Inhibition of Bactericidal and Bacteriolytic Activities of Poly-d-Lysine and Lysozyme by Chitotriose and Ferric Iron

GEOFFREY R. TOMPKINS,1 MARGARET M. O’NEILL,1 THOMAS G. CAFARELLA,1 AND GREG R. GERMAINE1,2*

Department of Oral Science, School of Dentistry,1 and Department of Microbiology, Medical School,2 University of Minnesota, Minneapolis, Minnesota 55455

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In a previous report from this laboratory (N. J. Laible and G. R. Germaine, Infect. Immun. 48:720–728, 1985), evidence was presented to suggest that the bactericidal actions of both reduced (i.e., muramidase-inactive) human placental lysozyme and the synthetic cationic homopolymer poly-o-lysine involved the activation of a bacterial endogenous activity that was inhibitable by N,N',N''-triacyctylechitotriose (chitotriose). In the present investigation however, we found that the bactericidal and bacteriolytic action of poly-o-lysine could be prevented only by some commercially available chitotriose preparations and not by others. Analysis by physical and chemical methods failed to distinguish protective chitotriose (CTa) and nonprotective chitotriose (CTi) preparations. CTi and CTa preparations displayed equal capacities to competitively inhibit binding of [3H]chitotriose by immobilized lysozyme and were indistinguishable in their abilities to block the lytic activity of lysozyme against Micrococcus lysodeikticus cells. Elemental analysis revealed significantly higher levels of phosphorus, calcium, iron, sodium, manganese, and copper in CTa. Removal of metals from CTa by chelate chromatography completely abolished the poly-o-lysine-protective capacity. Of the metals detected, only ferric iron (5 to 10 μM) mimicked the protective action of CTa. A Fe(III) concentration of 50 μM was required to inhibit lysozyme (5 μg/ml). Both Fe(III) and CTa (but not CTi) quantitatively blocked the labeling of poly-o-lysine by fluorescamine, suggesting that the primary amino groups of the lysine residues participate in iron binding. Thus, it appears that the poly-o-lysine-protective capacity of certain chitotriose preparations was due not to the chitotriose itself but to contaminating metal ions which interact directly with the polycationic agent. In contrast, Fe(III) cannot account for inhibition of either the bactericidal or bacteriolytic activity of lysozyme by chitotriose.

CATIONIC peptides are believed to exert their lethal effects primarily by inducing bacterial membrane permeabilization (35–37, 40, 45). However, several studies have determined that such agents may also activate bacterial autolytic systems (2, 13, 20, 42). In addition, the presence of an autolysin may increase bacterial sensitivity to lysis by lysozyme (39). Previous investigations conducted in this laboratory examined the bactericidal mechanisms of human placental lysozyme (HPL), dithiothreitol-reduced HPL (muramidase inactive), and the synthetic cationic homopolymer poly-d-lysine (22). Reduced HPL retained a bactericidal potency equal to that of native HPL. Both poly-d-lysine and reduced HPL induced lysis of Streptococcus faecium cells and the lethality of both of these agents against either S. faecium or Streptococcus sanguis (muramidase resistant) was completely abrogated by 5 mM N,N,N''-triacyctylechitotriose (chitotriose), an active-site inhibitor of muramidase-like enzymes (22). Chitotriose also inhibited trypsin-induced autolysis of S. faecium as well as the lytic activity of cell-free autolysin preparations (22). These findings suggested that chitotriose-inhibitable (i.e., muramidase-like) enzymes, expressed by both S. faecium and S. sanguis, may mediate the bactericidal action of cationic protein-induced bacterial death.

During the course of further investigations aimed at ascertaining the universality of chitotriose-inhibitable polycation-induced bacterial killing, we discovered that poly-o-lysine-protective activity was confined only to certain commercially available chitotriose preparations. We provide evidence here that the poly-o-lysine-protective activity is provided not by chitotriose itself but by micromolar quantities of iron present in some chitotriose preparations.

Saliva constitutes the primary defensive barrier opposing microorganisms entering the oral cavity and contains numerous proteins believed to possess antimicrobial functions (6, 26, 32). The enzyme lysozyme (EC 3.2.1.17) is present in human saliva at concentrations averaging around 4.0 μg/ml (34) and is assumed to confer a nonspecific defense against bacteria susceptible to its cell wall-lytic action. Gram-positive bacteria commonly isolated from the human oral cavity are relatively resistant to the lytic action of lysozyme when compared with bacteria isolated from other sources (11). Lysozyme exhibits N-acetylmuramidase glycanohydrolase (muramidase) activity but despite this well-defined enzymic action, the in situ antimicrobial mechanism of vertebrate lysozymes remains a controversial issue (12, 18, 32). Within the physiological pH range, lysozyme exists as an extremely cationic molecule (pI > 10.5), a feature which may confer an antimicrobial capability independent of the glycanohydrolase action (22). This cationic property may explain why certain bacteria possessing lysozyme-resistant cell walls are nevertheless killed by lysozyme (3, 17, 19, 32). In addition, synthetic polymers of cationic amino acids and some catonic proteins which clearly do not naturally function as antimicrobial agents (e.g., cytochrome c and pancreatic ribonuclease) also display antibacterial activity in vitro (21, 22, 27). Both saliva and phagocytic leukocyte granules contain arrays of cationic peptides which have no recognizable enzyme activity but appear to function as antimicrobial agents (25, 33, 40).

* Corresponding author.
MATERIALS AND METHODS

Bacterial strains. *Streptococcus mitis* ATCC 903 was provided by B. Rosan (University of Pennsylvania, Philadelphia, Pa.), and *S. faecium* ATCC 9790 was provided by G. Shockman (Temple University, Philadelphia, Pa.). Cultures were maintained by weekly subculture on tryptic soy broth (containing 0.25% glucose; BBL Microbiology Systems, Cockeysville, Md.) supplemented with 1.5% (wt/vol) agar (Difco Laboratories, Detroit, Mich.), 0.5% (wt/vol) yeast extract (Difco), and 5% (vol/vol) defibrinated sheep blood (Kroy Medical Inc., Stillwater, Minn.) and were renewed regularly from cultures frozen in skim milk. Bacterial cells were grown to mid-exponential phase at 37°C in tryptic soy broth (TSB) supplemented with 0.5% (wt/vol) yeast extract (TSB-YE), cooled on ice, and prepared for bactericidal assays as described previously (22).

Enzymes and chemicals. Bovine serum albumin (BSA), cytochrome c (horse heart, type III), poly-d-lysine hydrobromide (poly-d-lysine; degree of polymerization [DP] = 140), hen egg white lysozyme (HEWL) (3× crystallized), N,N'-diacetylchitobiose (chitobiose), N-acetylmuramyl-L-alanine [beta-(1→4)]-d-glucosamine (GlcNAc), d(+)-glucosamine, and 3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine) were from Sigma Chemical Co. (St. Louis, Mo.). Chitotriose lots 74F-4036, 45F-4022, and 48F-4030 were from Sigma, and lot 050526 was from E. Y. Laboratories Inc. (San Mateo, Calif.). HPL (twice crystallized) was from Green Cross Corporation (Osaka, Japan). Immunodye-activated membranes were obtained from Pall Biosupport Division (East Hills, N.Y.), and CoBind activated membranes were from Micro Membranes Inc. (Newark, N.J.).

A sample of chitotriose (Sigma lot 74F-4036) was titrated commercially by catalytic gas exchange (Amersham Corporation, Arlington Heights, Ill.). The specific activity of [3H]chitotriose was ~180 mCi/mmol. 55FeCl3 (24.4 mCi/mg) was from New England Nuclear Research Products (Du Pont Co., Boston, Mass.). [methyl-3H]thymidine (43 Ci/mmol) was from Amersham.

Bactericidal assay. Determination of the bactericidal activities of poly-d-lysine and lysozyme was essentially as described previously (22). Washed mid-exponential-phase cells were diluted to ~10^7 CFU/ml in 0.5 mM potassium phosphate buffer (pH 7.0) containing 10 μg of L-arginine per ml (assay solution) and any other required supplements (e.g., chitotriose). Following 5 min of preincubation at 37°C, either poly-d-lysine or lysozyme was added at the concentration required to reduce the viable count to 1 to 10% of the control (which was incubated in assay solution alone). Incubation continued for a further 60 min after which the cell suspensions were immediately cooled on ice, diluted serially in cold 10% (vol/vol) TSB without glucose (BBL), and spot plated onto TSB-YE agar to enumerate survivors. By using this method, the standard deviation of replicate bactericidal assays was determined to be 10.75% of the mean survival values. This assay was slightly modified for some experiments, the details of which are given in the appropriate section below.

Bacteriolysis assay. Lysozyme- and poly-d-lysine-induced damage of *S. faecium* cells, in the presence and absence of potential protective agents, was assessed by measuring the amount of radioactivity released from cells grown in TSB-YE supplemented with [methyl-3H]thymidine (10 μCi/ml). The [3H]thymidine-labeled bacteria were harvested and prepared as in the bactericidal assay. Following appropriate incubation, duplicate 100-μl samples were removed and brought to 0.2% (wt/vol) sodium dodecyl sulfate (SDS) to effect lysis of damaged cells. The SDS-treated samples were centrifuged (15,000 × g for 4 min) to remove intact cells, and 80 μl of the supernatant was added to 10 ml of scintillant fluid (EcoLite; ICN Biochemicals, Irvine, Calif.). The disintegration rate was determined by liquid scintillation spectroscopy, using a LS-1701 counter (Beckman Instruments, Fullerton, Calif.). To assess the effects of various agents on spontaneous autolysis, [3H]thymidine-labeled *S. faecium* cells were incubated in the absence of bactericidal agents for up to 3 h before SDS treatment. The effect of various chitotriose preparations on the lytic action of HEWL against *Micrococcus lysodeikticus* cells was determined as previously described (22).

Binding of chitotriose by immobilized lysozyme. The capacities of chitotriose preparations to compete with [3H]chitotriose for binding to HEWL immobilized on CoBind activated membranes were compared. The membrane was cut into squares (1 cm²) and soaked in 25 mM potassium phosphate buffer (pH 7.0) for 30 min at room temperature. The squares were blotted to remove excess moisture and soaked for 1 h at 0°C in 25 mM buffer alone or in buffer containing either HEWL (10 mg/ml) or cytochrome c (10 mg/ml). The membrane squares were blotted, washed three times (for 5 min each time) in buffer, blotted, and soaked in buffer containing BSA (10 mg/ml) for 2 h at 0°C to block unreacted binding sites. The squares were again blotted and finally soaked in 50 mM Tris hydrochloride (pH 7.0) containing 27 μM [3H]chitotriose and various concentrations of unlabeled chitotriose for 1 h at 0°C. The membrane squares were washed three times in buffer and allowed to dry at room temperature, and the bound radioactivity was determined by liquid scintillation spectroscopy.

Analytical comparison of chitotriose lots. Several analytical techniques were used to compare chitotriose preparations. 13C Nuclear magnetic resonance spectra of solutions containing either chitotriose, chitobiose, or GlcNAc at 20 mg per ml of D2O were obtained. Spectra were recorded with a Nicolet 300-MHz spectrometer.

Descending paper chromatography, employing Whatman 1 or 3MM paper, were developed in a pyridine-pentanol-water (8:8:7) solvent system (44). The chitin saccharides were visualized by UV fluorescence after treatment with 0.5 N NaOH in ethanol–1-propanol (6:4) followed by heating at 120°C for 5 min (38).

Reverse-phase high-pressure liquid chromatography (HPLC) of chitin saccharides was performed with an ODS-5S column (4 by 150 mm; Bio-Rad Laboratories, Richmond, Calif.) protected by an ODS-5S precolumn. Samples (20 μl) were applied to the column and developed by elution with distilled, deionized water (100 to 300 μl/min) at ambient temperature (4). The column effluent was monitored at 206 nm.

Mass spectra in the fast-atom bombardment ionization mode were recorded for Sigma chitotriose lots 74F-4036 and 45F-4022 on a VG 7070E-HF instrument (VG Analytical, Manchester, United Kingdom) operating at an accelerating voltage of 6 kV. The fast-atom bombardment ion gun was operated at 8 kV and 1 mA, and xenon served as the gas. Samples were deposited onto a stainless steel stage in a thioglycerol matrix with 1 μl of 0.1 M HCl added.

Elemental analysis of chitotriose. Chitotriose lots were analyzed by inductively coupled plasma atomic-emission spectroscopy (ICP-AES) using a model QA 137 instrument (Applied Research Laboratories, Valencia, Calif.). Samples were dissolved in 2 M HCl at a final concentration of 2.5
mg/ml. Aching of chitotriose samples prior to the addition of HCl did not appear to affect the results. Background levels of each element were determined from solvent controls and subtracted from sample measurements. The level of iron was also determined by the ferrozine method (8). Omission of the reduction step from the ferrozine procedure allowed the distinction of ferrous iron from total iron.

**Chelate chromatography.** Multivalent metal ions were removed from chitotriose by passage through a column (1-mL bed volume) of hydrated tris(carboxymethyl)-ethylenediamine (TED) agarose (Sigma). A 1.6-mL volume of 50 mM chitotriose (in water) was applied to the column which was developed at a flow rate of 0.1 mL/min with distilled water. The eluent was analyzed by ICP-AES, and the carbohydrate content was determined by a reducing sugar assay (30), using GlcNAc and chitotriose as standards.

**Interaction of ferric ions with poly-d-lysine and lysozyme.** Direct binding of Fe(III) by either poly-d-lysine or HEWL was examined by immobilizing either agent on a nylon (Immunodyne) membrane and measuring the amount of 55Fe(III) adsorbed. Membrane squares (1 cm²) were spotted with 5 µL of 0.5 mM potassium phosphate (pH 7.0) containing various concentrations of either poly-d-lysine, HEWL, or cytochrome c. The membranes were allowed to dry before rinsing three times in 50 mL of 0.5 mM potassium phosphate (pH 7.0) at room temperature. Excess moisture was removed by blotting. Remaining free membrane binding sites were blocked by gentle agitation of the squares for 1 h at 0°C in 0.5 mM potassium phosphate (pH 7.0) containing BSA (10 mg/mL). The membranes were blotted, and excess BSA was removed by rinsing in 0.5 mM potassium phosphate (pH 7.0). After the membranes were blotted, they were agitated for 1 h at 0°C in 0.5 mM potassium phosphate (pH 7.0) supplemented with 10 µM FeCl₃ (10⁶ cpm of 55FeCl₃ per mL [0.25 µM]). The membranes were rinsed in three successive 20-mL volumes of 0.5 mM potassium phosphate (pH 7.0) and dried. Adsorbed radioactivity was determined by scintillation spectrometry.

The effect of either chitotriose or FeCl₃ on the detection of poly-d-lysine by fluorescamine was examined by a modification of the method of Udenfriend et al. (41). Poly-d-lysine (1 µg/mL) was incubated with either chitotriose or FeCl₃ (total volume, 0.5 mL) at 37°C for 5 min in 0.5 mM phosphate buffer (pH 7.0). Fluorescamine (20 µL at 3.0 mg of acetone per mL) was then added; 1 min later, 0.5 mL of 0.5 M borate buffer (pH 8.5) was added. Preparations remained at room temperature for 30 min before fluorescence measurement in a spectrofluorometer (J4-8962 Amino-Bowman; American Instrument Co., Silver Springs, Md.). The excitation wavelength was 390 nm, and the emission wavelength was 475 nm.

In addition to the fluorescamine-blocking method described above, the interaction of HPL with Fe(III) was examined by determining the proportion of HPL that was sedimented by increasing concentrations of FeCl₃. Following incubation in 0.5 mM potassium phosphate (pH 7.0) for 60 min at 37°C, the reaction mixtures were centrifuged (15,000 × g for 5 min) and the amounts of iron and HPL present in the supernatant and redissolved pellet fractions were determined. The amount of protein was determined by a modified version of the fluorescamine method (41) in which EDTA (final concentration, 0.2 mM) was added before fluorescamine to prevent interference by iron (28). The amount of iron was measured by the ferrozine assay (8).

**RESULTS**

**Effect of chitotriose on the bactericidal action of poly-d-lysine.** The effects of various commercially available chitotriose preparations on the killing of S. faecium by poly-d-lysine (2.0 µg/mL) are shown in Fig. 1. Chitotriose lots clearly differed in their capacities to protect bacteria. Lot 74F-4036 (CTa) provided complete protection at a concentration of 5 mM, whereas to achieve a comparable effect, lot 45F-4022 [CTi(II)] was required at greater than 25 mM. Lots 48F-4030 [CTi(III)] and 050526 [CTi(III)] did not protect cells at 25 mM. Reducing sugar analyses confirmed that the various chitotriose preparations contained equivalent carbohydrate concentrations. S. mitis cells responded in essentially the same manner, although less poly-d-lysine (0.5 µg/mL) was required to achieve comparable cell death (data not shown).

The amount of CTa required to protect 100% of susceptible bacterial cells was proportional to the concentration of poly-d-lysine used in bactericidal assays (Fig. 2a).

**Comparison of chitotriose preparations.** Chitotriose preparations were analyzed by various methods to determine the biochemical basis for the inability of CTi preparations to protect bacteria from poly-d-lysine.

Fast-atom bombardment mass spectrometry of both CTa and CTi(II) exhibited the protonated molecular ion (CT + H⁺) at a m/z of 628.3 (as confirmed by high-resolution analysis) (Fig. 3a). Other fragments previously assigned (5) as protonated N,N'-diacetylchitobiose-H₂O (m/z = 407) and protonated GlcNAc-H₂O (m/z = 204) were also prominent in the spectra. Overall, the presence of several fragments in our spectra agreed with the previous report of the fragmentation pattern of the relevant chitooligosaccharides prepared by HF hydrolysis of chitin (5). An analysis of the difference spectrum of the two preparations of chitotriose suggested the presence of CT + Na⁺ (m/z = 650) in the CTa prepara-
tion. Therefore, we concluded that the two chitotriose preparations were essentially identical with respect to chitotriose.

Reverse-phase HPLC (Fig. 3b), paper chromatography, and $^{13}$C nuclear magnetic resonance (data not shown) also failed to distinguish chitotriose preparations. CTa and CTi preparations exhibited similar reducing sugar equivalents ($P = 0.66$; df = 14) and displayed identical capacities to prevent binding of $[^3]$Hchitotriose to immobilized HEWL (Fig. 4). In addition, the capacities of CTa and CTi(II) to block the lysis of M. lysodeikticus cells by HEWL were not significantly different. Lytic activity of HEWL (5 μg/ml) was reduced from a control rate (mean ± standard deviation) of 64.00 ± 5.89 to 28.67 ± 5.03 and 31.00 ± 4.24 U by CTa and CTi(III), respectively. However, when compared with CTi(I), CTa possessed 1.5 to 1.6 times the capacity to prevent the lytic action of HEWL (0.5 μg/ml) against $[^3]$Hthymidine-labeled S. faecium cells. Finally, analyses by ICP-AES revealed that CTa contained significantly greater concentrations of phosphorus, calcium, iron, sodium, manganese, and copper than did CTi preparations (Table 1).

Chelate chromatography of CTa. To determine the influence of contaminating polyvalent cations on protection from killing by poly-D-lysine, CTa was subject to TED-agarose chelate chromatography. Table 2 compares the phosphorus and metal concentrations detected in CTa before and after passage through the chelating column. Reducing the concentrations of di- and trivalent metal ions by chelate chromatography completely abolished the poly-D-lysine-protective activity (Fig. 5).

Effect of metal ions on the bactericidal action of poly-D-lysine. To investigate the possible effects that contaminating metal ions may have had on the bactericidal action of poly-D-lysine, the chloride salts of the relevant metals were tested for poly-D-lysine-protective activity using S. faecium as the test organism. Comparatively low concentrations of Fe(III) (6 μM) and Fe(II) (17 μM) interfered with poly-D-lysine-protective activity.

FIG. 2. Relationship between poly-D-lysine concentration and the chitotriose (CTa) concentration required to protect 100% of S. faecium 9790 cells.

FIG. 3. Comparison of CTa and CTi by fast-atom bombardment mass spectroscopy (a) and reverse-phase HPLC (b).
lysine-induced death, whereas the protective concentration of Ca (~20 mM) was in vast excess of that present in 5 mM CTa (Table 1). Protection of 50% of cells was not achieved with either Mn (1 mM) or Na (1 mM), and greater than 1 μM Cu was directly toxic to *S. faecium* cells. Only iron [specifically Fe(III)] was protective in the concentration range in which it was present in 5 mM CTa (Fig. 6). Analysis by the ferrozine method determined the presence of iron at an equivalent concentration of 5.8 ± 0.5 μM in 5 mM CTa and indicated that Fe(II) made up less than 20% of the iron present. Fe(III) at 5 μM also protected greater than 90% of *S. mitis* cells from the lethal action of poly-d-lysine (0.5 μg/ml).

It was previously reported that the chitin saccharide series chitotriose, chitobiose, and GlcNAc exhibited decreasing potency as inhibitors of poly-d-lysine bactericidal activity (22). Analysis of currently available chitin saccharides revealed 1.78, 0.29, 0.04, and 0.002 μM Fe present in 1.0 mM solutions of CTa, CTi, chitobiose, and GlcNAc, respectively. Examination of the concentrations of the chitin saccharides required to completely protect *S. mitis*, *S. faecium*, *Escherichia coli*, and *Candida albicans* from the bactericidal action of poly-d-lysine yielded 2 to 5, ≥30, 25 to 160, and >1,000 mM for CTa, CTi, chitobiose, and GlcNAc, respectively (22, 40a; unpublished data). A comparison of the association of Fe content and protective capacity of these chitin saccharides revealed that when CTi, chitobiose, and GlcNAc concentrations are adjusted to equal the Fe content of CTa, their predicted protective activities are not unlike their actual activities (observed/calculated values: ≥30/12 to 30 for CTi; >25 to 160/80 to 200 for chitobiose; and >1,000/1,800 to 4,500 for GlcNAc). Thus, it is plausible that any protection afforded by CTi, chitobiose, and GlcNAc preparations was also actually due to iron.

The effect of iron pretreatment on the sensitivity of *S. faecium* to the bactericidal action of poly-d-lysine was studied. Washed bacteria (10⁹ CFU/ml) were incubated in the presence or absence of 1 mM FeCl₃ at 37°C for 10 min, the suspensions were divided, and one aliquot from each treatment was washed three times in assay solution. Cell suspensions were then diluted 100-fold into assay solution with or without 1 μg of poly-d-lysine per ml. Following incubation at 37°C for 1 h, the number of surviving bacteria were determined by dilution and plating. Iron pretreatment (without washing) protected 75% of bacteria, whereas iron pretreatment with subsequent washing reduced survival to ~4%. The survival rates of washed and unwashed controls (i.e., without exposure to iron) exposed to poly-d-lysine were reduced to 9 and 6%, respectively. These results indicate that pretreatment with iron did not protect cells. In addition, bacteria that were pretreated with poly-d-lysine (5 min at 37°C) and then washed could not be rescued by addition of 10 μM FeCl₃.

**Interaction of Fe(III) with poly-d-lysine and lysozyme.** When immobilized on nylon fibers, both poly-d-lysine and lysozyme bound ⁵⁵Fe(III) (Fig. 7). Immobilized cytochrome c did not bind significantly greater quantities of ⁵⁵Fe(III) than did membrane controls (blocked with BSA). In

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**TABLE 1. Elemental analysis by ICP-AES and comparison of CTa and CTi**

<table>
<thead>
<tr>
<th>Element</th>
<th>Equivalent concn (μM) present in 5 mM chitotriose</th>
<th>p*</th>
<th>Conc’n (μM) required for 50% reduction of poly-d-lysine (1 μg/ml) lethal activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTa*</td>
<td>CTi*</td>
<td></td>
</tr>
<tr>
<td>Phosphorus</td>
<td>63.98 ± 22.34</td>
<td>12.67 ± 4.70</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Calcium</td>
<td>11.93 ± 3.59</td>
<td>3.76 ± 1.56</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Iron</td>
<td>8.90 ± 4.91</td>
<td>1.45 ± 0.49</td>
<td>0.002</td>
</tr>
<tr>
<td>Sodium</td>
<td>137.8 ± 60.7</td>
<td>17.60 ± 11.30</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Manganese</td>
<td>0.434 ± 0.09</td>
<td>0.197 ± 0.08</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Copper</td>
<td>0.519 ± 0.33</td>
<td>0.192 ± 0.01</td>
<td>0.014</td>
</tr>
</tbody>
</table>

* Determined by Student’s t test (n ≥ 7).
* Sigma chitotriose lot 74F-4036.
* Sigma chitotriose lots 45F-4022, 48F-4030, and 050526.
* NA, Not applicable because assays were performed in 500 μM phosphate.

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**TABLE 2. Removal of major elemental contaminants from CTa by TED agarose chelate chromatography**

<table>
<thead>
<tr>
<th>CTa fraction</th>
<th>Metal concn (μM) present in 5 mM CT</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
</tr>
<tr>
<td>Pre-TED</td>
<td>59.9</td>
</tr>
<tr>
<td>Post-TED</td>
<td>34.5</td>
</tr>
</tbody>
</table>

* The percent depletion values of metals were 42.4% for P, 83.6 for Ca, 100% for Fe, and 13.7% for Na. Determined by ICP-AES.
a similar experiment, FeCl₃ (40 μM) was unable to prevent binding of [³H]chitotriose to immobilized HEWL (data not shown).

Initial experiments demonstrated that FeCl₃ (up to 5 μM) cosedimented poly-D-lysine from solution in 0.5 mM potassium phosphate, suggesting a direct interaction between the two agents (data not shown). Addition of higher concentrations of iron increased the solubility of both poly-D-lysine and iron. At 10 μM FeCl₃, neither the iron nor the poly-D-lysine was sedimentable by centrifugation (15,000 × g, 5 min). However, CTa did not induce sedimentation of poly-D-lysine, and an alternative approach was adopted to examine the possible interaction of these agents. CTa and FeCl₃, but not CTi(II), were found to quantitatively block the reaction of fluorescamine with poly-D-lysine (Fig. 8). Note that the chitotriose concentrations are expressed as iron equivalents. CTa and FeCl₃ blocked the fluorescamine reaction at the same iron-to-lysine residue molar ratio (0.9). The control in which 5 mM CTa alone was tested for fluorescamine reactivity indicated that this preparation was contaminated with a component bearing a free amino group. At the highest CTa concentrations, the contaminating fluorescamine-reactive material appeared to account for all of the detected fluorescence (Fig. 8). However when compared with d(+)-glucosamine, CTa contained less than 0.03 mol% fluorescamine-reactive equivalents. CTi(II) at 5 mM produced no detectable reaction with fluorescamine.

FeCl₃ also interfered with fluorescamine labeling of HPL (Fig. 9). With 10 μM FeCl₃, virtually 90% of the fluorescence was abolished but bactericidal potency was only marginally reduced. To quantitate the interaction between the protein and iron, the effect of FeCl₃ on sedimentation of HPL was examined (Fig. 9). With 10 μM FeCl₃, there was negligible sedimentation of HPL and at least 50 μM FeCl₃ was required to sediment greater than 80% of the protein (iron-to-lysine ratio = 1:1). Protein sedimentation and bactericidal potency (indicated by viability) were inversely related (Fig. 9).

Effect of chitotriose on poly-D-lysine-induced cell wall damage. Initial experiments to determine the effect of poly-D-lysine on the integrity of S. faecium cells indicated that cell wall damage occurred only at specific poly-D-lysine concentrations (Fig. 10a). Optimal poly-D-lysine concentrations were 0.5 to 1.0 μg/ml (for 10⁷ CFU/ml). Above this concentration, cell wall damage appeared to diminish eventually to control levels. However, this effect may have been due to restricted [³H]thymidine solubility caused by the interaction of the poly-D-lysine with cytoplasmic constituents. This notion is supported by studies in which the addition of 5 μg of poly-D-lysine per ml to bacterial cells previously incubated for 60 min with 0.5 μg of poly-D-lysine per ml (which would normally result in maximal lysis) severely lowered the
amount of radiolabel released into the supernatant solutions (Fig. 10a). Incubation of bacterial cells with 5 mM CtI(II) did not prevent either sensitization to SDS-induced lysis or autolysis induction by poly-d-lysine (Fig. 10b). In addition, experiments in which [3H]thymidine-labeled cells were incubated for 3 h in the absence of sensitizing agents indicated that neither CtI(II) nor Fe(III) was able to prevent the spontaneous sensitization of S. faecium cells to SDS-induced lysis (data not shown).

In the foregoing experiments, the maximum radiolabel released by poly-d-lysine-SDS treatment was always greater than that apparently added to the reaction mixture (determined from controls without poly-d-lysine treatment). This observation was probably due to quenching of 3H within intact cells by cytoplasmic and cell wall components.

**DISCUSSION**

**Comparison of protective and nonprotective chitotriose lots.** Following the previous finding that chitotriose prevents the bactericidal action of some polycationic agents, perhaps by blocking an essential bacterial enzyme such as a muramidase or glucosamidase involved in cell wall development (22), the current investigation originally undertook to determine the universality of this protective effect among a variety of bacteria. However, the survey (40a) was interrupted by the inconsistent protection afforded by different lots of commercially available chitotriose against the bactericidal action of poly-d-lysine. Whereas CTA at 5 mM completely prevented the bactericidal effect of poly-d-lysine, other preparations (CtI) exhibited either markedly reduced or no protective capacity. Attention was therefore focused on determining the reason for differences in protective capacity among chitotriose lots. CTA and CtI preparations were indistinguishable in their capacities to inhibit binding of [3H]chitotriose to immobilized lysozyme and to prevent the lytic action of lysozyme against M. lysodeikticus cells. Extensive physical and chemical comparisons of chitotriose lots failed to reveal any differences in carbohydrate composition. However, elemental analysis by ICP-AES indicated that significantly greater levels of P, Ca, Fe, Na, Mn, and Cu were present in CTA than in CtI. Chelate chromatography of CTA removed the protective capacity, implying that contaminating metals, and not the chitotriose itself, were responsible for the protective effect. Of the elements present in CTA, only Fe(III) provided significant protection to susceptible cells at the concentration at which it was present in 5 mM CTA. We therefore postulate that Fe(III) is responsible for the observed protective action. According to laboratory records, Sigma chitotriose lot 33F-4006 was principally used in the studies of Laible and Germaine (22), but unfortunately this lot number is no longer available and we have been unable to determine its metal content.

FeCl3 (5 to 10 μM) protected S. mitis and S. faecium from the lethal effect of poly-d-lysine, but it was generally less effective and more variable than CTA. CTA (5 mM) consistently saved greater than 90% of cells from the lethal action of up to 2 μg of poly-d-lysine per ml, whereas FeCl3 at the equivalent iron concentration (~10 μM) protected cells from only 0.5 to 1.0 μg of poly-d-lysine per ml. Thus, FeCl3 was approximately 50% as effective as CTA in protecting bacterial cells. The variability in the protection provided by the iron salt may have been partly due to its poor solubility and tendency to polymerize on hydrolysis at physiological pH (7). The form of the iron contaminant in CTA is not known, but it could conceivably be in a more soluble or accessible form than that provided as FeCl3, and therefore be more...
previous proposed model which postulated that chitotriose protects bacterial cells from bactericidal cationic proteins and polypeptides by blocking the activity of some putative endogenous enzyme possibly involved in either cell wall remodeling or autolysis (22). The model relied heavily on the ability of chitotriose to protect test bacteria from the lethal action of poly-D-lysine. As shown here, micromolar quantities of iron present in some chitotriose preparations account for protection against poly-D-lysine (but not lysozyme) under the conditions employed previously.

Protective mechanism of iron. The question remained as to how iron prevents the bactericidal action of poly-D-lysine. Two possibilities were initially considered; iron interacts either with the bacterial cell or with the polycationic agent. In support of the first possibility, Fe(III) has been found to interfere with the autolytic systems of both E. coli (16) and Bacillus subtilis (9). Alternatively, in support of the second possibility, Fe(II) has been reported to protect various organisms, including S. faecalis, from the lethal action of a leukocyte lysosomal cationic protein (15). Against Staphylococcus spp., Fe(III) was also protective (S. faecalis was not tested). Fe(II) precipitated the cationic protein [Fe(III) was not examined], suggesting that the protective effect was due to a direct interaction of Fe(II) with the cationic agent (15). Pretreatment of the bacteria with iron, however, did not prevent killing (15).

In the present study, pretreatment of S. faecium with Fe(III) failed to protect from killing by poly-D-lysine. In addition, 55Fe(III) was found to bind to immobilize poly-D-lysine. These findings suggest that the protective action of Fe(III) is due to the interaction of the hydrolyzed Fe(III) complexes with the polycationic agent rather than with the bacterial cell itself. Both FeCl3 and CTA (but not CTi) quantitatively blocked the reaction of fluorescamine with poly-D-lysine. This would explain the unexpected linear relationship between poly-D-lysine concentration and CTA concentration required to protect bacteria (Fig. 2a). The principal sites of fluorescamine interaction with peptides are the primary (i.e., side chain) amino groups (41) and therefore it seems likely that iron binding by poly-D-lysine also involves these groups. Indeed, the capacity of poly-D-lysine to bind metal ions has previously been attributed to the primary amino groups (31). Consequently, it may be expected that Fe(III) and poly-D-lysine would interact at a Fe-to-lysine residue ratio approaching 1. By converting the chitotriose concentrations plotted in Fig. 2a to their iron equivalents (Fig. 2b), the Fe-to-lysine residue ratio required to completely prevent poly-D-lysine-induced killing was 0.88. The Fe-to-lysine residue ratio calculated from the fluorescamine-blocking experiment at 100% inhibition of fluorescamine was 0.9 for both FeCl3 and CTA. These data suggest that each lysine residue of poly-D-lysine is capable of binding iron.

Ferric iron was also found to interact with HPL, as judged by the fluorescamine inhibition assay. Ninety percent loss of fluorescence occurred at an iron-to-HPL molar ratio of ~27. Amino acid residues bearing primary amino groups available for interaction with fluorescamine in HPL number about 36 (10 Asn, 6 Gln, 5 Lys, 14 Arg, plus amino terminus (24)). Fluorescamine reactivity and fluorescence vary among different amino acids (41); therefore, we would expect the iron-to-HPL molar ratio at 90 to 100% loss of fluorescence to be less than 36. No significant loss of antibacterial activity occurred under conditions in which Fe(III) caused up to a 90% loss of HPL reactivity with fluorescamine. Since loss of HPL bactericidal activity did not relate to inhibition of fluorescamine binding (in contrast to poly-D-lysine), a sedi-

**FIG. 10.** Effect of metal-free chitotriose on poly-D-lysine-induced cell wall damage of S. faecium 9790 cells. (a) Determination of optimal poly-D-lysine concentration. [3H]thymidine-labeled S. faecium cells were incubated for 60 min at 37°C in various concentrations of poly-D-lysine. SDS was then added to 0.2% (wt/vol) to effect lysis, and preparations were centrifuged to pellet intact cells and cell wall debris. The radiolabel remaining in the supernatant was determined and is expressed as percentage of total radioactivity added to the assay. Symbols: □, poly-D-lysine added immediately before incubation; ■ poly-D-lysine added immediately before incubation and more poly-D-lysine (to a final concentration of 5 μg/ml) added at 60 min. Error bars indicate standard deviations. (b) Effect of CTi. [3H]thymidine-labeled S. faecium cells were incubated for 60 min at 37°C with no supplements (control), 0.5 μg of poly-D-lysine per ml (PDL), 0.5 μg of poly-D-lysine per ml and 5 mM chitotriose [PDL+CTi(II)], and 5 mM chitotriose [CTi(II)]. Preparations were processed as described for panel a.
anmentation assay was used to further study the Fe(III)-HPL interaction. The inhibition of the bactericidal activity of 5 µg of HPL per ml commenced at greater than 10 µM FeCl₃ and coincided with the transformation of HPL from a soluble to a sedimentable form. Essentially complete inhibition of HPL bactericidal activity occurred when ~80% of the enzyme became sedimentable. At this point, 50 µM FeCl₃ was present and the molar ratio of iron to amino acid residues was 1:12. Thus, under our assay conditions, inhibition of the HPL bactericidal activity by CTa cannot be due to contaminating iron, because CTa at 5 mM contains less than 10 µM Fe. In the study of Gladstone and Walton (15), Fe(II) was not found to prevent the bactericidal action of lysozyme despite a higher molar ratio of iron to lysozyme (~367) than that found to be protective for Fe(III) in the current investigation (~140). Possibly the use of either Fe(II) or a citrate buffer in the former study discouraged the interaction. The findings of the present study suggest that Fe(II) possesses only about one-third of the poly-D-lysine-protective capacity of Fe(III). Were this difference applied to protection from HPL, a molar ratio of around 420 would be required to achieve ~80% protection.

In contrast to the inhibition of either the bacteriolytic action of lysozyme against M. lysodeikticus cells or the bactericidal action against S. sanguis (40b), CTa was clearly more effective than CTI in preventing lysozyme lytic action against [³H]thymidine-labeled S. faecium. Only very low concentrations (500 ng/ml) of lysozyme were required to cause lysis of S. faecium in this assay. Thus, with 5 mM CTa (~10 µM Fe), the Fe-to-lysozyme ratio was 10 times greater than that used in either M. lysodeikticus lysis assays or S. mitis bactericidal assays which both used 5 mM chitotriose and lysozyme at 5 µg/ml. Note that, as in previous studies (22), S. mitis was not used in lysis studies, as it is relatively resistant to the lytic action of lysozyme and to sensitization to SDS-induced lysis by poly-D-lysine.

Detection of cell wall damage caused by poly-D-lysine was crucially dependent on the concentration of the cationic agent. At concentrations above the optimal range, poly-D-lysine did not appear to sensitize the cells to SDS-induced lysis but in fact suppressed the otherwise spontaneous sensitization of S. faecium cells. Similar observations have been made with polylysine against Staphylococcus aureus (13), with HEWL against S. mutans (32), and with human salivary lysozyme against S. faecium (10). However, in the present study, addition of excess poly-D-lysine to cells that were previously sensitized with the optimal poly-D-lysine concentration demonstrated a dramatic apparent desensitization to SDS-induced lysis. Possibly the cells did in fact lyse, but the highly cationic poly-D-lysine may have precipitated the radiolabeled cytoplasmic contents despite the presence of SDS.

Significance of iron protective action against bactericidal cationic agents. We are not aware of any studies reporting the concentrations of free iron in either saliva or plaque fluid, but it seems highly unlikely that iron would be available in concentrations high enough to interfere with salivary defensive factors such as lysozyme and other cationic proteins. Most biological fluids, including plasma (43) and saliva (6), contain specific iron-binding proteins which reduce the concentrations of free iron to vanishingly small values. For example, the concentration of free iron in plasma has been estimated to be less than 10⁻¹⁴ M (43). Similarly, phagocytic leukocyte granules, in addition to lysozyme and other cationic proteins, contain the iron-binding protein lactoferrin (1, 23). However, Gladstone (14) found iron-saturated lactoferrin to be more effective than free iron in blocking the bactericidal action of rabbit polymorphonuclear leukocyte cationic proteins. Further studies will be required to assess the potential of salivary iron complexes to interfere with the bactericidal action of lysozyme and other cationic peptides in situ.

Finally, the observations reported here serve to caution that micromolar quantities of iron can markedly affect the bactericidal potency of some cationic proteins and polypeptides in the laboratory. Thus, investigations of such bacterial agents should take into account the modulating effects of contaminating metals such as iron in addition to the more widely recognized divalent cations Ca and Mg (40).

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