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Bactericidal Activity of Human Lysozyme, Muramidase-Inactive Lysozyme, and Cationic Polypeptides against *Streptococcus sanguis* and *Streptococcus faecalis*: Inhibition by Chitin Oligosaccharides

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The basis of the bactericidal activity of human lysozyme against *Streptococcus sanguis* was studied. Experiments were designed to evaluate the role of lysozyme muramidase activity in its bactericidal potency. Inactivation of the muramidase activity of lysozyme was achieved by reduction of essential disulfides with dithiothreitol (DTT) or by incubation with the chitin oligosaccharides chitotriose and chitobiose. Muramidase-inactive lysozyme, prepared by reduction with DTT, was equal in bactericidal potency to native lysozyme. Solutions of native chicken egg white lysozyme and human lysozyme exhibited equal bactericidal potency yet differed ca. fourfold with respect to lytic (muramidase) activity. The above results suggested that the bactericidal activity of lysozyme is not dependent upon muramidase activity. Chitotriose and chitobiose were found to inhibit both lytic and bactericidal activities of lysozyme. The bactericidal activity of muramidase-inactive lysozyme (reduction with DTT) was also inhibited by chitotriose and chitobiose. Further investigations demonstrated that chitotriose and chitobiose were also potent inhibitors of the bactericidal activity of the cationic homopolypeptides poly-L-arginine and poly-D-lysine. These latter results suggested that the essential bactericidal property of lysozyme was its extreme cationic nature and that some bacterial endogenous activities, inhibitable by chitotriose and chitobiose, were essential for expression of the bactericidal activity of either native or muramidase-inactive lysozyme or of the cationic homopolypeptides. Experiments with *Streptococcus faecalis* whole cells, cell walls, and crude autolysin preparations implicated endogenous autolytic muramidases as the bacterial targets of chitotriose and chitobiose. The essentially identical responses of *S. sanguis* and *S. faecalis* to chitotriose in bactericidal assays with muramidase-inactive lysozyme and polylysine suggested that muramidase-like enzymes exist in *S. sanguis* and, furthermore, play an essential role in cationic protein-induced loss of viability of the oral microbe.

Saliva is known to have a profound influence on the microbial composition of the human oral flora and on the susceptibility to development of caries and oral mucosal infections (4, 6, 13, 34, 35, 37). Salivary secretions contain several substances with potential antibacterial activity. Principal antibacterial constituents of saliva include immunoglobulins, lactoperoxidase, lactoferrin, lysozyme, and several cationic proteins (4, 36, 37). With the exception of immunoglobulins, the antibacterial armamentarium of saliva is very similar to that of polymorphonuclear leukocytes (51).

Our principal objective was to determine the role of lysozyme in modulation of the composition of the oral flora. Lysozyme is an especially interesting member of the antibacterial system of saliva. The most widely recognized attribute of lysozyme is its bacteriolytic activity. Other lesser known activities of lysozyme, however, may harbor significant potential to affect the oral microflora. For example, lysozyme aggregates oral bacteria (3, 25, 40). Bacterial aggregation may reduce the efficiency of adherence to oral surfaces and promote clearance of microbes from the oral cavity (31, 39). Lysozyme is present in acquired enamel pellicle and plaque (9, 28), and adherence of some streptococci to saliva-coated hydroxyapatite is markedly reduced when lysozyme is present in the saliva pellicles (L. M. Tellefson, and G. R. Germaine, J. Dent. Res. 63:188, 1984). Thus, lysozyme may have profound effects on adherence of some bacteria to oral surfaces. Lysozyme, with a pI > 10.5, is also a rather extreme example of a cationic protein. Cationic proteins are known bactericidal agents against both gram-positive and gram-negative organisms (15, 17, 38, 41, 49, 50). Two modes of bactericidal action are thus available to lysozyme: a muramidase-dependent mode and a cationic-dependent mode. The oral streptococci are typically resistant to direct lysis by either human or chicken lysozyme (3, 25, 40). Lysozyme is, however, bactericidal for the major species of oral streptococci (3, 22, 25, 40; N. J. Laible, Ph.D. dissertation, University of Minnesota, Minneapolis, 1984). Although oral streptococci are not directly lysed by lysozyme, they are damaged by exposure to lysozyme such that subsequent addition of salt or detergent results in immediate lysis (3, 25, 40). In the absence of salt or detergent, lysozyme-treated *Streptococcus mutans* exhibits areas of cell wall dissolution (7). Lysozyme-dependent sensitization of *S. mutans* to salt-induced lysis also occurred when the muramidase activity of lysozyme was inactivated by an active-site inhibitor (40). This observation, in addition to earlier work with *Staphylococcus aureus* that employed lysozyme and several nonmuramidase cationic proteins, suggested that autolysins were involved in lysis of bacteria by lysozyme and that lysis by lysozyme may actually depend more on its cationic nature than on its muramidase activity (29, 44, 55).

In previous studies, we showed that adsorption of lysozyme from human saliva by oral streptococci and acti-

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nomyces exhibited a wide (ca. eightfold) range within and across species but a narrow range within serotypes or biotypes of species (30). Adsorption of lysozyme by *Streptococcus sanguis* 903 was independent of pH (range, 3.9 to 8), temperature (0 and 37°C) at low ionic strength, and specific salts (NaCl, $CaCl_2$, K_2HPO_4-KH_2PO_4) but was dependent on ionic strength. In contrast, bactericidal activity of lysozyme against *S. sanguis* 903 was temperature dependent (did not occur at 0°C), specific-salt dependent (particularly sensitive to Ca^{2+}), and ionic-strength dependent (N. J. Laible and G. R. Germaine, Abstr. Annu. Meet. Am. Soc. Microbiol, 1983, B42, p. 30; Laible, Ph.D. dissertation). The temperature dependence of bactericidal activity in light of the temperature independence of lysozyme adsorption suggested that some enzymatic process(es) was essential for bactericidal activity. We therefore sought to elucidate the role of the muramidase activity of lysozyme in its bactericidal action against the oral streptococci. In this paper, we report evidence that the bactericidal action of lysozyme is independent of its muramidase activity and requires the action of some bacterial endogenous muramidase-like activity that is inhibited by the chitin oligosaccharides chitotriose and chitobiose.

MATERIALS AND METHODS

Bacteria. Organisms used included *S. sanguis* 903 and B4 (from B. Rosan, University of Pennsylvania, Philadelphia), *S. mutans* BHT (laboratory stock), and *Streptococcus faecalis* 9790 and its autolysis-deficient mutant lyt-14 (G. D. Shockman, Temple University, Philadelphia). Culture stocks were maintained at −70°C in 10% skim milk broth. Exponential-phase cells were harvested from 3% Trypticase soy (BBL Microbiology Systems, Cockeysville, Md.)–0.5% yeast extract broth containing 0.25% glucose (TSYG) by centrifugation (6,000 × *g* for 10 min at 4°C) and washed twice with 1 volume each time of 10 mM phosphate buffer containing 1 mM $MgCl_2$, pH 7. Washed bacteria were suspended to an A_{540} of 1.0 and held in ice until needed (less than 30 min). Bacteria radiolabeled in their DNA were harvested as above in the exponential phase after at least four generations of growth in TSYG supplemented with 10 μCi of [*methyl*-^{3}H]thymidine (25 Ci/mmol; RPI Corp., Elk Grove Village, Ill.) per ml.

Lysozyme. Human placental lysozyme (HPL) (2× crystallized; Alpha Therapeutics, Los Angeles, Calif.) and chicken egg white lysozyme (HEWL) (3× crystallized; Calbiochem-Behring, La Jolla, Calif.) were used in these studies. HPL and HEWL were shown to be homogenous preparations by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Lysozyme lytic activity was estimated by a spectrophotometric method in 10 mM potassium phosphate buffer containing 0.9% NaCl, pH 7 (30). Dried *Micrococcus luteus* (Sigma Chemical Co., St. Louis, Mo.) suspended to an A_{540} of 0.35 served as substrate. Rates of absorbance decrease over the first minute (or in some cases over several minutes) of the reaction were determined. Occasionally, lytic activity was assessed by determination of lysis after 30 min of incubation. One unit of lytic activity decreased the absorbance 0.001 units per min at 37°C. Inactivation of HPL muramidase activity was accomplished by reduction with dithiothreitol (DTT). Thus, 100 μg of HPL per ml was reduced with 10 mM DTT in 0.5 mM potassium phosphate buffer (pH 7) at 37°C. Samples of the reduction mixture were periodically assayed for lytic activity (see above) to monitor the course of the inactivation (see Fig. 2). With the exception of the data in Fig. 2, HPL was treated with DTT for at least 18 h at 37°C. Inactivated HPL was tested for lytic activity by incubation of 12.5 μg of the preparation with *M. luteus*. In all cases of long-term reduction, no lysis of *M. luteus* was observed after 2 h of incubation at 37°C.

Bactericidal activity of lysozyme, PDL, and polyarginine. Washed stock suspensions of bacteria were diluted 1/100 into prewarmed (37°C) 0.5 mM potassium phosphate (pH 7.0) containing any desired supplements (e.g., lysozyme, poly-D-lysine [PDL], poly-L-arginine). Inhibition of HPL with chitotriose (Sigma; >99%), chitobiose (Calbiochem), or *N*-acetyl-D-glucosamine (Sigma; crystalline) included a 5-min incubation of inhibitor and HPL before addition of bacteria. In all cases, 10 μg of arginine per ml was also included in reaction mixtures. Arginine was found to prevent loss of viability of controls (no added bactericidal agents) over the course of incubation but had no effect on the action of bactericidal agents. Survival of *S. sanguis* in control samples without arginine was 17.5 ± 18.3% (n = 7). In contrast, survival in the presence of arginine was 94.9 ± 29.4% (n = 20). Comparison of survival of the *S. faecalis* strains with and without arginine was not done. Arginine was included in all viability experiments with *S. faecalis*. Control survival of *S. faecalis* strains 9790 and lyt-14 was 85.8 ± 23.0% (n = 6) and 108 ± 2.9% (n = 4), respectively. At both zero time and 60 min, samples were diluted (0.5% yeast extract), and viability determined in duplicate by enumeration on TSYG pour plates (1% agar).

Autolysis of *S. faecalis* and *S. sanguis*. Autolysis of whole cells was monitored at 37°C by either absorbance decrease of turbid cell suspensions (A_{540} ~ 0.7) or by release of [^{3}H]DNA from nonturbid suspensions (A_{540} ~ 0.007) of radiolabeled bacteria. Release of [^{3}H]DNA was estimated by centrifugation of samples (5 min at 4°C in a Beckman Microfuge B) and assay of the supernatant solution for cell-free radioactivity. In cases in which cationic agents were used to induce autolysis, polyglutamic acid was added (100 μg/ml) to each sample just before centrifugation to reduce trapping of released DNA in the pellet fraction. Several control experiments verified that this simple assay method accurately reflected whole-cell autolysis. For example, (i) dual assay of turbid suspensions by both the absorbance and the [^{3}H]DNA-release methods gave identical autolysis results; (ii) conditions (e.g., autolysis of *S. faecalis* 9790 and treatment of strains 9790 and lyt-14 with trypsin, PDL, HPL, and DTT-treated HPL) which promoted lysis of turbid suspensions also promoted [^{3}H]DNA release by nonturbid suspensions; (iii) conditions (e.g., autolysis of *S. faecalis* lyt-14 and *S. sanguis* 903; treatment of *S. faecalis* with excess HPL and PDL) which yielded no lysis of turbid suspensions failed also to result in [^{3}H]DNA release; and (iv) inclusion of polyglutamic acid increased [^{3}H]DNA release into the supernatant by about twofold (1.9 ± 0.8) when cationic agents, especially PDL, were present but had no effect in their absence.

Cell walls. Exponential-phase cultures of *S. faecalis* 9790 and *S. sanguis* 903 were harvested by centrifugation and washed three times in ice-cold water, and the cells were lyophilized. Cells (ca. 300 mg) were disrupted by being shaken with glass beads in a cell mill (Vibrogen; Edmond Buhler, Tubingen, Federal Republic of Germany) at 0°C. Cell walls were recovered by differential centrifugation and then washed four times with ice-cold water and lyophilized. Autolysins associated with *S. faecalis* 9790 cell walls were inactived with SDS (Eastman Kodak Co., Rochester, N.Y.)

(47). Cell walls (2 mg/ml) were stirred with 2% SDS at 4°C for 1 h and recovered by centrifugation, and the SDS treatment was repeated. Walls were then washed four times with ice-cold water, suspended in 20 mM phosphate buffer (pH 7), and stored at −20°C.

Crude autolysin. *S. faecalis* 9790 cell walls (700 μg/ml) in 20 mM phosphate buffer (pH 7) were activated with 50 ng of trypsin (Sigma) per ml at 37°C. After 2 to 3 h, the original absorbance of the wall suspension had declined by more than 90%. The autolysate was chilled and centrifuged (25,000 × *g* for 15 min), and the supernatant solution (crude autolysin preparation) was stored in ice until needed (10).

RESULTS

Requirement for muramidase activity. The role of muramidase in the bactericidal activity of HPL against *S. sanguis* was examined by inhibition of the enzymatic activity with chitotriose. Chitotriose is a well-known active-site inhibitor of muramidases (5, 12, 14, 42). HPL preincubated with increasing concentrations of chitotriose (0 to 15 mM) lost activity as judged by lysis of *M. luteus* whole cells (Fig. 1b). Up to 1 M *N*-acetyl-D-glucosamine had no effect on lytic activity. Examination of the bactericidal activity of chitotriose-inhibited HPL solutions clearly indicated that loss of bactericidal activity (Fig. 1a) was associated with loss of muramidase activity (Fig. 1b). Chitotriose inhibition of the bactericidal action of HPL has also been observed with *S. sanguis* B4 and *S. mutans* BHT (Laible, Ph.D. dissertation). Thus, muramidase activity seemed essential for HPL bactericidal activity.

Additional evidence for the essential involvement of muramidase in the bactericidal action of HPL was sought. In this case, muramidase activity was destroyed by reduction of

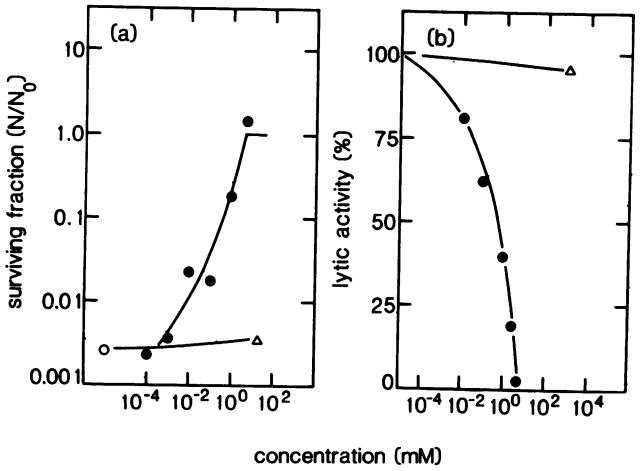

FIG. 1. Effect of chitotriose on HPL lytic and bactericidal activity. (a) Bactericidal activity of HPL preincubated with the indicated concentrations of chitotriose (●), *N*-acetyl-D-glucosamine (△), or no additions (○). HPL at 10 μg/ml in 0.5 mM potassium phosphate (pH 7) containing 10 μg of arginine per ml was preincubated for 5 min at 37°C. *S. sanguis* 903 was then added to 7 × 10^6 CFU/ml, and survivors were determined after 60 min at 37°C. Control survival (no chitotriose) over 60 min was 78%. (b) Lytic activity of HPL preincubated with the indicated concentrations of chitotriose (●) or *N*-acetyl-D-glucosamine (△). HPL at 5 μg/ml in 0.5 mM potassium phosphate (pH 7) was preincubated as in panel a followed by addition of *M. luteus* and determination of bacteriolysis. Absorbance decrease in the absence of inhibitor was 0.195 over 30 min.

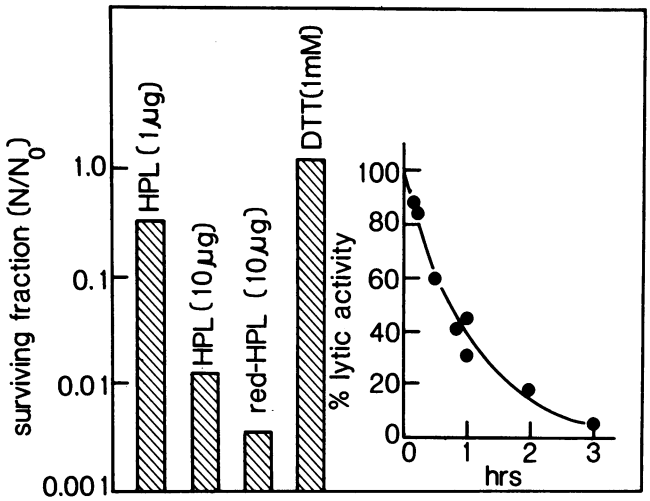

FIG. 2. Bactericidal activity of reduced HPL. Bactericidal activity of native HPL and reduced HPL (red-HPL). *S. sanguis* 903 at 1.5 × 10^7 CFU/ml was incubated for 60 min at 37°C with the indicated concentrations of HPL or red-HPL followed by determination of survivors. Control survival (no treatments) over 60 min was 93%. Inset: DTT reduction of HPL was accomplished by incubation of HPL (100 μg/ml) in 0.5 mM potassium phosphate (pH 7) with 10 mM DTT for the indicated times. *M. luteus* was then added to diluted samples of reduced HPL, and the rates of lysis were determined.

essential disulfide bonds with DTT. Inactivation of muramidase activity by DTT and subsequent bactericidal potency is shown in Fig. 2. The inset shows the loss of lytic activity over time as judged with the *M. luteus* lysis assay. After 3 h, 96.5% of the initial activity was lost. The bactericidal potency of DTT-inactivated HPL (red-HPL) and active HPL were comparable (99.6 and 98.8% of the population killed, respectively). In an analysis of several experiments, we found a mean (± standard deviation) survival of 4.05 ± 8.18% (n = 18) and 0.79 ± 0.59% (n = 2) after exposure of the organism to either 10 μg of HPL or 10 μg of red-HPL per ml, respectively. The red-HPL preparation contained 0.35 μg of active enzyme per ml in the bactericidal assay. The bactericidal potency of 1 μg of active HPL per ml is also shown in Fig. 2. A comparison of the bactericidal activity of 1 μg of active HPL per ml with that of 10 μg of red-HPL per ml indicated that the active HPL (0.35 μg/ml) remaining in the red-HPL preparation cannot account for the bactericidal activity of the red-HPL. Finally, since 1 mM DTT is carried over from the muramidase inactivation step to the bactericidal assay step, a DTT control was also included. DTT was without effect. Thus, muramidase activity appeared nonessential for HPL bactericidal activity. This result is in direct conflict with that of the previous experiment (Fig. 1). Therefore, another way of assessing the role of muramidase activity was sought.

Human lysozyme and HEWL are similar in molecular weight yet differ up to fourfold in specific activity (24, 54, 56). These facts suggested the basis for yet another means of asking whether muramidase activity was an essential bactericidal attribute of lysozyme with the oral streptococci. If muramidase activity was the major determinant of bactericidal potency, solutions of HPL and HEWL equal in muramidase activity should exhibit equivalent bactericidal activity. If, on the other hand, bactericidal activity is independent of muramidase activity and thus perhaps dependent on the cationic property of each lysozyme, solutions of HPL and HEWL equal in protein concentration should exhibit equal

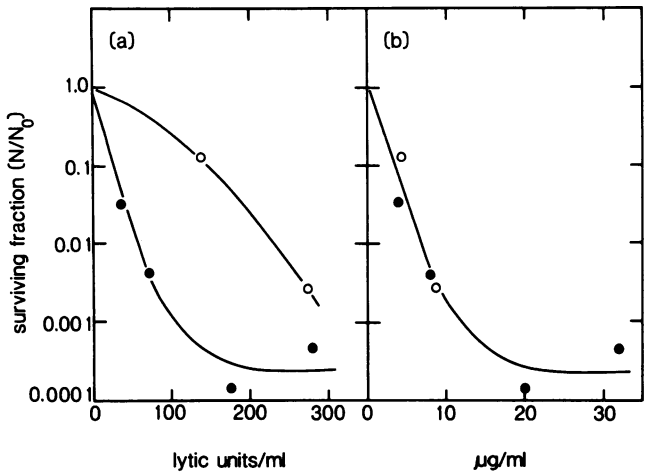

FIG. 3. Comparison of the bactericidal activity of HPL and HEWL. Solutions of HPL (●) and HEWL (○) in 0.5 mM potassium phosphate (pH 7) containing 10 μg of arginine per ml were incubated for 60 min at 37°C with *S. sanguis* 903 present at 1.2×10^7 CFU/ml, followed by determination of survivors. HPL and HEWL concentrations are expressed in terms of lytic units (a) or protein (b). Specific activities of HPL and HEWL were 27.5 and 7.0 U/μg, respectively.

TABLE 1. Chitotriose protection of *S. sanguis* and *S. faecalis* from the bactericidal activity of HPL, red-HPL, and cationic polypeptides[a]

Organism[b]	Cationic agent (μg/ml)	% Survivors[c]	
		−CT	+CT[d]
S. sanguis 903	HPL (10)	<1.0	92
	Red-HPL (10)	1.2	120
	PDL (10)	<0.01	157
	Polyarginine (10)	<0.01	115
S. faecalis 9790	HPL (10)	0.54	21[e]
	Red-HPL (10)	32	65
	Red-HPL (20)	0.7	70
	PDL (1)	0.6	137
	PDL (2)	0.04	11
	PDL (5)	0.0002	4.6
S. faecalis lyt-14	Red-HPL (10)	6.6	78
	PDL (1)	11	41
	PDL (2)	0.17	6.6
	PDL (5)	0.024	0.27

[a] Data from eight experiments.
[b] 10^7/ml.
[c] After 60 min of incubation. CT, Chitotriose.
[d] 5 mM.
[e] 2.5 mM chitotriose.

bactericidal potency. The results of such a comparison are shown in Fig. 3. Only when solutions of equal protein concentration (and thus unequal muramidase activity) were compared was equivalent bactericidal potency observed. The data suggest that the bactericidal potency of lysozyme is independent of muramidase activity.

To summarize, two experiments (Fig. 2 and 3) suggest that muramidase is nonessential for killing of *S. sanguis*. One experiment (Fig. 1), which utilized chitotriose to inhibit muramidase activity, suggested that enzymatic activity was essential for bactericidal activity.

Model for muramidase-independent bactericidal action of lysozyme. These seemingly contradictory conclusions can be resolved if one assumes that (i) chitotriose targets include lysozyme and some cell-associated activity that is required for loss of bacterial viability and that (ii) the essential bactericidal property of lysozyme is its extreme cationic character. The aggregate results of Fig. 1, 2, and 3 could then be reinterpreted to suggest that muramidase-inactive lysozyme was bactericidal by virtue of its cationic nature. Furthermore, the chitotriose inhibition of the bactericidal activity of HPL seen in Fig. 1 would be based on its inhibition of some endogenous bacterial activity and not due to inhibition of the muramidase activity of HPL. If these assumptions are true, one must be able to show that (i) death is cationic protein dependent and that (ii) a bacterial target of chitotriose exists. The first prediction is supported by the data in Fig. 2 and 3 and can be further tested by using nonenzyme cationic polypeptides as bactericidal agents. The second prediction can be tested by determining whether chitotriose is also protective against cationic-polypeptide-induced bacterial death. In this latter case, the only possible chitotriose target is microbe associated since no muramidases are added.

Experiments to test the above predictions with *S. sanguis* are shown in Table 1. It is apparent that the nonenzyme cationic polypeptides PDL and polyarginine as well as inactivated HPL are potent bactericidal agents against *S. sanguis*. Furthermore, with *S. sanguis*, chitotriose afforded complete protection from the bactericidal cationic polypeptides and inactivated HPL. The results with *S. sanguis* thus support the notion that some endogenous bacterial chitotriose-sensitive mechanism is essential for loss of viability. Since chitotriose is a muramidase inhibitor, it seemed plausible that the bacterial target(s) of chitotriose might include autolysins with muramidase activity.

Muramidases exhibit decreasing sensitivity to the series chitotriose, chitobiose, *N*-acetyl-D-glucosamine (5, 12, 14, 42). If muramidase-like autolysins in *S. sanguis* are indeed

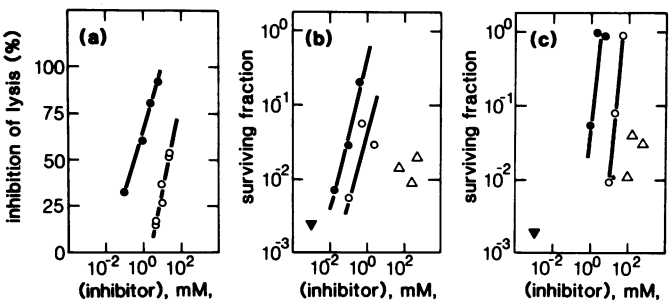

FIG. 4. Effect of chitotriose, chitobiose, and *N*-acetyl-D-glucosamine on HPL lytic activity and HPL and PDL bactericidal activity. (a) HPL at 5 μg/ml in 0.5 mM potassium phosphate (pH 7) was preincubated for 5 min at 37°C with the indicated concentrations of inhibitors followed by addition of *M. luteus* and determination of bacteriolysis after 30 min. (b) HPL at 10 μg/ml was preincubated with inhibitors as described in the legend to Fig. 1 followed by addition of *S. sanguis* 903 to 9×10^6 CFU/ml. After 60 min at 37°C, the number of survivors was determined. (c) *S. sanguis* 903 at 9×10^6 CFU/ml was preincubated with inhibitors in 0.5 mM potassium phosphate (pH 7) containing 10 μg of arginine per ml. Next, PDL was added to 1 μg/ml, and incubation continued for 60 min at 37°C. The number of survivors was then determined. Survival of the organism in the absence of any additions over 60 min was 93%. Control samples for panels b and c included bacteria incubated in buffer only (i.e., 100% survival) and in buffer with the bactericidal agent but no inhibitors (▼). Symbols: ●, chitotriose; ○, chitobiose; △, *N*-acetyl-D-glucosamine.

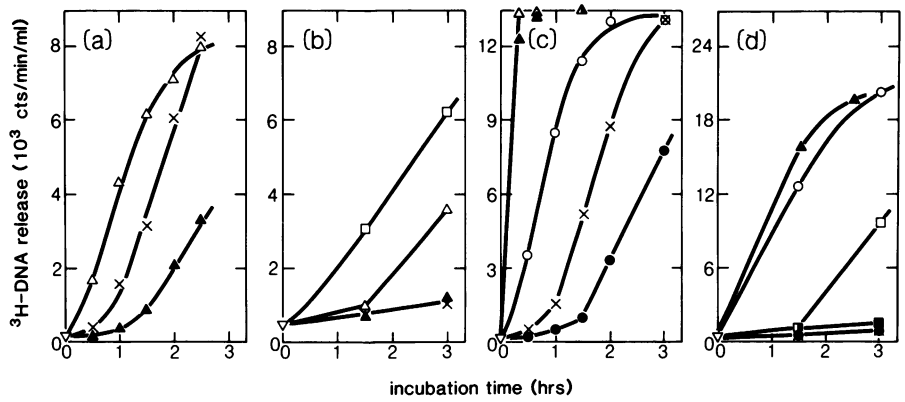

FIG. 5. Effect of chitotriose on whole-cell lysis of *S. faecalis* in the presence of reduced HPL or PDL. (a) *S. faecalis* 9790 labeled with [^{3}H]thymidine was diluted into prewarmed (37°C) 0.5 mM potassium phosphate (pH 7) containing no additions (×), 0.2 μg of PDL per ml (△), or 0.2 μg of PDL per ml and 5 mM chitotriose (▲). (b) *S. faecalis* lyt-14 labeled with [^{3}H]thymidine was incubated as in panel a with no additions (×), 0.2 (△) or 0.4 (□) μg of PDL per ml, and 0.2 μg of PDL per ml plus 5 mM chitotriose (▲). (c) *S. faecalis* 9790 was incubated as in panel a with no additions (×), HPL at 0.1 (▲) and 1.0 (△) μg/ml, 1 μg of DTT-reduced HPL per ml (○), and 1 μg of DTT-reduced HPL per ml plus 5 mM chitotriose (●). (d) *S. faecalis* lyt-14 was incubated as in panel b with no additions (×), HPL at 0.1 μg/ml (▲), 10 ng of trypsin per ml (□), 10 ng of trypsin per ml plus 5 mM chitotriose (■), 2 μg of DTT-reduced HPL per ml (○), and 2 μg of DTT-reduced HPL per ml plus 5 mM chitotriose (●). DTT reduction of HPL occurred over 18 h and resulted in less than 0.25% of the original lytic activity. The HPL data included in panel c are derived from another experiment in which the labeling efficiency of the cells was 25% greater than that of the cells used for the remaining data. Thus, to accomodate all data in this panel, the HPL data were normalized to the specific labeling efficiency of the control cells shown.

involved in loss of viability, one might anticipate that a similar order of effectiveness of the *N*-acetyl-D-glucosamine series would also be observed in protection from the bactericidal activity of cationic polypeptides. This appears to be true (Fig. 4). The effects of chitotriose, chitobiose, and *N*-acetyl-D-glucosamine in HPL lysis of *M. luteus* (Fig. 4a) and HPL (Fig. 4b) and PDL (Fig. 4c) bactericidal activity against *S. sanguis* indicate that chitotriose > chitobiose > *N*-acetyl-D-glucosamine in lysis inhibition and protection from loss of viability. Bactericidal protection therefore qualitatively mimics inhibition of muramidase activity. Emphasis was then focused on the possible role of bacterial autolysins in loss of viability.

Role of autolysins. Initial attempts to use *S. sanguis* 903 to investigate the role of autolysins in cationic-protein-induced death were disappointing. Autolysis of whole cells occurred only to about 25 to 30% over 2 h and did not change significantly with 19 h of additional incubation. Control cells incubated in ice exhibited no autolysis. Cell walls of the organism exhibited only 20% autolysis over 2 h. Autolysis of walls was not stimulated by trypsin (100 ng/ml) or PDL (10 μg/ml). In addition, attempts to observe autolysis of [^{3}H]thymidine-labeled *S. sanguis* 903 by [^{3}H]DNA release from cell suspensions containing $<10^7$ cells per ml also were disappointing. Thus, only 10% release of [^{3}H]DNA was observed, and neither trypsin (100 ng/ml) nor PDL (100 to 400 ng/ml) stimulated autolysis.

Since we wanted to further investigate the mechanism of chitotriose protection and its possible involvement with autolytic phenomena, we included *S. faecalis* in our studies. *S. faecalis* has a well-described autolytic system whose principal component is a muramidase (2, 26, 27). Initially, we sought evidence that *S. faecalis* responded to cationic polypeptides and muramidase-inactive HPL in a manner similar to that of *S. sanguis*. A wild type (9790) and an autolysis-defective strain (lyt-14) (2, 11) exhibited sensitivity to the cationic agents and were also protected by chitotriose (Table 1). The wild-type strain (9790) exhibited sensitivity to HPL. Chitotriose increased survival 40-fold. The bactericidal potency of red-HPL was weak compared with that of HPL, and chitotriose further reduced its activity. For 20 μg of red-HPL per ml, chitotriose increased survival 100-fold. The cationic polypeptide PDL also was a potent bactericidal agent. Chitotriose increased survival 230- to 23,000-fold. Similar results were obtained with the autolysis-defective strain (lyt-14). Strain lyt-14 was not tested with HPL.

Since *S. faecalis* was qualitatively similar to *S. sanguis* in response to cationic polypeptides and chitotriose, its amenable autolytic system was used to further elucidate the mechanism of chitotriose protection. If autolysins are involved in cationic-polypeptide-induced death and are also the target of chitotriose, one should be able to demonstrate (i) whole-cell autolysis induced by muramidase-inactive HPL and by PDL and (ii) inhibition of autolysis by chitotriose. The summary of results of such experiments with strains 9790 and lyt-14 are shown in Fig. 5. The effect of PDL is shown for strains 9790 and lyt-14 in Fig. 5a and b. Control autolysis (i.e., no additions) of strain 9790 commenced after a 15-min lag and proceeded linearly over the next 2.5 h. In the presence of 200 ng of PDL per ml, the lag in onset of autolysis was abolished but the rate of lysis was similar to that of the control. Inclusion of 5 mM chitotriose with 200 ng of PDL per ml resulted in an increase in the lag to ca. 60 min followed by a reduced rate (54% of control) of lysis. The effect of PDL on autolysis is more dramatic in the autolysis-defective strain because this strain has an extremely slow control rate of autolysis (Fig. 5b). Lags in onset of control autolysis of strain lyt-14 typically exceed 60 min. Incubation of the organism with PDL markedly stimulated autolysis. Note that with 400 ng of PDL per ml, no lag in onset of autolysis was apparent. Inclusion of 5 mM chitotriose with 200 ng of PDL per ml resulted in reduction of autolysis to the control rate. The effects of HPL and red-HPL on autolysis of strains 9790 and lyt-14 are shown in Fig. 5c and d, respectively. Active HPL was particularly potent in causing lysis of strain 9790. Note that as little as 100 ng of HPL per ml caused total lysis in 20 min. Incubation of the organism with 1 μg of red-HPL per ml stimulated autolysis as evidenced by the absence of a lag period and a 36% increase in lysis rate compared with that of control autolysis. Inclusion of 5 mM

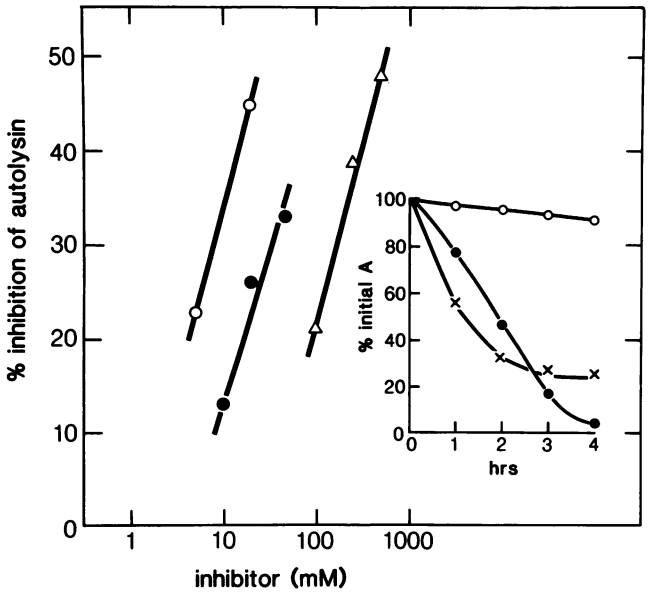

FIG. 6. Inhibition of *S. faecalis* crude autolysin by chitotriose, chitobiose, and *N*-acetyl-D-glucosamine. Crude autolysin (90%, vol/vol) obtained from autolyzed cell walls was incubated with autologous SDS-treated cell walls as substrate (see the text). Inhibitors were present at the indicated concentrations: chitotriose (○), chitobiose (●), and *N*-acetyl-D-glucosamine (△). Each data point represents a rate of cell wall lysis. Inset: Controls demonstrating (i) lack of autolysis of substrate SDS-treated cell walls (○) and (ii) susceptibility of SDS-treated cell walls to lysis by crude autolysin (●) or 5 μg of HPL per ml (×). Rates of lysis were 0.016 and 0.158 absorbance units per h for control autolysis and autolysin-mediated lysis, respectively.

chitotriose with red-HPL resulted in a prolonged lag period (80 to 90 min) and subsequent reduced rate of lysis (47% of the rate with red-HPL alone). Lysis of strain lyt-14 (Fig. 5d) with 100 ng of HPL per ml was less rapid than with the wild-type strain (Fig. 5c). Autolysis of cells of strain lyt-14 incubated with 2 μg of red-HPL per ml commenced with essentially no lag period and was reduced to the control autolysis response when 5 mM chitotriose was also present. Stimulation of autolysis of strain lyt-14 with 10 ng of trypsin per ml is also shown. Note that trypsin activation of autolysis was also inhibited by 5 mM chitotriose. In summary, whole-cell autolysis of either strain of *S. faecalis* was stimulated by the cationic agents, and such stimulation was inhibited by chitotriose. Therefore, whole-cell autolysis is indeed stimulated by the cationic agents and is inhibited by chitotriose.

Finally, evidence that *S. faecalis* autolysins were directly inhibited by chitotriose was sought. Crude autolysin prepared from *S. faecalis* 9790 cell walls was assayed with SDS-treated 9790 cell walls as substrate. The substrate walls were without active autolysins and susceptible to degradation by crude autolysin and by HPL (Fig. 6, inset). Next, crude autolysin was incubated with chitotriose, chitobiose, and *N*-acetyl-D-glucosamine together with SDS-treated walls, and the rates of wall lysis were monitored. The results (Fig. 6) clearly indicate that the crude autolysin preparation was sensitive to chitotriose, chitobiose, and *N*-acetyl-D-glucosamine and that the order of effectiveness of the inhibitors was the same as that observed earlier with HPL lysis of *M. luteus* and with the bactericidal activity of HPL and PDL against *S. sanguis*.

DISCUSSION

The experiments reported here demonstrated that the principal bactericidal action of HPL against *S. sanguis* and *S. faecalis* was independent of its muramidase activity. Similarities in the bactericidal activity of PDL and muramidase-inactive HPL supported the notion that the essential bactericidal feature of lysozyme was its cationic property. PDL and lysozyme shared a common bactericidal mechanism as evidenced by the inhibitory effects of chitotriose and chitobiose with each bactericidal agent. Inhibition of PDL-induced death by the chitin oligosaccharides clearly demonstrated the existence of some microbe-associated target of chitotriose and chitobiose and its essential involvement in loss of bacterial viability. The experiments with *S. faecalis* whole cells, cell walls, and crude autolysin preparations implicated endogenous muramidases as the bacterial target of chitotriose. Because of the similarity in bactericidal response of *S. sanguis* and *S. faecalis* to the cationic agents and the chitin oligosaccharides, it seems likely that similar chitotriose targets exist in the organisms. We suggest, therefore, that a chitotriose-inhibitable autolysin(s) plays an essential role in expression of the bactericidal activity of lysozyme, muramidase-inactive lysozyme, PDL, and polyarginine.

S. faecalis possesses perhaps two autolytic muramidases that are the principal enzymes responsible for autolysis of whole cells or cell walls (2, 11, 26, 47). Inhibition of muramidase activity by chitin oligosaccharides is characteristic of lysozymes (5, 12, 14, 42) and, based on our work, apparently a property also of *S. faecalis* autolytic muramidases. Chitotriose inhibition of the bactericidal outcome of incubation of *S. faecalis* with muramidase-inactive lysozyme or cationic polypeptides provides strong evidence for the essential role of autolytic muramidases in loss of cell viability in the enterococcus. In support of this notion is the observation that lysozyme sensitivity and autolytic capacity are positively related in *S. faecalis* (48). Indeed, the data in Table 1 indicate an apparent decreased sensitivity of the autolysis-deficient strain, lyt-14, to cationic agents compared with that of the wild-type strain. This latter observation is also consistent with the view that autolytic enzymes are the primary determinant of sensitivity of *S. faecalis* to bactericidal cationic agents. The essentially identical responses of *S. sanguis* and *S. faecalis* to chitotriose in bactericidal assays with lysozyme, muramidase-inactive lysozyme, and cationic polypeptides support the speculation that muramidase-like enzymes exist in oral microbes and, furthermore, also play an essential role in cationic-protein-induced loss of viability. As noted above, *S. mutans* BHT is also protected by chitotriose from the bactericidal effects of lysozyme. Thus, it seems plausible that the chitotriose phenomenon may be common in the oral streptococci.

Few oral streptococci have been shown to exhibit efficient or extensive autolysis, and no autolysins have been isolated from these organisms (32, 33, 52). We also were unable to observe extensive autolysis of *S. sanguis*. As noted earlier, whole cells or cell walls of the organism exhibited only 20 to 30% lysis in the first few hours of incubation. Further lysis was not observed with prolonged incubation. These results are in apparent conflict with those of another report (52). In this study, 65% lysis of *S. sanguis* 903 (also known as *Streptococcus mitis* 903) was reported over 20 h of incubation. Note, however, that these authors used 1 M phosphate buffer as the suspending medium. In our studies, we used low-ionic-strength buffer to favor association of the cationic

substances with the test bacteria. We have previously shown that the interaction between oral streptococci and small quantities of lysozyme is very sensitive to ionic strength (30). We suggest that our failure to observe the extent of autolysis of *S. sanguis* cited above is most likely due to the marked difference in suspending buffer concentration used in each study.

The involvement of autolysins in the bactericidal action of lysozyme and other cationic proteins against gram-positive and gram-negative bacteria has been suggested several times (21, 29, 40, 44, 53, 55). The binding of lysozyme and other cationic proteins to bacterial surfaces may be mediated, in part, by surface molecules that participate in the regulation of autolytic enzymes. For the oral streptococci, we suggested that lysozyme binding to cells relied on electrostatic interactions (30). Foremost among the potential candidates of surface-associated molecules that could bind lysozyme and also regulate autolysins are the lipoteichoic acids (LTA). The polyanionic LTA molecules have been shown to directly affect autolysin activity and to influence autolysin activation in gram-positive bacteria (8, 16, 18, 19). Lysozyme has also been shown to promote release of LTA from staphylococci (43) and to bind to streptococcal cell-free LTA (23). It has been suggested that in gram-positive bacteria, loss of LTA or its biochemical modification results in activation of cell wall-associated autolysins and may lead to bacterial death or lysis or both (20, 45, 46). Activation of bacterial autolysins by cationic proteins thus may occur via loss or modification of LTA and teichoic acids. In the latter case, modification of LTA may result from effects of cationic proteins on enzymes responsible for LTA modification.

Autolysin involvement in the bactericidal action of cationic proteins is not unexpected in cases in which death is accompanied by lysis. The apparent requirement for autolysins in cases of death without lysis, as exhibited by the oral streptococci, is less readily understood. The essential role of some chitotriose-sensitive autolysin in cationic-protein-induced death without lysis may be direct or indirect. A direct role implies that the actual death event results from the activity of the essential autolysin without the requirement of any other intervening activities. An indirect role might be manifest as a temporal sequence of events in which autolysin involvement is early in the sequence but does not represent the actual death event. The identification of the actual mechanisms involved in the phenomenon and elucidation of the specific role of chitotriose-sensitive endogenous activities requires further study.

Throughout our studies we noted that the efficiency of chitotriose inhibition of lysozyme or PDL bactericidal activity against *S. sanguis* and *S. faecalis* was generally related to the extent of bacterial death obtained in the absence of chitotriose. The efficiency of inhibition decreased as the proportion of survivors decreased. It was also apparent that chitotriose inhibition of whole-cell autolysis of *S. faecalis* was transient at the inhibitor concentrations used. Inhibition was manifest as a delay in onset of autolysis and also as a reduced rate of autolysis. In contrast, inhibition of crude autolysin activity by the chitin oligosaccharides exhibited no transient phenomena. The efficiency of chitotriose inhibition of the bactericidal effects of lysozyme and PDL depends on the time of sampling and rate of killing. These observations may reflect (i) some metabolism of chitotriose over time so that inhibition is relieved or (ii) additional bactericidal mechanisms which are not chitotriose sensitive (e.g., irreversible membrane damage), or both. Further investigations are required to explain these aspects of the inhibitory process.

As noted earlier, whole cells or cell walls of oral streptococci are not readily lysed by lysozyme. We were unable to demonstrate lysis of *S. sanguis* undergoing lysozyme or cationic-polypeptide-induced death. Therefore, the oral microbe exhibited death without lysis. In contrast, *S. faecalis* whole cells (Fig. 5) and cell walls (Fig. 6) were readily lysed by muramidase, and the organism did lyse under the conditions of the bactericidal experiments. Some evidence suggested that muramidase-active lysozyme operated both enzymatically and as a cationic protein on *S. faecalis* 9790. Lysozyme lysis of whole cells of *S. faecalis* 9790 was rapid and complete. In contrast, lysozyme did not fully degrade SDS-treated cell walls of this strain, in agreement with a previous report (1). The relative resistance of SDS-treated walls to lysozyme may be due to their lack of functional autolysins (10). Conversely, the efficient lysozyme lysis of whole cells may be attributable to the participation of autolysins. Additional evidence that lysozyme functioned as both a muramidase and a cationic protein with the enterococcus was provided by comparing lysozyme effects on *S. faecalis* 9790 and lyt-14. With identical lysozyme treatment, lysis of the autolysis-defective mutant was slower than lysis of strain 9790. lyt-14 has a normal quantity of total autolysin but a reduced proportion (2% of total) of active autolysins (2, 11). Because cell walls of lyt-14 are chemically indistinguishable from those of the parental strain, the slower lysozyme lysis of lyt-14 may be due to its reduced autolytic activity. The nonoral streptococcus, therefore, could succumb to lysozyme by at least two mechanisms: muramidase-dependent lysis and muramidase-independent, autolysin-dependent lysis. Under appropriate conditions death without lysis may also occur with *S. faecalis*, although we have not observed this phenomenon in our studies.

It is of interest to note that the potency of HPL and PDL differed. Comparable bactericidal activity or stimulation of autolysis required about 10 times more HPL than PDL on a weight basis and about 5 times more HPL on a molar basis. The net positive charge density of PDL per unit of molecular weight is about 12 times that of HPL. It seems plausible that charge density contributes to the difference in potency of PDL and HPL.

To our knowledge, our results represent the first demonstration of a nongenetic, direct, and specific method to manipulate the sensitivity of bacteria to the potent bactericidal cationic proteins and homopolypeptides. We anticipate that further studies with chitotriose and similar inhibitors will greatly aid in the understanding of the bactericidal mechanism(s) of host cationic proteins.

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