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Hypochlorite Scavenging by *Pseudomonas aeruginosa* Alginate

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Alginic acid was purified from a mucoid clinical isolate of *Pseudomonas aeruginosa*. Luminol-dependent chemiluminescence of phorbol myristate acetate-stimulated neutrophils was inhibited by this alginate, but oxygen consumption was unaffected. Further studies indicated that this effect was due to the ability of the pseudomonal alginate to scavenge hypochlorite. A seaweed alginate was less effective and dextran T500 was ineffective in hypochlorite scavenging. It appears that the uronic acid core and the *O*-acetyl groups of pseudomonal alginate are involved in its hypochlorite-scavenging ability. The relevance of this phenomenon was demonstrated by the greater resistance to killing by hypochlorite of mucoid *P. aeruginosa* compared with a nonmucoid revertant, and the addition of purified alginate to the nonmucoid revertant protected the organism from hypochlorite. Thus, this extracellular polysaccharide may enhance the virulence of *P. aeruginosa* by scavenging the phagocyte-generated oxidant HOCl. This enhanced virulence may be involved in disease processes in which mucoid organisms predominate, such as cystic fibrosis.

Cystic fibrosis is a common inherited disease of unknown biochemical basis. Today this disease is the most common lethal genetic disease of the Caucasian population, with an incidence of 1 in every 1,600 live births (13). Pulmonary infection presumably results from impaired clearance mechanisms, with infection by *Pseudomonas aeruginosa* as the leading cause of morbidity and mortality in patients with the disease (18, 20, 28). The organisms are repeatedly isolated from the sputum in large numbers (35) and are often mucoid in nature because of alginic acid, a viscous, polyanionic polysaccharide they produce (16). It should be noted that mucoid and nonmucoid are relative terms in that strains that appear to be nonmucoid actually produce small amounts of extracellular alginate (2).

Mucoid *P. aeruginosa* is isolated almost exclusively from cystic fibrosis patients with pulmonary infections (14), and much attention has been given to alginic acid, or alginate, and its possible role in the pathogenesis of cystic fibrosis lung pathology. The material has been shown to be antiphagocytic (34), to inhibit bacterial binding to macrophages (26), to stimulate antibody responses in patients (10, 36) and experimental animals (29), and to enhance adherence of the organism to mucosal surfaces (31). Presented in this paper are data indicating that this alginate actively scavenges hypochlorite, a major oxidant generated by the activated phagocyte. Removal of phagocyte-generated hypochlorite may help the organism survive the oxidative onslaught of the phagocyte and may promote the establishment of a chronic pulmonary infection.

MATERIALS AND METHODS

Microorganisms. A mucoid *P. aeruginosa* was isolated at autopsy from a patient with cystic fibrosis. This mucoid isolate was termed RS303. A nonmucoid revertant, termed RS303N, was isolated from RS303 by serial subculture on nutrient agar (Difco Laboratories, Detroit, Mich.) at 37°C and selection for nonmucoid colonies. Identification of mucoid and nonmucoid organisms as *P. aeruginosa* was performed by using standard morphologic and biochemical tests (15).

Purification of pseudomonal alginate. RS303 was cultured on MacConkey agar (Difco) with 3% glycerine (16) at 25°C for 96 h. Confluent growth was scraped off the agar surface and suspended in sterile saline and 2 mM EDTA (disodium salt). The suspension was centrifuged at 20,000 × *g* for 1 h, and the supernatant was centrifuged at the same force for 2 h. The alginate was precipitated by the addition of 3 volumes of isopropanol to the supernatant fluid, washed with isopropanol, and suspended in deionized water. This process was repeated twice. The resultant material was dried, weighed, suspended in deionized water, and dialyzed for 48 h against four changes of 1 mM EDTA in deionized water and for 48 h against four changes of deionized water. The preparation was precipitated and washed as described above and dried in vacuo (23).

Seaweed alginate (from *Macrocystis pyrifera*; Sigma Chemical Co., St. Louis, Mo.) was purified as described above, except that the final product was suspended in deionized water and lyophilized.

Pseudomonal alginate was deacetylated by exposure to NaOH (16). Alginate was suspended in water at 5 mg/ml, and 500 μl of 0.3 N NaOH per ml was added. The solution was stirred at 25°C for 1 h, and the pH was lowered to 7.0 with 0.3 N HCl; then the solution was dialyzed against 30 volumes of water at 4°C for 24 h. The retained material was precipitated with isopropanol, suspended in water, and lyophilized.

Purity of the alginate preparations was checked by visible and UV scans from 760 to 200 nm with a Beckman 25 spectrophotometer (Beckman Instruments, Inc., Palo Alto, Calif.); protein content was determined by the method of Bradford (Bio-Rad Laboratories, Richmond, Calif.) (7), and endotoxin contamination was determined by *Limulus* amoebocyte lysate assay (Pyrostat; Millipore Corp., Bedford, Mass.) (38). Uronic acid content was determined by the borate–sulfuric acid–*O*-phenylphenol method (6) with mannuronic acid lactone (Sigma) used as the standard.

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TABLE 1. Effect of pseudomonal alginate on CL and oxygen consumption of PMN[a]

PMN treatment	CL[b]	O_2 consumed (nmol/10^6 PMN per min)[c]
Resting	0	0.2
Alginate (1,000 μg/ml)	2	0.2
PMA (10 ng/ml)	116	2.4
PMA with alginate (μg/ml)		
100	60	2.2
333	46	2.4
1,000	2	2.1

[a] Data are from one representative experiment.
[b] Total integrated CL during the first 24 min of oxygen consumption.
[c] Oxygen consumption was measured for 24 min.

Isolation of PMN. Human polymorphonuclear leukocytes (PMN) were isolated by dextran sedimentation and countercurrent elutriation. Blood from informed volunteers (with approval of an institutional review board for protection of human subjects) was drawn into acid-citrate-glucose anticoagulant, and the erythrocytes were sedimented at 1 × *g* for 45 min at 37°C with dextran T500 (Pharmacia Fine Chemicals, Piscataway, N.J.). The PMN-rich upper layer was diluted fourfold with 10 mM phosphate-buffered normal saline (PBS) (pH 7.4) and pumped with a peristaltic pump at 7.5 ml/min into a Beckman J6-B centrifuge equipped with a JE-6B elutriator rotor and a standard chamber spun at 2,250 rpm. When the diluted cell suspension was completely pumped into the rotor, the contaminating erythrocytes, monocytes, and lymphocytes were flushed from the chamber with PBS by a gradual increase in the flow rate to 15 ml/min. When all visible erythrocytes were flushed from the chamber, the centrifuge was stopped, and the PMN remaining in the chamber were removed; purity was >93% PMN. The PMN were washed once with PBS and suspended to working concentration in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Sigma) balanced salt solution with 1 mM Ca^{2+}, 1 mM Mg^{2+}, 145 mM NaCl, 5 mM KCl, and 5 mM glucose (pH 7.4).

Effect of pseudomonal alginate on CL of PMN. Luminol-dependent chemiluminescence (CL) was measured with a photometer (Turner Designs, Mountain View, Calif.) with the photometer chamber maintained at 37°C. Samples (100 μl each) of PMN at 4 × 10^6/ml, pseudomonal alginate or HEPES balanced salt solution, and 5-amino-2,3-diphthalazinedione(luminol; Sigma) at 4 × 10^{-6} M were added to a polypropylene cuvette (8 by 50 mm). The cuvette was placed in the photometer chamber, and the reaction was started by injecting 100 μl of 40-ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma). CL was recorded during the period of oxygen consumption (measured separately) and expressed as total integrated counts.

Effect of pseudomonal alginate on oxygen consumption by PMN. Oxygen consumption by PMN exposed to PMA or pseudomonal alginate or both was measured simultaneously with CL by using a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). PMN were isolated as described above and suspended in HEPES balanced salt solution to 1.6 × 10^7/ml. The reaction mixture consisted of 500 μl each of PMN, alginate or buffer, and PMA or buffer for a total volume of 2.0 ml. Data were recorded for 24 min after oxygen consumption began and were expressed as nanomoles of O_2 consumed per 10^6 PMN per min. The electrode was calibrated with phenazine methosulfate (Sigma) and NADH (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) (32).

Effect of pseudomonal alginate on the enzymatic action of MPO. To determine if alginate suppression of luminol-dependent CL by PMA-stimulated neutrophils was due to an inhibition of myeloperoxidase (MPO), we tested the ability of the alginate to inhibit the oxidation of 2,2'-azino-di-[3-ethyl-benzothiazoline-(6)-sulfonic acid] (ABTS; Sigma) by MPO and H_2O_2. The reaction mixture contained 3.32 mM H_2O_2, 6.8 mM ABTS, partially purified MPO (the ratio of A_{430}/A_{280} was 0.43), and Sorensen buffer (pH 6.0) or pseudomonal alginate at 1 mg/ml. The A_{405} was measured continuously.

Inhibition by polysaccharides of CL produced by co-oxidation of luminol by NaOCl with H_2O_2. Inhibition of luminol-dependent CL by native and deacetylated pseudomonal alginate, seaweed alginate, and dextran T500 was measured with a Turner Designs photometer. Samples (100 μl each) of 4 × 10^{-6} M luminol, 4 × 10^{-5} M H_2O_2, and 20 mM phosphate buffer (PB) (pH 7.4) or polysaccharide were added to a polypropylene cuvette (8 by 50 mm), and the cuvette was inserted into the photometer chamber. The reaction was initiated by injection of 100 μl of 4 × 10^{-5} M NaOCl, and integrated CL was recorded for 5 s. The concentration of H_2O_2 was calculated by using the extinction coefficient of 81/M per cm at 230 nm (5). The concentration of sodium hypochlorite was calculated by using the extinction coefficient of 360/M per cm at 290 nm (24).

To be certain that scavenging of hypochlorite, and not of H_2O_2 or some product of their interaction with luminol, was responsible for the inhibition of CL by pseudomonal alginate, one set of experiments was performed without H_2O_2. The concentration of luminol had to be increased to 4 × 10^{-5}

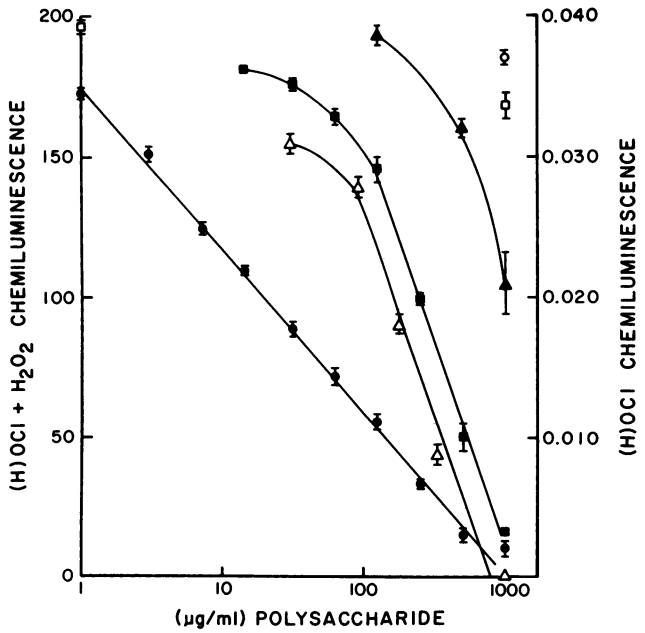

FIG. 1. Inhibition by polysaccharides of the CL produced by the co-oxidation of luminol by NaOCl and H_2O_2. CL is expressed as the mean of triplicate experiments ± 1 standard deviation. Left-hand ordinate [(H)OCl + H_2O_2]: ●, pseudomonal alginate; ■, deacetylated pseudomonal alginate; ▲, seaweed alginate; ○, dextran T500; □, PB. Right-hand ordinate [(H)OCl]: △, pseudomonal alginate.

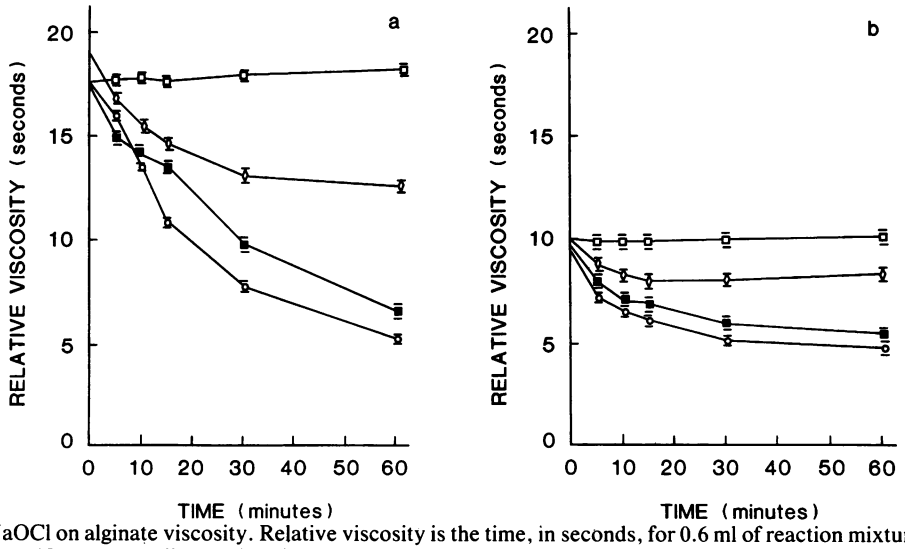

FIG. 2. Effect of NaOCl on alginate viscosity. Relative viscosity is the time, in seconds, for 0.6 ml of reaction mixture to pass from a 2.5-ml glass syringe through an 18-gauge needle. Each point represents the mean of triplicate readings ± 1 standard deviation. Symbols indicate pseudomonal alginate (a) and deacetylated pseudomonal alginate (b) with buffer control (□) or with 1 mM (◇), 2.5 mM (■), or 5 mM (○) NaOCl.

M, but otherwise the reaction was run as described above, with PB replacing the H_2O_2.

Effect of NaOCl on alginate viscosity. Native and deacetylated pseudomonal alginate and seaweed alginate at 2.0 mg/ml were suspended in PB at 25°C. NaOCl was diluted in PB. At time zero, 2.0 ml each of alginate and NaOCl or PB were mixed. Viscosity was determined at 0, 5, 10, 15, 30, and 60 min by measuring the time, in seconds, necessary for 0.6 ml of reaction mixture to pass from a 2.5-ml glass syringe through an 18-gauge needle (22).

Detection of residual NaOCl in reaction mixtures with polysaccharides. Residual NaOCl in mixtures with polysaccharides was measured directly by the decrease in its A_{290}. Blanks were set with appropriate concentrations of polysaccharide or buffer. The reaction was carried out in 0.01 M sodium phosphate buffer (pH 7.54, which is the pK_a of $HOCl\text{-}OCl^-$ at 25°C) (24). The reaction was initiated by the addition of 3,080 nmol of $HOCl\text{-}OCl^-$ to a 2-ml volume containing polysaccharide or buffer, and the disappearance of $HOCl\text{-}OCl^-$ was monitored continuously.

Killing of *P. aeruginosa* RS303 and RS303N by NaOCl. RS303 and RS303N were grown in an iron-limited defined medium (30) with shaking at 37°C to late log phase. The organisms were harvested by centrifugation at 15,000 × *g* for 45 min, suspended to a Klett reading of 150 in 10 mM PBS with 0.1% glucose (PBS-glucose), and allowed to rest for 30 min at 37°C. Bacterial suspension (1 ml) was added to 9 ml of NaOCl (25 μM final) in PBS-glucose, and the mixture was vortexed briefly and allowed to stand for 15 min. Samples were taken and serially diluted with nutrient broth (Difco) for the first dilution tube and sterile water for the remaining tubes. Surviving organisms were counted by using pour plate techniques with Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.). Plate cultures were incubated for 18 h at 37°C and counted. Washed organisms were prepared from the organisms described above by vigorously vortexing of the suspensions for 1 min, centrifugation at 15,000 × *g* for 10 min, suspension of the organisms in PBS-glucose to a Klett reading of 150, and immediate performance of the killing experiments as described above.

In another experiment, the protective effect of alginate on hypochlorite killing of nonmucoid organisms (RS303N) was studied. Organisms were grown, harvested, and suspended to a Klett reading of 100. The microorganisms were suspended in PBS-glucose or purified alginate and incubated at room temperature for 5 min. Hypochlorite was then added at a final concentration of 25 μM, and after 15 min, the organisms were diluted and surviving organisms were enumerated by using pour plates as described above.

RESULTS

Characterization of purified pseudomonal alginate. Preparations of pseudomonal and seaweed alginates contained less than 1 μg of protein per ml as determined by Bradford assay and A_{280}. Nucleic acid was not detectable by A_{260}. Endotoxin contamination of pseudomonal alginate was less than 100 pg/mg of alginate as determined by using a U.S. reference endotoxin from *Escherichia coli*. Uronic acid content of pseudomonal alginate was determined to be >95%.

Effect of pseudomonal alginate on CL and oxygen consumption of PMN. Luminol-dependent CL requires generation by the activated PMN of HOCl (12), which is produced by the oxidation of chloride by H_2O_2 via MPO (17). Since H_2O_2 is produced via reduction of oxygen, HOCl production (thus CL) is associated with oxygen consumption. When PMN were exposed to alginate, no activation was evident as measured by increased oxygen consumption or CL (Table 1), but the soluble stimulant PMA resulted in a large burst of CL and oxygen consumption. When PMN were simultaneously exposed to alginate and PMA, CL was decreased in a concentration-dependent manner. Oxygen consumption, however, was unaffected. Pseudomonal alginate was not cytotoxic to PMN as determined by trypan blue exclusion (data not shown).

Effect of pseudomonal alginate on the enzymatic action of MPO. In the absence of alginate, the activity of MPO was 0.477 ± 0.031 U/liter. In the presence of 1 mg of pseudomonal alginate per ml, the MPO activity was 0.434 ± 0.014 U/liter, an insignificant difference ($P = 0.173$ by the one-way analysis of variance).

Inhibition by polysaccharides of the CL produced by co-oxidation of luminol by NaOCl and H_2O_2. H_2O_2 greatly

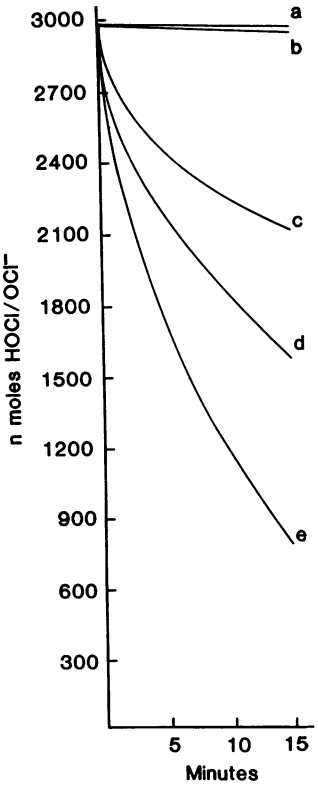

FIG. 3. Consumption of (H)OCl by alginate. Consumption of hypochlorite was monitored at 290 nm. The reaction was run at the pK_a of hypochlorite; thus, the (H)OCl nomenclature is used to indicate equimolar concentrations of HOCl and OCl^-. HOCl was present as follows: alone (a), with dextran T500 at 1 mg/ml (b), or with pseudomonal alginate at 0.25 (c), 0.5 (d), or 1 (e) mg/ml.

enhances the CL produced by the oxidation of luminol by NaOCl (8). This reaction may resemble conditions found in the activated phagocyte. This CL system was chosen for examination of scavenging of NaOCl by alginate. Pseudomonal alginate greatly inhibited CL in a concentration-dependent manner (Fig. 1). Deacetylated pseudomonal alginate was less active in inhibiting CL than was the native material but more active than seaweed alginate. Dextran T500 was not effective.

Pseudomonal alginate also inhibited CL due to the oxidation of luminol by hypochlorite in the absence of H_2O_2. Without H_2O_2, the level of CL is several orders of magnitude less, but the concentration-dependent inhibition of CL suggests that it is the hypochlorite, and not H_2O_2 or some intermediate in their co-oxidation of luminol, that reacts with pseudomonal alginate.

Effect of NaOCl on alginate viscosity. Previous work has shown that a decrease in viscosity can be used as a measure of depolymerization of polysaccharides (22). The addition of NaOCl to pseudomonal alginate dramatically reduced its viscosity in a time- and concentration-dependent manner (Fig. 2a). Deacetylation of pseudomonal alginate decreased its viscosity, but the pattern of further viscosity reduction by NaOCl was similar to that of the native alginate (Fig. 2b).

Detection of residual NaOCl in reaction mixtures of polysaccharides. If NaOCl is being consumed by its reaction with alginate, a decrease in the concentration of NaOCl should occur in the reaction mixture. Residual NaOCl was determined by a decrease in its A_{290}. Pseudomonal alginate consumed HOCl-OCl^- and was concentration dependent (Fig. 3). In the absence of polysaccharide, no measurable decline in the concentration of HOCl-OCl^- was noted and dextran T500 was only a very weak scavenger. The concentration of HOCl-OCl^- was determined by reported molar extinction coefficients and by running the reaction at the pK_a at room temperature (24).

TABLE 2. Killing of *P. aeruginosa* RS303 and RS303N by NaOCl

Condition and strain	CFU/ml (10^6)[a]	P[b]
Unwashed		
RS303	476 ± 30	
RS303 + NaOCl[c]	363 ± 82	0.005
RS303N	353 ± 34	
RS303N + NaOCl	0.03 ± 0.004	<0.001
Washed[d]		
RS303	496 ± 62	
RS303 + NaOCl	0.04 ± 0.004	0.001
RS303N	530 ± 36	
RS303N + NaOCl	0.07 ± 0.001	<0.001

[a] Mean ± standard deviation; $n = 4$.
[b] Statistical significance (P) is reported as the two-tailed probability calculated by the paired t test of each value compared with its untreated control.
[c] NaOCl, when added, was 25 μM.
[d] See Materials and Methods for explanation of washing procedures.

Killing of *P. aeruginosa* RS303 and RS303N by NaOCl. The ability of alginate to scavenge NaOCl suggests that the mucoid RS303 should be more resistant to killing by NaOCl than is the nonmucoid RS303N. In preliminary experiments, the optimal concentration of NaOCl for bacterial killing was found to be 25 μM. RS303 was found to be more resistant to NaOCl toxicity than was its nonmucoid revertant (Table 2), as RS303N showed a 4-log decrease in survival in 25 μM NaOCl. This protective effect could be partially removed by vigorous washing of the bacteria, as washed RS303 showed a 4-log decrease in survival. Thus, washed RS303 showed a sensitivity to NaOCl similar to that of the nonmucoid RS303N.

The addition of purified pseudomonal alginate to nonmucoid organisms (RS303N) resulted in a concentration-dependent protection from killing by hypochlorite (Table 3).

DISCUSSION

Previous studies have demonstrated that the polysaccharide alginate of *P. aeruginosa* inhibits phagocytosis (34) and

TABLE 3. Alginate protection of killing of RS303N by NaOCl[a]

RS303N treatment	CFU/ml (10^6)[b]	P[c]
Without alginate or NaOCl	70.60 ± 4.2[c]	0.001
Without alginate, with NaOCl	0.12 ± 0.007	
With NaOCl and alginate (μg/ml)		
25	0.11 ± 0.015	0.522
100	6.62 ± 0.225	0.001
1,000	68.72 ± 5.2	0.001

[a] NaOCl, when present, was at 25 μM.
[b] Mean ± the standard error of the mean; $n = 4$.
[c] Statistical significance (P) is reported as the two-tailed probability calculated by the paired t test. Values are compared with organisms treated with NaOCl but not with alginate.

the binding of microbes to macrophages (26) and enhances bacterial adherence to tracheal cells (31). Other polysaccharides, such as the capsule of *Streptococcus pneumoniae* (3) and gastric mucin (25), are antiphagocytic and can act as virulence factors. We present data here that suggest an additional role for extracellular polysaccharide, i.e., the scavenging of hypochlorite.

The scavenging of hypochlorite by pseudomonal alginate is supported by the following: (i) the ability of pseudomonal alginate to inhibit luminol-dependent CL, but not oxygen consumption, of PMA-stimulated PMN; (ii) the ability of pseudomonal alginate to inhibit CL generated by the co-oxidation of luminol by NaOCl and H_2O_2; (iii) the reduction of alginate viscosity by NaOCl; (iv) the direct observation of hypochlorite scavenging as measured by the disappearance of NaOCl in alginate mixtures; and (v) the greater resistance to killing by NaOCl of an alginate-producing strain of *P. aeruginosa*, compared with its nonmucoid revertant. The alginate is loosely associated with the exterior of the bacterium (21) and presumably its removal by washing rendered the bacteria more vulnerable to killing. Furthermore, the addition of pseudomonal alginate to nonmucoid organisms protected them from hypochlorite.

Neutrophil stimulation is accompanied by consumption of oxygen (33), production of superoxide (4) and hydrogen peroxide (27), and synthesis of HOCl via the oxidation of chloride by H_2O_2 and MPO (17). This MPO-H_2O_2-chloride system has been shown to be bactericidal (19) because of the generation of toxic chlorinated phagocyte and bacterial components (37). Killing of bacteria by PMN can be either intra- or extracellular. Extracellular killing is dependent on the presence of oxygen (39) and thus on the generation of oxidants. Alginate, by inhibiting phagocytosis and scavenging hypochlorite, may assist the bacterium to survive this phagocyte-generated oxidative burst and to establish a chronic infection. Rabbit antibody to purified alginate does mediate phagocytosis and killing of mucoid *P. aeruginosa* in vitro (1), but whether a comparable antibody is present and active in the lungs of patients with cystic fibrosis is unknown.

Seaweed alginate was less inhibitory than was pseudomonal alginate of CL in the co-oxidation of luminol by NaOCl and H_2O_2. Dextran T500 had very little effect on either the inhibition of CL or the consumption of HOCl-OCl^-.

The differences in viscosity and NaOCl scavenging of the two alginates may be due to differences in their primary structure. The activity described here correlates with uronic acid structure as evidenced by the lack of activity of the polyglucose dextran T500. Both pseudomonal and seaweed alginates are composed of homopolymeric blocks of mannuronic acid interspersed with heteropolymeric lengths of mannuronic and guluronic acids (16, 23). The percentage of mannuronic acid in samples of both alginates varies, with pseudomonal alginate composed of from 26 to 91% mannuronic acid. From 3 to 14% of the mannuronic acid residues of pseudomonal alginate are O acetylated (11), and such acetylation is correlated with viscosity (16). The role of such *O*-acetyl side groups in scavenging NaOCl was investigated with deacetylated pseudomonal alginate. The viscosity of deacetylated pseudomonal alginate was less than that of native material. However, the reaction of deacetylated pseudomonal alginate with NaOCl showed a similar pattern of decreasing viscosity, which may represent depolymerization. The deacetylated product was less active than was native pseudomonal alginate in inhibiting CL from the co-oxidation of luminol by NaOCl and H_2O_2. Thus, such *O*-acetyl groups appear to play a role in hypochlorite scavenging, but some activity is likely associated with the uronic acid core of the molecule, as the deacetylated pseudomonal alginate is partially effective.

The effect of polysaccharides on hypochlorite scavenging goes further than what we have covered in this study. Yeast mannan can inhibit the release of MPO from neutrophils, further reducing the extracellular production of HOCl (41), and it appears to interact with MPO through its phosphate groups (40). A similar phenomenon has been observed with beef lung heparin, which reduces MPO release from stimulated neutrophils and inhibits luminol-dependent CL, presumably by hypochlorite scavenging (9).

In cystic fibrosis lung disease, the role of *P. aeruginosa* and its myriad of products is unclear (20). In these pulmonary infections, the host of lytic enzymes, toxins, and lipopolysaccharide and the immune response of the patient to these products probably all contribute to the pulmonary injury seen. While the mechanism of establishment and maintenance of such chronic infections with *P. aeruginosa* in these patients is unknown, the protective effect of its alginate may be one of the ways by which this organism establishes and maintains such a host-parasite relationship.

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