensions in which the leukocytes had been activated than in the suspensions with nonactivated leukocytes (Table 1). This difference could be explained by an oxidation of sulfide after activation of the respiratory burst of the leukocytes. In Fig. 1 the oxidation rate of sulfide in the leukocyte suspension is shown (curve a). This rate increased significantly after the activation of the respiratory burst by PMA (curve b). This capacity to oxidize sulfide after activation of the respiratory burst was almost the same after the leukocytes had been stored for 1 h in a solution with an initial sulfide level of 1 mmol/liter (curve c).

TABLE 1. Sulfide levels in PMN suspensions (10^7 cells per ml) 1 h after the exposure to sodium sulfide[a]

Initial suspension	Sulfide level (μM) in:		
	Cells activated by zymosan	Cells not activated by zymosan	Suspension of zymosan without cells
1,000	50 ± 27	490 ± 120	800 ± 38
2,000	165 ± 80	1,550 ± 300	1,690 ± 110

[a] Means and standard deviations are given for three independent experiments.

Myeloperoxidase and catalase. The effect of sulfide on catalase activity of PMN was studied because of the well-established inhibitory effect of sulfide on this enzyme protein (3, 24). When PMN were stored in a solution with an initial sulfide level of 1 mmol/liter, the intracellular catalase and myeloperoxidase activities of the PMN after 1 h were similar to the activities in cells stored in a sulfide-free solution (Tables 2 and 3). With an initial sulfide level of 2 mmol/liter, the intracellular catalase and myeloperoxidase activities decreased during 1 h by about 60% (Tables 2 and 3). When 5 mM sulfide was tried, there was extensive lysis of the PMN.

Compared with nonactivated PMN, cells activated by serum-opsonized zymosan had after 1 h a 25% lower intracellular catalase activity and 50% lower intracellular myeloperoxidase activity (Tables 2 and 3). However, there were higher extracellular levels of myeloperoxidase in the suspension of the activated cells than in the suspensions of nonactivated cells. The extracellular levels of catalase were the same in the activated and nonactivated suspensions. When the activated leukocytes were exposed to sulfide, no myeloperoxidase activity could be detected either intracellularly or extracellularly after 1 h. Under the same conditions no extracellular catalase activity was detected. Intracellularly the catalase activity was decreased by 35 and 50% when activated cells were exposed to 1 and 2 mM sulfide, respectively, for 1 h (Tables 2 and 3).

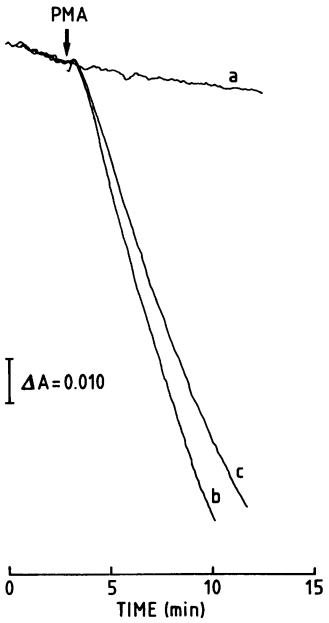

FIG. 1. Oxidation of sulfide in PMN suspensions: decrease in sulfide level (a) in resting PMN suspensions, (b) after activation of the respiratory burst by PMA, and (c) after activation of the respiratory burst of leukocytes, which had been exposed to 1 mM sulfide 1 h before the activation.

TABLE 2. Effect of sulfide on myeloperoxidase of PMN 1 h after the exposure of 10^7 cells to sodium sulfide[a]

Addition	Myeloperoxidase activity[b] in:			
	Cells activated by zymosan		Cells not activated by zymosan	
	Intracellular	Extracellular	Intracellular	Extracellular
None	1.7 ± 0.38	0.48 ± 0.12	3.4 ± 0.95	0.16 ± 0.11
1 mM sulfide	0	0	3.2 ± 0.90	0
2 mM sulfide	0	0	1.4 ± 0.21	0

[a] Means and standard deviations are given for three independent experiments.
[b] Nanomoles of dichlorodimedon produced by 10^6 cells per min.

Myeloperoxidase in cell extracts was separated from catalase by ion-exchange chromatography and exposed to various levels of sulfide. More than 90% of the activity was lost when 1 μmol of sulfide per liter was added to the reaction mixture (Fig. 2). Almost identical results were obtained when purified myeloperoxidase (Calbiochem) was used (data not shown). In these reaction mixtures there are, however, many more possible interactions among the ingredients that between sulfide and myeloperoxidase. Sulfide may be oxidized to sulfate by hydrogen peroxide (19) and may react with hypochlorite produced by myeloperoxidase. To test for the overall effect of these interactions, myeloperoxidase was substituted with an syringe pump delivering hypochlorite into the reaction mixture. The addition of sulfide (1 μmol/liter) to the reaction mixture did not decrease the rate of MCDM chlorination by more than 10% (Fig. 3). The decrease in chlorination of MCDM by sulfide demonstrated in Fig. 2 could thus be ascribed to an inactivation of myeloperoxidase by sulfide.

Phagocytosis and killing of bacteria. Both the LD and the HD variants of *S. agalactiae* type III were readily phagocytosed and killed in the presence of 5% serum. In a sulfide-free reaction mixture 0.3 to 0.4% of the bacteria of the initial inoculum survived for 1 h (Table 4). With an initial sulfide level of 1 mmol/liter, the survivors increased to 1.2% for the HD variant and to 10.2% for the LD variant (Table 4). In reaction mixtures without PMN and with or without sulfide (1 mmol/liter), the number of living bacteria did not decrease (data not shown). This indicated that phagocytosis and killing of *S. agalactiae* worked quite efficiently even in the presence of this high sulfide level.

TABLE 3. Effect of sulfide on catalase of PMN 1 h after the exposure of 10^7 cells to sodium sulfide[a]

Addition	Catalase activity[b] in:			
	Cells activated by zymosan		Cells not activated by zymosan	
	Intracellular	Extracellular	Intracellular	Extracellular
None	1.2 ± 0.30	0.30 ± 0.07	1.6 ± 0.50	0.30 ± 0.12
1 mM sulfide	0.78 ± 0.24	0	1.4 ± 0.53	0
2 mM sulfide	0.56 ± 0.08	0	0.59 ± 0.14	0

[a] Means and standard deviations are given for three independent experiments.
[b] Micromoles of O_2 produced by 10^6 cells per min at an initial hydrogen peroxide concentration of 0.33 mmol/liter.

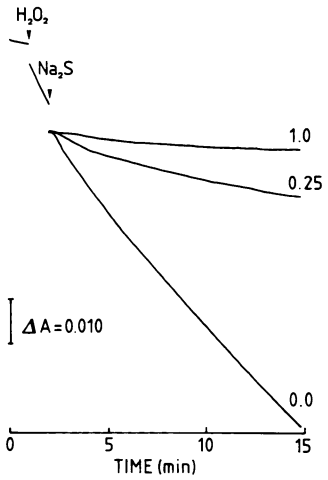

FIG. 2. Effect of sulfide on myeloperoxidase activity. The reaction was initiated by hydrogen peroxide, and 1 min thereafter sodium sulfide was added. The chlorination of MCDM was recorded. Three superimposed recordings after initial concentrations of 0, 0.25, and 1 μM sulfide are shown.

DISCUSSION

Hydrogen sulfide gas is extremely toxic to humans. Exposure to air containing 0.7 mg of hydrogen sulfide per liter (500 ppm) is rapidly fatal. Death results from an effect of sulfide on the central nervous system, with paralysis of the respiratory center. The toxic effect is believed to be a consequence of an inactivation of cellular cytochrome oxidase. A splitting of essential disulfide bonds of proteins and a binding of sulfide to various metal ions may also contribute to the toxicity (for reviews see references 2 and 34).

It was an unexpected finding that sulfide had such a limited detrimental effect on the function of PMN. The PMN phagocytosed and killed bacteria efficiently in the presence of 1 mM sulfide. One-hour exposure of the PMN to sulfide affected neither their myeloperoxidase activity nor their respiratory burst. This must mean that such vital functions of the cell as the generation of NADPH in the hexose monophosphate pathway and the activation of the NADPH oxidase by the protein kinase c-dependent pathway (8, 31, 33) were unaffected by the sulfide exposure.

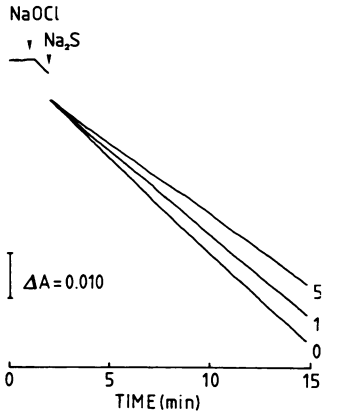

FIG. 3. Effect of sulfide on the chlorination of MCDM in the presence of hydrogen peroxide. In the reaction mixture for determination of myeloperoxidase activity, myeloperoxidase was substituted with a syringe pump, which delivered sodium hypochlorite into the reaction mixture. Three superimposed recordings after initial concentrations of 0, 1, and 5 μM sulfide are shown.

TABLE 4. Phagocytosis and killing of *S. agalactiae* M732[a]

Variant	Sulfide concn (mM)	% Viable bacteria		
		Total	In supernatant	In pellet
HD	0	0.39 ± 0.11	0.08 ± 0.03	0.22 ± 0.06
	1	1.18 ± 0.66	0.24 ± 0.25	0.82 ± 0.47
LD	0	0.34 ± 0.17	0.13 ± 0.12	0.14 ± 0.14
	1	10.2 ± 3.0	1.8 ± 1.2	6.0 ± 2.2

[a] After 1 h of incubation of bacteria with PMN, the total number of bacteria in the reaction mixture was determined as well as the number of bacteria in the supernatant fluid and in the pellet after centrifugation of the reaction mixture at 110 × *g* for 10 min. The number of viable bacteria is expressed as a percentage of the initial inoculum. Means and standard deviations are given for six independent experiments with the HD variant of the strain and five independent experiments with the LD variant.

The current view that mammalian cells are easily permeated by hydrogen sulfide (34) was not corroborated. As a source of hydrogen sulfide, sodium sulfide was used in the present study. This compound is promptly and completely hydrolyzed in aqueous solutions with the formation of hydrogen sulfide. Hydrogen sulfide has two acid dissociation constants. Dissociation of the first proton results in the formation of hydrosulfide anions (pK_a, 7.04) and of the second proton in the formation of sulfide anions (pK_a, 11.96). This means that 40% of the hydrogen sulfide in our reaction mixtures (pH 7.2) was in the form of undissociated acid. Since nonionic species are thought to diffuse more readily through biological membranes than anionic species, our experimental conditions should have prepared for the permeation of the PMN by hydrogen sulfide (34). The preserved activity of myeloperoxidase and catalase in the sulfide-exposed leukocytes clearly showed that hydrogen sulfide did not enter the cells in significant amounts.

The inactivation of catalase by sulfide is well established (3, 24). In the present study myeloperoxidase was shown to be extremely sensitive to hydrogen sulfide. More than 90% of its activity was lost upon exposure to 1 μM sulfide. PMN activated by serum-opsonized zymosan and exposed to sulfide lost all their myeloperoxidase activity. The cell membrane thus seemed to be an efficient barrier for the permeation of hydrogen sulfide into the PMN. The cell wall and protoplast membrane of bacteria, on the other hand, do not seem to provide such a barrier to sulfide permeation. In bacteria sulfide rapidly inactivates catalase (6) and induces mutations and killing in the presence of hydrogen peroxide (4, 5).

This appears to be the first time an inhibition of myeloperoxidase by sulfide has been demonstrated. The effect of sulfide on other peroxidases varies. Hydrogen sulfide is a competitive inhibitor of the selenoprotein glutathione peroxidase (G. Cohen and P. Hochstein, Fed. Proc. **24**:605, 1965) and a substrate for the protohemoprotein horseradish peroxidase (24, 27). Lactoperoxidase, like myeloperoxidase, is inactivated by sulfide and reacts with sulfide like other protohemoproteins such as catalase, hemoglobin, and myoglobin, forming sulfhemoproteins (22, 24). It appears likely that myeloperoxidase forms an inactive sulfhemoprotein, but this has not yet been demonstrated. The high sulfide sensitivity of myeloperoxidase is comparable to the sensitivity of cytochrome oxidase to sulfide (25).

The activation of the respiratory burst resulted in an oxidation of hydrogen sulfide. It is likely that hydrogen sulfide reacted with the superoxide anions and hydrogen peroxide (19) produced in the NADPH oxidase reaction. The possibility cannot be excluded, however, that hydrogen sulfide actually substituted for NADPH as a substrate for NADPH oxidase. It was not possible to test for this substitution in cell extract because of a high reactivity of sulfide with cytochrome *c*, the indicator of superoxide anion production by NADPH oxidase. In support of a reaction mainly between sulfide and oxygen products of the NADPH oxidase was an observation that sulfide completely quenched the luminol-dependent chemiluminescence of PMN phagocytosing *S. agalactiae* (unpublished results).

Sulfide was more effective in preventing the phagocytosis and killing of the LD variant of *S. agalactiae* than of the HD variant. The main difference between these two variants is that the LD variant has a larger polysaccharide capsule than the HD variant (12–14). Encapsulated variants of *S. agalactiae*, like other encapsulated pathogens, are more dependent on antibodies and complement than variants without capsule for an efficient phagocytosis and killing (1, 17). It is possible that sulfide, by its capacity to split disulfide bonds (7, 10), changed the configuration of antibodies and/or complement factors and that this influenced the opsonization.

High levels of sulfide have been reported in sites of polymicrobial anaerobic infections, such as in gingival pockets of periodontal disease (16, 21, 28; S. Persson, R. Claesson, and J. Carlsson, Oral Microbiol. Immunol., in press). In the center of such sites the respiratory burst and myeloperoxidase will not work because of the anaerobic conditions. The PMN have to rely on their oxygen-independent mechanisms for microbial killing. The present study indicates that in areas where the anaerobic infected zone reaches oxygenated tissue the PMN will be able to use their respiratory burst and may work efficiently even in the presence of high levels of sulfide. The products of the respiratory burst actually help in the detoxification of sulfide by oxidizing sulfide.

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