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Lysozyme-Mediated Aggregation and Lysis of the Periodontal Microorganism *Capnocytophaga gingivalis* 2010

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The ability of lysozyme to aggregate and lyse the gram-negative capnophilic periodontal microorganism *Capnocytophaga gingivalis* 2010 was monitored optically at 540 nm. Both hen egg white and chromatographically purified human lysozymes had significant but similar aggregation potentials for both logarithmic- and stationary-phase bacteria. In general, an increase in enzyme concentration resulted in a graded increase in both the initial and maximum changes in turbidity which occurred during the reaction period. The greatest change in turbidity occurred within the initial minutes of interaction of lysozyme and the cells, and the extent of aggregation paralleled a rapid depletion of lysozyme by the suspensions during the first minute of its incubation with the bacteria. Interestingly, the muramidase inhibitors *N*-acetyl-D-glucosamine and histamine did not block aggregation, whereas maleylation of lysozyme completely inhibited its aggregating ability. Demaleylation, however, restored aggregation activity comparable to the native enzyme, indicating that maleylated lysozyme retained its integrity and that aggregation was primarily dependent on charge. The addition of up to physiological concentrations of $NaHCO_3$ and NaCl to cell aggregates resulted in varying degrees of deaggregation and lysis. Surprisingly, ultrastructural analysis of lysozyme-treated cells revealed morphological changes with or without the addition of salt. Damage appeared to occur at the blunted polar end of the cells where there was a large spherical outpouching bordered by a damaged cell envelope. Damaged cells uniformly contained dense granular cytoplasmic debris. In effect, the cationic enzyme lysed *C. gingivalis* 2010, which was not apparent in the spectrophotometric assay. The paradoxical finding that during bacterial aggregation there was lysis may be of significance to the further elucidation of lysozyme's antibacterial role in the gingival sulcus.

The oral microflora has generally been regarded to have a role in the initiation and progression of periodontal diseases, and some bacteria have been implicated as being more important than others in the pathogenesis of individual diseases (59, 62). Although no cause-effect relationships have as yet been established, there do appear to be strong associations between certain bacterial groups and specific disease entities. One such group is the gram-negative, capnophilic bacillus *Capnocytophaga*. Species of *Capnocytophaga* have been isolated from granulocytopenic (15, 17) and immunocompromised (42) patients. They have been associated with advanced periodontitis in juvenile diabetics (43) and have been implicated in the development of periodontal disease in patients with Papillon-Lefevre syndrome (52). Moreover, these microorganisms have several features which would warrant their being considered periodontal pathogens. They have been shown to produce an immunosuppressive exopolysaccharide (5, 6), to adversely affect neutrophil function (58), and to produce a bone-resorbing lipopolysaccharide (31), and they have been implicated to have tissue-invasive capabilities (11). In addition, they have been shown to hydrolyze immunoglobulins A and G (33) and to produce a superoxide dismutase (25). Paradoxically, in spite of its apparent ability to cause periodontal tissue destruction, *Capnocytophaga* sp. has been routinely isolated from the dental plaque of apparently normal, healthy subjects (35). It is therefore probable that its virulence potential is well regulated by other plaque bacteria, environmental conditions, and host defense factors (18).

A host defense factor which may be of significance in the regulation of *Capnocytophaga* and other periodontal microorganisms is the cationic protein lysozyme. Lysozyme is a major secretory product of macrophages (13) and is found in both the primary and secondary granules of neutrophils (8, 36). Moreover, it is a crevicular fluid component whose concentration increases with the severity of periodontal inflammation (7) and whose fluid level is significantly elevated in individuals with localized juvenile periodontitis (periodontosis) (16). Lysozyme's antibacterial role has generally been attributed to its muramidase property in the hydrolysis of bacterial cell wall peptidoglycan (55). However, it has been suggested that lysozyme may participate in the regulation of the oral microflora through its ability to inhibit bacterial growth (28) and to mediate bacterial aggregation (29, 49). Furthermore, the molecule's cationic property is receiving great attention in the literature, in relation not only to a possible mechanism for bacterial aggregation, but also to lysozyme's ability to bind to bacterial cell membranes, activate autolytic enzymes, and effect lysis (21, 39, 49, 57, 63, 64, 67). In this regard, it has recently been shown that oral *Veillonella* species and the gram-negative periodontal pathogen *Actinobacillus actinomycetemcomitans* Y4 are lysed in the presence of physiological concentrations of lysozyme (3, 27, 63). We wished to determine if this lytic susceptibility was unique to these microorganisms or, perhaps, characteristic of other periodontal bacteria. The focus of this study was, therefore, the interaction of lysozyme with a human strain of *Capnocytophaga gingivalis*, a species isolated from the gingival crevice area (35). We report that both hen and human lysozymes have significant but similar aggregation potentials for *C. gingivalis* and that the micro-

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organism is lysed by lysozyme possibly through activation of autolysins.

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MATERIALS AND METHODS

Lysozyme sources and preparation. Human lysozyme (HuL) was obtained from the urine of chronic monocytic leukemic patients (47) and purified to homogeneity by immunoadsorption (40). The concentration of HuL in stock solutions was determined from an extinction coefficient, $E_{1\ cm}^{1\%}$, of 25.5 at 280 nm (10). Hen egg white lysozyme (HEWL; 3× crystallized) was obtained from the Sigma Chemical Co. (St. Louis, Mo.). HEWL concentration was determined spectrophotometrically at 280 nm, using an extinction coefficient, $E_{1\ cm}^{1\%}$, of 26.4 (60). Lysozyme hydrolytic activity was determined turbidimetrically by measuring the decrease in absorbance at 700 nm of a suspension of *Micrococcus lysodeikticus* cells, using either purified HuL or HEWL as a standard (10; B. J. MacKay, Ph.D thesis, State University of New York at Stony Brook, 1982).

Microbial strain and culture conditions. *C. gingivalis* 2010, originally isolated from a deep periodontal pocket, was kindly provided by S. Socransky (Forsyth Dental Center, Boston, Mass.) and maintained in the lyophilized state in the Stony Brook culture collection. Cultures were reconstituted and grown in a modified fluid thioglycolate broth containing 0.4% sodium bicarbonate, as previously described (27, 61). For turbidimetric assays of aggregation and lysis, cultures were grown to either mid-logarithmic phase (optical density of 0.5 at 700 nm) or stationary phase (optical density of 1.0 at 700 nm). Cells were harvested by centrifugation (4,810 × *g*, 30 min, 10°C), washed twice in 0.01 M Tris-hydrochloride (pH 7.4; Tris buffer), washed once in cold distilled water, and lyophilized. Freshly harvested cells, washed three times in Tris buffer, were used for ultrastructural analyses.

Spectrophotometric assay. The interaction of both HuL and HEWL with *C. gingivalis* 2010 was studied by a spectrophotometric procedure, essentially as described previously (29, 30). Briefly, assays consisted of the addition of 100 μl of Tris buffer or Tris buffer containing various amounts of lysozyme to microcuvettes containing bacterial cells in 900 μl of Tris buffer (optical density at 540 nm of the cell suspension, 0.520) at 37°C. Immediately upon addition of lysozyme, the suspensions were stirred for 1 min and the optical density was monitored at 540 nm for 120 min at 37°C. The cell suspensions were then stirred for 1 min and the optical density was determined for an additional 6 min. The resulting curves were termed either optical density-time curves or aggregation curves (30).

The change in optical density caused by lysozyme was calculated by subtraction of the optical density of the control suspension (cells plus Tris buffer) from the optical density of the lysozyme-containing suspensions at appropriate time points. These included (i) the initial point immediately after mixing the cells (1 min), (ii) the time at which the suspensions attained maximum optical density, and (iii) after resuspension of the cells at the end of the 120-min reaction period. Student's *t* test was used to evaluate the significance of the differences between changes in optical density caused by HEWL and HuL with both logarithmic- and stationary-phase cells.

To examine the effects of salts on lysozyme-treated cells, 100 μl of aqueous solutions of $NaHCO_3$ or NaCl was added to the suspensions (final salt concentration, 0.025 to 0.15 M) at the end of the aggregation assay. The cuvettes were then stirred for 1 min and incubated at 37°C for an additional 12 min, at which time they were again stirred, and the suspensions were examined for reduction in optical density. The percent reduction in optical density was calculated from the differences between the optical density of the lysozyme-treated suspensions with and without the addition of salt.

Lysozyme-binding assay. To determine if aggregation of *C. gingivalis* 2010 cells monitored during the spectrophotometric assay was a function of the amount of lysozyme adsorbed onto the cells, we used a modification of the lysozyme-binding assay described by Laible and Germaine (34). The amount of lysozyme bound to the cells was essentially determined to be a measure of the depletion of the enzyme from the supernatants of lysozyme-bacterial cell suspensions. Briefly, analyses consisted of the addition of 100 μl of Tris buffer containing as much as 250 μg of lysozyme to microtest tubes (Bio-Rad Laboratories, Rockville Centre, N.Y.) containing *C. gingivalis* 2010 cells suspended in 900 μl of Tris buffer at 37°C (optical density at 540 nm, approximately 0.520). Control tubes contained lysozyme without bacteria. Reaction tubes were stirred for 1 min and incubated at 37°C for 1, 5, 30, 60, and 120 min. Immediately after incubation, the tubes were centrifuged (12,000 × *g*, 10 min, 4°C) and replicate 50-μl samples were removed from the supernatants and assayed for lysozyme hydrolytic activity. The amount of lysozyme bound by the bacterial cells at each time point was determined from the differences in hydrolytic activity between control and test samples. Tris buffer and supernatants obtained from suspensions of *C. gingivalis* 2010 cells without added lysozyme had no detectable hydrolytic activity. Student's *t* test was applied for testing the significance of the differences between the amounts of HEWL and HuL bound by both logarithmic- and stationary-phase cells.

Inhibition of lysozyme. The interaction of lysozyme with bacterial cells may be dependent on either the molecule's cationic nature or its muramidase property or both. It was therefore important to determine which aspect of this host defense factor was critical in its interaction with *C. gingivalis* 2010. The muramidase inhibitors *N*-acetyl-D-glucosamine (Sigma Chemical Co.) and histamine (free base; Calbiochem-Behring, La Jolla, Calif.), which have been shown to competitively inhibit lysozyme hydrolytic activity on *M. lysodeikticus* cells (37, 56), were studied in the spectrophotometric assay. A wide range of concentrations (up to 150 mM) of these inhibitors was preincubated with 100 μg of HEWL for 30 min at 37°C in 0.02 M sodium acetate buffer (pH 4.5) as described by Glazer and co-workers (22). Enzymatically inactive enzyme was then used in the aggregation assay as described above. Differences in optical density change caused by inactive enzymes as compared with the native enzyme were calculated.

To determine the role of charge in the aggregation process, HEWL was maleylated to block positively charged free amino groups before reacting with *C. gingivalis* 2010. Maleylation was carried out in 0.2 M sodium borate (pH 9.0) at 4°C, as described previously (9), using a 20-fold molar excess of maleic anhydride over the total ε-amino group content of the lysozyme preparation. Completion of the reaction was determined spectrophotometrically from the relative extinction coefficients of the maleylamino group at 250 ($\varepsilon 250 = 3{,}360$) and 280 ($\varepsilon 280 = 308$) nm, respectively (9). The positive charges were restored by demaleylation, using 1% (vol/vol) pyridine–5% (vol/vol) acetic acid, pH 3.5, at 37°C for 24 h (9).

Cells from mid-logarithmic-phase cultures were used in all inhibition studies due to the potential for interference from extruded surface components, loosely fitting surface-associated appendages, and exocellular debris often found to a greater extent in older cultures (48).

Electron microscopy. Electron microscopy was used in conjunction with the spectrophotometric assay of aggregation and lysis to assess damage to the *C. gingivalis* 2010 cells. In these studies, freshly harvested and washed cells were suspended in 9 ml of Tris buffer (optical density at 540 nm, 0.520) in screw-cap tubes (17 by 10 mm) to which were added various amounts of HuL and HEWL in 1 ml of Tris buffer. Immediately upon addition of lysozyme, the tubes were vortexed for 1 min and the optical density at 540 nm was followed at 37°C essentially as described above for the spectrophotometric assay. Upon completion of the assay, glutaraldehyde (final concentration, 3%) was added, and the suspensions were incubated for 2 h at room temperature with end-over-end mixing on a blood tube rotator. At the end of the fixation period, the suspensions were centrifuged (27,000 × *g*, 1 h, 4°C) in thick-walled glass centrifuge tubes (15 ml), and the cell pellet was washed overnight in 0.5 M phosphate buffer (pH 6.2) containing 0.08 M potassium chloride–0.01 M magnesium acetate. The cells were then osmium postfixed, embedded, stained with uranyl acetate and lead citrate, and sectioned, essentially as described by Higgins and Shockman (26). All thin sections were examined and photographed in a Phillips 200 electron microscope (accelerating voltage, 60 kV).

RESULTS

Aggregation and lysis with HuL and HEWL. HEWL was tested for its ability to aggregate logarithmic-phase *C. gingivalis* 2010 by the spectrophotometric assay. The optical densities of cell suspensions for up to 121 min after addition of 100 μl of Tris buffer with 0, 10, 25, 50, and 100 μg of HEWL are shown in Fig. 1. Each of the curves obtained from the reaction of lysozyme and cells was characterized by an initial change in optical density which peaked within 30 min for the larger amounts of enzyme tested. Both the increase in turbidity and the subsequent fall in the curves were found to be proportional to the amount of enzyme added. Moreover, the curves for the greatest concentrations of lysozyme fell most rapidly. For 25 and 50 μg of HEWL, a sharp fall in the curves did not occur within 2 h and, like the suspension containing no lysozyme, only a slight decrease in turbidity was observed throughout the reaction period after the initial changes upon stirring the cuvettes. To determine whether the fall in the curves was due to lysis of the cells or to settling of aggregates, we stirred the suspensions at the end of the 2-h reaction period and recorded the resulting optical density changes (Fig. 1). It became apparent that the fall in the curves was due to settling of aggregates because, after mixing, the optical density readings reached maximum levels of turbidity. In addition, bacterial clumps were clearly discernible upon visual inspection of the cuvettes.

To determine the relative contribution of bacterial growth phase to the changes in optical density and to compare the aggregation abilities of HEWL and HuL, spectrophotometric assays were done with both logarithmic- and stationary-phase *C. gingivalis* 2010 cells in the presence of a wide range of concentrations of both lysozymes. There was a dose-dependent response to lysozyme treatment regardless of enzyme source and bacterial growth phase (data not shown). In general, an increase in enzyme concentration (≦500 μg) resulted in a graded increase in both the initial and the maximum changes in optical density which occurred during the 121-min reaction period. Furthermore, the greatest change in optical density for each reaction mixture (i.e., as much as 100% of the maximum change) occurred within the initial minutes of interaction of lysozyme and cells. The similarity in aggregation potential of HEWL and HuL was examined further by multiple spectrophotometric assays with a single concentration of enzyme (100 μg) and both logarithmic- and stationary-phase cells. There were no significant differences ($P > 0.5$) between any of the reaction mixtures (Table 1), with the exception of HEWL and stationary-phase cells at that assay point when the suspensions attained maximum optical density ($P < 0.02$). In contrast, therefore, to potential differences in other biological functions, the aggregation potentials of both HEWL and HuL in the spectrophotometric assay were very similar.

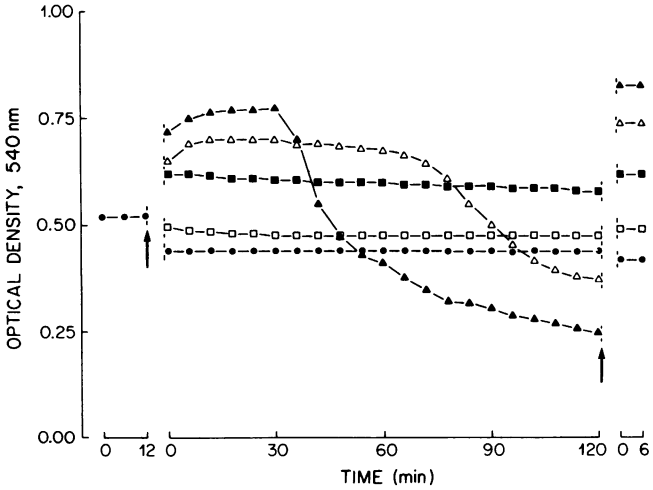

FIG. 1. Aggregation curves obtained by the spectrophotometric procedure. Analyses consisted of the addition of 100 μl of Tris buffer containing various amounts of lysozyme to 900 μl of logarithmic-phase *C. gingivalis* 2010 cells in Tris buffer at 37°C (first vertical arrow). After the suspensions were stirred for 1 min, the optical density was monitored at 540 nm for 120 min, at which time the suspensions were again stirred for 1 min (second vertical arrow), and the optical density was monitored for an additional 6 min. Symbols: (●) cells plus Tris; (□) plus 10 μg of HEWL; (■) plus 25 μg of HEWL; (△) plus 50 μg of HEWL; (▲) plus 100 μg of HEWL.

Since it has been reported that physiological concentrations of sodium salts promote lysis of lysozyme-treated cells (49), aqueous solutions of $NaHCO_3$ and NaCl were tested for their effects on lysozyme-aggregated logarithmic- and stationary-phase *C. gingivalis* 2010 cells at the end of the spectrophotometric assay, and the percent reductions in optical density were determined. The addition of up to 0.15 M $NaHCO_3$ or 0.15 M NaCl to aggregated cells caused a 61 or 60% reduction in optical density, respectively, which was related to the concentration of the salt rather than to the enzyme source or growth phase of the cells. Moreover, differences between the effects of $NaHCO_3$ and NaCl were not significant ($P > 0.5$). Interestingly, upon visual examination of the cuvettes, a clearing of the suspensions was noted for all concentrations of both salts tested, and, upon close inspection of gram-stained samples, cellular damage was detected even without salt addition (data not shown).

Lysozyme binding during the spectrophotometric assay of aggregation. We next sought to determine if the lysozyme-induced rapid increase in turbidity of whole-cell suspensions was dependent on enzyme binding, calculated as the amount

TABLE 1. Effect of HEWL and HuL on change in optical density at 540 nm[a] of logarithmic- and stationary-phase *C. gingivalis* 2010 cells

Assay point[b]	ΔOD_{540}			
	Logarithmic-phase cells with 100 μg of:		Stationary-phase cells with 100 μg of:	
	HEWL	HuL	HEWL	HuL
Initial	0.253 ± 0.040[c]	0.263 ± 0.033	0.344 ± 0.059	0.198 ± 0.017
Maximum	0.326 ± 0.016	0.351 ± 0.019	0.450 ± 0.036[d]	0.451 ± 0.066
Resuspension	0.478 ± 0.044	0.431 ± 0.029	0.566 ± 0.033	0.526 ± 0.034

[a] Determined by substraction of the optical density of control suspensions (cells plus Tris buffer) from the optical density of lysozyme-containing suspensions at various time points during the spectrophotometric assay of aggregation.
[b] Assay points included the initial point immediately after mixing the cells (1 min), the time at which the suspensions attained maximum optical density, and after resuspension of the cells at the end of the 120-min assay.
[c] Each value represents the mean ± standard error for four (HEWL) and five (HuL) determinations.
[d] $P < 0.02$, HEWL with stationary-phase cells versus HEWL with logarithmic-phase cells.

of lysozyme removed by the bacteria during the spectrophotometric assay. Therefore, the aggregation assay was done with both HEWL and HuL and logarithmic- and stationary-phase cells and the kinetics of enzyme binding were determined (Table 2). As expected, lysozyme was depleted by each of the reaction mixtures. Surprisingly, however, of the amount of enzyme bound during the entire assay, the greatest percentage of the total bound occurred during the first minutes of reaction with the cells (Table 2). With stationary-phase cells, upon the addition of 100 μg of HEWL or HuL, virtually all bound within 1 min of stirring the cell suspensions. Of interest, a similar amount bound to the stationary-phase cells after the addition of 250 μg of HEWL, i.e., 107.4 μg. However, during the course of the 120-min reaction period, there was a gradual increase in the amount removed which did not reach 250 μg. This would suggest a limit in enzyme binding at <200 μg for the cell suspension used in the assay (Table 2). To be certain, these data reflect the observation that the maximum changes in optical density did not get appreciably larger upon the addition of more than 250 μg of lysozyme to the cell suspensions. For example, the maximum optical densities attained by stationary-phase cell suspensions after the addition of 500, 750, and 1,000 μg of HEWL were 0.663, 0.663, and 0.698, respectively.

The data obtained with logarithmic-phase cells were likely a more accurate reflection of enzyme binding to the cells due to the presence of fewer extracellular vesicles and surface fibrils as compared with stationary-phase cells. There were no significant differences in the amount of lysozyme depleted by logarithmic-phase cells at 1 min of reaction with 100 or 250 μg of enzyme (Table 2). However, the value was 50% less than that removed by stationary-phase cells. From the initial minutes of reaction to the end of the assay, there was a gradual increase in the amount of enzyme bound to approximately 100 μg for both HEWL and HuL (Table 2). The pattern of rapid binding of lysozyme by both logarithmic- and stationary-phage cells paralleled the initial dramatic changes in optical density which occurred within 1 min after addition of the enzyme to the cell suspensions (Fig. 1).

Effect of lysozyme inhibitors on aggregation. Experiments were designed to determine if recognized inhibitors of muramidase activity could block lysozyme-mediated aggregation of *C. gingivalis* 2010. When the aggregation effected by 100 μg of enzyme on logarithmic-phase cells was compared with that caused by muramidase-inactive lysozyme, the differences in change in optical density throughout the spectrophotometric assay were insignificant (data not shown). Of note, the differences between maximum optical densities attained by cell suspensions treated with lysozyme (100 μg) and those attained by treatment with lysozyme inhibited with either 150 mM *N*-acetyl-D-glucosamine or histamine were only 0.002 and 0.070 optical density units.

The inability of muramidase inhibitors to block aggregation suggested that aggregation was a charge-related phenomenon. This concept was supported by experiments in which the lysyl residues of lysozyme were blocked by maleylation before use in the spectrophotometric assay. The maleylated enzyme failed to aggregate *C. gingivalis* 2010 (Fig. 2). Demaleylation, however, restored aggregation activity comparable to the native enzyme, indicating that maleylated lysozyme retained its integrity and that aggregation was dependent upon charge (Fig. 2).

TABLE 2. Lysozyme bound by logarithmic- and stationary-phase *C. gingivalis* 2010 cells during the spectrophotometric assay of aggregation with HEWL and HuL

Reaction time (min)	Lysozyme bound (μg)[a]					
	Logarithmic-phase cells with:			Stationary-phase cells with:		
	HEWL (100 μg)	HEWL (250 μg)	HuL (100 μg)	HEWL (100 μg)	HEWL (250 μg)	HuL (100 μg)
	(A)[b]	(B)	(C)	(A')	(B')	(C')
1	49.1 ± 1.5 (6)	48.3 ± 11.9 (5)	51.7 ± 1.7 (6)	100.0 ± 0.0 (6)	107.4 ± 37.9 (4)	100.0 ± 0.0 (6)
5	49.9 ± 2.0 (6)	83.3 ± 18.6 (3)	65.0 ± 4.4 (4)	100.0 ± 0.0 (6)	151.6 ± 8.7 (3)	98.0 ± 0.7 (3)
30	62.8 ± 1.6 (6)	90.9 ± 4.5 (3)	100.0 ± 0.0 (6)	100.0 ± 0.0 (6)	184.0 ± 5.6 (6)	100.0 ± 0.0 (6)
60	76.4 ± 1.0 (5)	75.0 ± 0.0 (3)	100.0 ± 0.0 (6)	100.0 ± 0.0 (6)	141.4 ± 15.5 (3)	100.0 ± 0.0 (6)
	(D)	(E)	(F)	(D')	(E')	(F')
120	78.3 ± 0.9 (6)	108.7 ± 12.8 (4)	100.0 ± 0.0 (6)	100.0 ± 0.0 (6)	164.2 ± 4.5 (6)	100.0 ± 0.0 (6)

[a] Each value represents the mean ± standard error for number of determinations given in parentheses.
[b] $P < 0.001$: A ≠ A', C ≠ C', D ≠ D', D ≠ F, D' ≠ E', A ≠ D, C ≠ F. $P < 0.01$: E ≠ E'. $P < 0.02$: D ≠ E, B ≠ E.

Electron microscopy. The observation of cellular damage in Gram-stained *C. gingivalis* 2010 cells sampled at the end of the spectrophotometric assay with either HEWL or HuL led us to examine lysozyme-aggregated bacteria in thin sections by electron microscopy. Shown in Fig. 3 are electron micrographs of logarithmic-phase cells at the end of the spectrophotometric assay with 0, 25, 50, and 100 μg of human enzyme. In comparison to intact control cells, cellular damage was apparent with as little as 25 μg of lysozyme and was observed in virtually all cells treated with 100 mg of enzyme. Dense granular cytoplasmic material was observed in many of the lysing organisms, and extensive damage to the outer membrane-cell wall complex was clearly evident. Of significance was the severe damage which apparently occurred at the blunted polar end of the cells. In these regions there was a large spherical outpouching bordered by varying degrees of damaged outer membrane, cell membrane, and periplasmic space.

DISCUSSION

The interaction of lysozyme with *C. gingivalis* 2010 was investigated with a spectrophotometric procedure initially developed to analyze antibody-mediated bacterial cell aggregation (30). Under standardized conditions of pH (7.4), buffer molarity (0.01 M Tris-hydrochloride), temperature (37°C), and cell concentration (optical density of 0.520 at 540 nm), aggregation was dependent on lysozyme concentration and duration of incubation (Fig. 1). Similar to that observed in studies of antibody and oral microorganisms (30), aggregation occurred very rapidly. Indeed, in most instances, the maximum rate of change in turbidity occurred after 1 min of the interaction of lysozyme with cells, and turbidity values peaked within 30 min (Fig. 1). Of note, both HEWL and HuL aggregated *C. gingivalis* 2010 cells to a similar extent, and there was little difference in the aggregation of either logarithmic- or stationary-phase cells (Table 1). The speed of the aggregation process correlated with the rapid rate and extent of enzyme incorporation during the reaction period (Table 2). Moreover, as followed spectrophotometrically, the concentration dependence was rapidly saturated and reached a plateau at concentrations of lysozyme of <250 mg (Table 2). Interestingly, although turbidity values did not markedly differ, a greater initial depletion of lysozyme was observed in reaction suspensions of stationary-phase than logarithmic-phase cells (Tables 1 and 2). It is likely that this was due to an increased amount of surface fibrils and extracellular vesicles and debris, usually observed in stationary-phase cultures (48), which would have competed with the cells for the enzyme.

Lysozyme-mediated aggregation of periodontal microorganisms may provide protection against both bacterial adherence and colonization in the gingival sulcus (41). However, the size of the bacterial aggregate would be of critical importance in this potential protective mechanism (19). In this regard, it has been demonstrated that small aggregates, as compared with large aggregates, may actually enhance bacterial adherence to dental surfaces (38). In our studies, lysozyme-aggregated *C. gingivalis* 2010 cells presented as visible clumps. However, the actual size of cell aggregates that would occur in the gingival sulcus in vivo has as yet to be determined. Furthermore, aggregation may serve as more than just a clearance mechanism against bacterial colonization in the gingival sulcus. The aggregation of *C. gingivalis* cells by lysozyme may prepare them for phagocytosis by sulcular neutrophils (29, 41, 50). On the other hand, too great an agglutination may result in intense phagocytosis with extracellular release of neutrophil lysosomal enzymes, leading to inflammation (66).

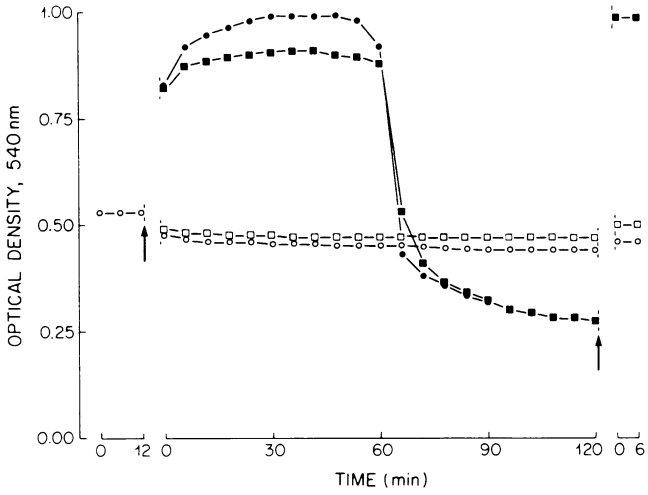

FIG. 2. Effect of maleylation of lysozyme-mediated aggregation of *C. gingivalis* 2010. Analyses consisted of the addition of 100 μl of Tris buffer and Tris buffer containing native enzyme, maleylated, or demaleylated enzyme to 900 μl of logarithmic-phase cells in Tris buffer at 37°C (first vertical arrow). After the suspensions were stirred for 1 min, the optical density was monitored at 540 nm for 120 min, at which time the suspensions were again stirred for 1 min (second vertical arrow), and the optical density was monitored for an additional 6 min. Symbols: (○) cells plus Tris; (□) plus 100 μg of maleylated HEWL; (■) plus 100 μg of demaleylated HEWL; (●) plus 100 μg of HEWL.

The mechanism by which lysozyme aggregated *C. gingivalis* intrigued us. Current evidence suggests that lysozyme may aggregate oral microorganisms (i) as a lectin by its interaction with surface carbohydrate through its enzymatic site (28, 50, 51); (ii) as a cationic protein which binds to anionic surface molecules (44, 50, 53); and (iii) as a hydrophobic molecule which interacts with membrane lipids and amphiphiles (32). Unlike that observed with oral streptococci, aggregation of *C. gingivalis* 2010 was not blocked by inhibitors of lysozyme's muramidase property, including *N*-acetyl-D-glucosamine and histamine (50). Moreover, previous studies on the aggregating ability of lysozyme for oral bacteria indicated that there was a pH and ionic strength dependence for the reaction (29, 44, 50). This dependence suggested that lysozyme binding and subsequent aggregation of *C. gingivalis* 2010 would be mediated through charge-to-charge interactions. This hypothesis was supported by experiments in which lysozyme's positive charge was blocked by maleylation. The maleylated enzyme did not aggregate *Capnocytophaga* cells (Fig. 2). Demaleylation restored aggregation activity, indicating that the maleylated enzyme was not denatured and that aggregation was primarily charge dependent (Fig. 2). However, since maleylation reduced enzymatic activity, our results do not rule out the possibility that lysozyme-mediated aggregation is initially a function of rapid neutralization of bacterial surface charge followed by binding through the enzymatic cleft of the lysozyme (50).

Similar to our observations with other gram-negative (63) and gram-positive (49, 50) bacteria, the addition of up to physiological concentrations of sodium salts to lysozyme-aggregated *C. gingivalis* 2010 resulted in deaggregation and lysis. Surprisingly, ultrastructural analysis of lysozyme-treated cells revealed morphological changes even in the absence of salt (Fig. 3). This would be the first evidence that

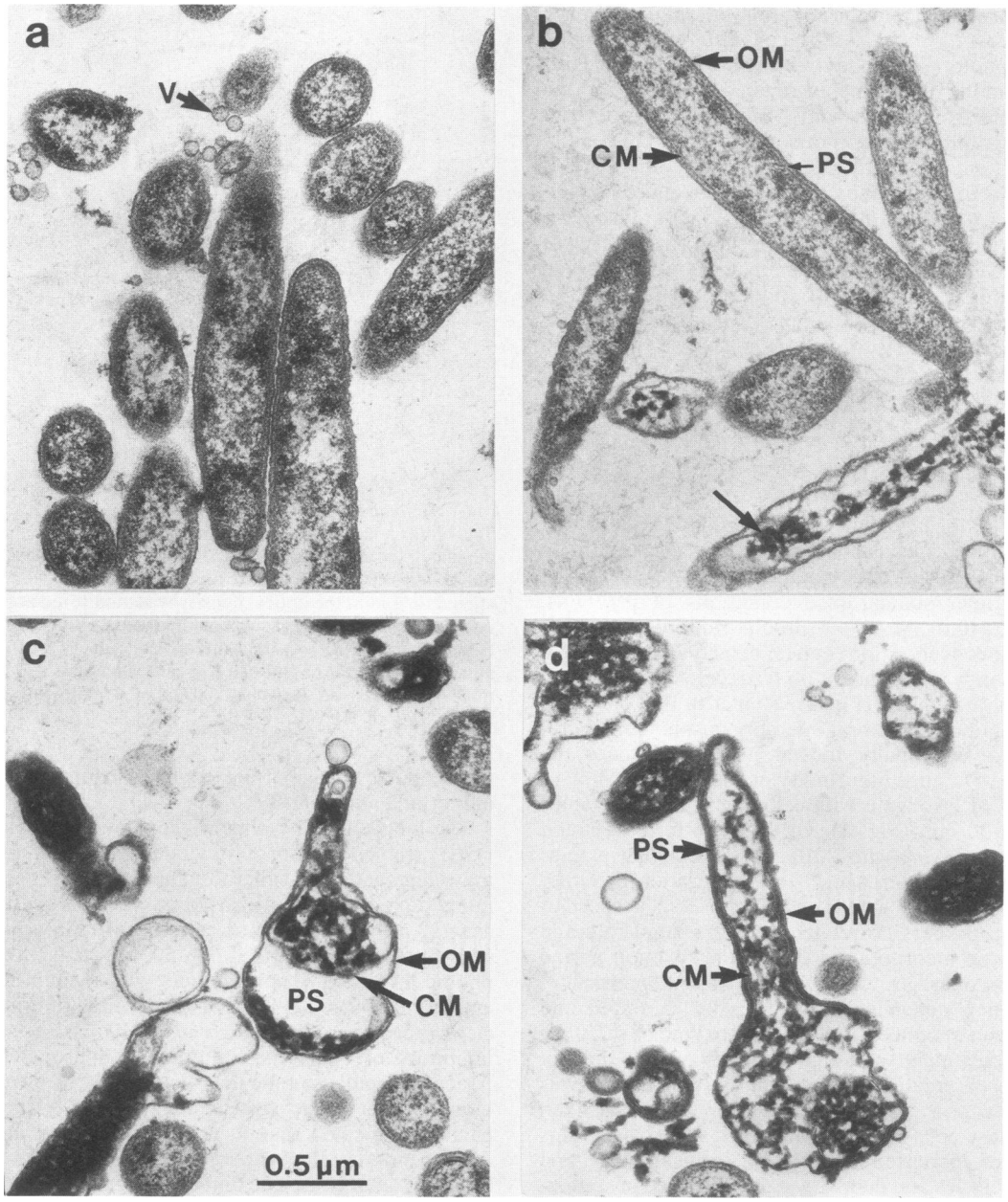

FIG. 3. Electron photomicrographs of logarithmic-phase *C. gingivalis* 2010 cells at the end of the spectrophotometric assay of aggregation with human enzyme. (a) Control cells in the absence of lysozyme; (b) cells plus 25 μg of HuL; (c) cells plus 50 μg of HuL; (d) cells plus 100 μg of HuL. V, Extruded vesicle; OM, outer membrane; PS, periplasmic space; CM, cytoplasmic membrane. Unlabeled arrow indicates aggregated cytoplasmic constituents.

C. gingivalis peptidoglycan can be degraded by lysozyme, at least during prolonged exposure to the enzyme (Fig. 3). Our earlier investigations (23, 28, 29, 50), as well as those of others (21, 39, 57, 64, 67), have suggested that for many bacteria lysozyme-mediated lysis is, in part, a function of autolysin activation. The pattern of lysis (Fig. 3) and the requirement for lysozyme's cationic charge (Fig. 2) would support this hypothesis. Damage appeared to be more severe at the blunted polar end of the cells where there was a large spherical outpouching bordered by varying degrees of damaged cell envelope (Fig. 3). Damage to the end plate is significant in that this region may provide the enzyme with either a site for facilitated access to a sensitive substrate (peptidoglycan) or an area for autolysin deregulation. The possibility that lysis of *C. gingivalis* 2010 was independent of lysozyme's muramidase property and solely dependent on charge remains to be determined. However, if lysis is a charge-related phenomenon, it could be of biological importance in light of the presence of lysozyme and other cationic proteins in neutrophils (45, 54, 65). It is conceivable that since the neutrophil is the predominant inflammatory cell in the gingival sulcus during the initiation and progression of periodontal disease (1), activation of autolysins by secreted cationic molecules may be an important effector mechanism for bacterial death and lysis in vivo.

Lysozyme sensitivity of the cell wall of *Capnocytophaga* species, and other gram-negative periodontal bacteria, may be of significance in the expression of the microorganism's

virulence potential. Depending on the degree of peptidoglycan hydrolysis, released cell wall fragments from lysing bacteria can have many biological activities, e.g., complement activation (24), pyrogenicity (46), polyclonal B-cell activation, and secretion of immunoglobulins (2, 14). Moreover, persistence of resistant and undigested cell walls in periodontal tissues may lead to a prolonged inflammatory response (12, 20). This latter biological effect may be of importance in light of recent studies which have indicated that several periodontal microorganisms, including *Capnocytophaga* species, are present in diseased periodontal tissues (11). Cell integrity in periodontal tissues may, in part, reflect resistance to lysozyme. On the other hand, released antigenic substances from lysing cells could compete with viable bacteria in binding to antibodies and complement, allowing the bacteria to spread further into the tissues before eventually being destroyed (4).

Further studies of the mechanism of lysozyme-mediated lysis will tend to clarify the in vivo functions of this cationic host defense factor. Moreover, the paradoxical finding that during aggregation there is lysis is very significant in light of earlier studies which tended to downplay the ability of the enzyme to lyse oral microorganisms (see references 28 and 50). Indeed, from the results of this study and our previous work with *Veillonella alcalescens* (63) and *A. actinomycetemcomitans* (27), it could be concluded that lysozyme is likely a major lytic factor in the gingival crevicular region.

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LITERATURE CITED

1. **Attstrom, R.** 1971. Studies on neutrophil polymorphonuclear leukocytes at the dento-gingival junction in gingival health and disease. J. Periodontal Res. Suppl. **8**:1–15.
2. **Babu, U. M., and A. R. Zeiger.** 1983. Soluble peptidoglycan from *Staphylococcus aureus* is a murine B-lymphocyte mitogen. Infect. Immun. **42**:1012–1016.
3. **Bladen, H. A., and S. E. Mergenhagen.** 1964. Ultrastructure of *Veillonella* and morphological correlation of an outer membrane with particles associated with endotoxic activity. J. Bacteriol. **88**:1482–1492.
4. **Blumel, P., W. Uecker, and P. Giesbrecht.** 1981. *In vitro* studies on the possible role of cell wall turnover in *Staphylococcus aureus* during infection. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Suppl. **10**:435–439.
5. **Bolton, R. W., and J. K. Dyer.** 1983. Suppression of murine lymphocyte mitogen responses by exopolysaccharide from *Capnocytophaga ochracea*. Infect. Immun. **39**:476–479.
6. **Bolton, R. W., J. K. Dyer, R. A. Reinhardt, and D. K. Okano.** 1983. Modulation of *in vitro* human lymphocyte responses by an exopolysaccharide from *Capnocytophaga ochracea*. J. Dent. Res. **62**:1186–1189.
7. **Brandtzaeg, P., and W. V. Mann, Jr.** 1964. A comparative study of the lysozyme activity of human gingival pocket fluid, serum, and saliva. Acta Odontol. Scand. **22**:441–455.
8. **Bretz, U., and M. Baggiolini.** 1974. Biochemical and morphological characterization of azurophil and specific granules of human neutrophilic polymorphonuclear leukocytes. J. Cell Biol. **63**:251–269.
9. **Butler, P. J. G., J. I. Harris, B. S. Hartley, and R. Leberman.** 1969. The use of maleic anhydride for the reversible blocking of amino groups in polypeptide chains. Biochem. J. **112**:679–689.
10. **Canfield, R. E., J. C. Collins, and J. H. Sobel.** 1975. Human leukemia lysozyme, p. 63–70. *In* E. F. Osserman, R. E. Canfield, and S. Beychok (ed.), Lysozyme. Academic Press, Inc., New York.
11. **Carranza, F. A., Jr., R. Saglie, M. G. Newman, and P. L. Valentin.** 1983. Scanning and transmission electron microscopic study of tissue-invading microorganisms in localized juvenile periodontitis. J. Periodontol. **54**:598–617.
12. **Cromartie, W. J.** 1981. Arthropathic properties of the peptidoglycan-polysaccharide complexes of microbial origin, p. 24–38. *In* H. Deicher and L. C. I. Schultz (ed.), Arthritis. Models and mechanisms. Springer, Berlin.
13. **Davies, P., R. J. Bonney, J. L. Humes, and F. A. Kuehl.** 1980. The role of macrophage secretory products in chronic inflammatory processes. J. Invest. Dermatol. **74**:292–296.
14. **Dziarski, R.** 1980. Polyclonal activation of immunoglobulin secretion in B lymphocytes induced by staphylococcal peptidoglycan. J. Immunol. **125**:2478–2483.
15. **Forlenza, S. W., A. I. Lipsey, S. B. Siegel, and U. Blachman.** 1980. *Capnocytophaga* sepsis: a newly recognized clinical entity in granulocytopenic patients. Lancet **i**:567–568.
16. **Friedman, S. A., I. D. Mandel, and M. S. Herrera.** 1983. Lysozyme and lactoferrin quantitation in the crevicular fluid. J. Periodontol. **54**:347–350.
17. **Gandola, C., T. Butler, S. Badger, E. Cheng, and S. Beard.** 1980. Septicemia in a granulocytopenic patient with glossitis. Arch. Intern. Med. **140**:851–852.
18. **Genco, R. J., and J. Slots.** 1984. Host responses in periodontal diseases. J. Dent. Res. **63**:441–451.
19. **Gibbons, R. J.** 1984. Adherent interactions which may affect microbial ecology in the mouth. J. Dent. Res. **63**:378–385.
20. **Ginsburg, I.** 1979. The role of lysosomal factors of leukocytes in the biodegradation and storage of microbial constituents in infectious granulomas, p. 327–406. *In* P. Jaque and J. Dingle (ed.), Lysozymes in applied biology and therapeutics. North-Holland Publishing Co., Amsterdam.
21. **Ginsburg, I., M. Lahav, and P. Giesbrecht.** 1982. Effect of leukocyte hydrolases on bacteria. XVI. Activation by leukocyte factors and cationic substances on autolytic wall enzymes in *Staphylococcus aureus*: modulation by anionic polyelectrolytes in relation to survival of bacteria in inflammatory exudates. Inflammation **6**:401–407.
22. **Glazer, A. N., A. O. Barel, J. B. Howard, and D. M. Brown.** 1969. Isolation and characterization of fig lysozyme. J. Biol. Chem. **244**:3583–3589.
23. **Goodman, H., J. J. Pollock, V. J. Iacono, W. Wong, and G. D. Shockman.** 1981. Peptidoglycan loss during hen egg white lysozyme-inorganic salt lysis of *Streptococcus mutans*. Arch. Oral Biol. **14**:277–294.
24. **Greenblatt, J., R. J. Boackle, and J. H. Schwab.** 1978. Activation of the alternate complement pathway by peptidoglycan from streptococcal cell wall. Infect. Immun. **19**:296–303.
25. **Gregory, E. M., W. E. C. Moore, and L. V. Holdeman.** 1978. Superoxide dismutase in anaerobes: survey. Appl. Environ. Microbiol. **35**:988–991.
26. **Higgins, M. L., and G. D. Shockman.** 1970. Early changes in the ultrastructure of *Streptococcus faecalis* after amino acid starvation. J. Bacteriol. **103**:244–254.
27. **Iacono, V. J., P. R. Boldt, B. J. MacKay, M. I. Cho, and J. J. Pollock.** 1983. Lytic sensitivity of *Actinobacillus actinomycetemcomitans* Y4 to lysozyme. Infect. Immun. **40**:773–784.
28. **Iacono, V. J., B. J. MacKay, S. DiRienzo, and J. J. Pollock.** 1980. Selective antibacterial properties of lysozyme for oral microorganisms. Infect. Immun. **29**:623–632.
29. **Iacono, V. J., B. J. MacKay, J. J. Pollock, P. R. Boldt, S. Ladenheim, B. L. Grossbard, and M. L. Rochon.** 1982. Roles of lysozyme in the host response to periodontopathic microorganisms, p. 318–342. *In* R. J. Genco and S. E. Mergenhagen (ed.), Host-parasite interactions in periodontal diseases. American Society for Microbiology, Washington, D.C.
30. **Iacono, V. J., M. A. Taubman, D. J. Smith, and E. C. Moreno.** 1976. A spectrophotometric procedure for quantitation of antibody directed to bacterial antigens. Immunochemistry **13**:235–243.
31. **Iino, Y., and R. M. Hopps.** 1984. The bone-resorbing activities

in tissue culture of lipopolysaccharides from the bacteria *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis*, and *Capnocytophaga ochracea* isolated from human mouths. Arch. Oral Biol. **29**:59–63.

32. **Katona, L. I., J. J. Pollock, V. J. Iacono, and G. D. Shockman.** 1981. Hydrophobic interaction chromatography: a method for purification of lipoteichoic acid, p. 51–67. *In* A. J. Wicken and G. D. Shockman (ed.), Chemistry and biological activities of bacterial surface amphiphiles. Academic Press, Inc., New York.
33. **Kilian, M.** 1981. Degradation of immunoglobulins A1, A2, and G by suspected principal periodontal pathogens. Infect. Immun. **34**:757–765.
34. **Laible, N. J., and G. R. Germaine.** 1982. Adsorption by lysozyme from human whole saliva by *Streptococcus sanguis* 903 and other oral microorganisms. Infect. Immun. **36**:148–159.
35. **Leadbetter, E. R., S. C. Holt, and S. S. Socransky.** 1979. *Capnocytophaga*: new genus of gram-negative gliding bacteria. I. General characteristics, taxonomic considerations and significance. Arch. Microbiol. **122**:9–16.
36. **Leffell, M. S., and J. K. Spitznagel.** 1972. Association of lactoferrin with lysozyme in granules of human polymorphonuclear leukocytes. Infect. Immun. **6**:761–765.
37. **Lehrer, S. S., and G. D. Fasman.** 1966. The fluorescence of lysozyme and lysozyme substrate complexes. Biochem. Biophys. Res. Commun. **23**:133–138.
38. **Liljemark, W. F., C. S. Bloomquist, and G. R. Germaine.** 1981. Effect of bacterial aggregation on the adherence of oral streptococci to hydroxyapatite. Infect. Immun. **31**:935–941.
39. **Logardt, I. M., and H. Y. Neujahr.** 1975. Lysis of modified walls from *Lactobacillus fermentum*. J. Bacteriol. **124**:73–77.
40. **MacKay, B. J., V. J. Iacono, J. M. Zuckerman, E. F. Osserman, and J. J. Pollock.** 1982. Quantitative recovery, selective removal and one-step purification of human parotid and leukemic lysozymes by immunoadsorption. Eur. J. Biochem. **129**:93–98.
41. **Mandel, I. D.** 1979. In defense of the oral cavity, p. 473–491. *In* I. Kleinberg, S. A. Elison, and I. D. Mandel (ed.), Proceedings: Saliva and Dental Caries (a special supplement to Microbiology Abstracts), vol. 3. Information Retrieval, Inc., Washington, D.C.
42. **Mashimo, P. A., Y. Yamamoto, M. Nakamura, and J. Slots.** 1983. Selective recovery of oral *Capnocytophaga* spp. with sheep blood agar containing bacitracin and polymyxin B. J. Clin. Microbiol. **17**:187–191.
43. **Mashimo, P. A., Y. Yamamoto, J. Slots, B. H. Park, and R. J. Genco.** 1983. The periodontal microflora of juvenile diabetics: culture, immunofluorescence, and serum antibody studies. J. Periodontol. **54**:420–430.
44. **Metcalf, R. H., and R. H. Diebel.** 1969. Differential lytic response of enterococci associated with addition order of lysozyme and anions. J. Bacteriol. **99**:674–680.
45. **Modrzakowski, M. C., and J. K. Spitznagel.** 1979. Bactericidal activity of fractionated granule contents from human polymorphonuclear leukocytes: antagonism of granule cationic proteins by lipopolysaccharide. Infect. Immun. **25**:597–602.
46. **Oken, M. M., P. K. Petersen, and B. J. Wilkinson.** 1981. Endogenous pyrogen production by human blood monocytes stimulated by staphylococcal cell wall components. Infect. Immun. **31**:208–213.
47. **Osserman, E. F., and D. P. Lawlor.** 1966. Serum and urinary lysozyme (muramidase) in monocytic and myelomonocytic leukemia. J. Exp. Med. **124**:921–955.
48. **Poirier, T. P., S. J. Tonelli, and S. C. Holt.** 1979. Ultrastructure of gliding bacteria: scanning electron microscopy of *Capnocytophaga sputigena*, *Capnocytophaga gingivalis*, and *Capnocytophaga ochracea*. Infect. Immun. **26**:1146–1158.
49. **Pollock, J. J., H. Goodman, P. K. Elsey, and V. J. Iacono.** 1983. Synergism of lysozyme, proteases, and inorganic monovalent anions in the bacteriolysis of oral *Streptococcus mutans* GS-5. Arch. Oral Biol. **28**:865–871.
50. **Pollock, J. J., V. J. Iacono, H. Goodman, B. J. MacKay, L. I. Katona, L. B. Taichman, and E. Thomas.** 1976. The binding, aggregation, and lytic properties of lysozyme, p. 325–352. *In* H. M. Stiles, W. J. Loesche, and T. C. O'Brien (ed.), Proceedings: Microbial Aspects of Dental Caries (a special supplement to Microbiology Abstracts), vol. 3. Information Retrieval, Inc., Washington, D.C.
51. **Pollock, J. J., and N. Sharon.** 1970. Studies on the acceptor specificity of the lysozyme-catalyzed transglycosylation reaction. Biochemistry **9**:3913–3925.
52. **Rateitschak-Pluss, E. M., and H. E. Schroeder.** 1984. History of periodontitis in a child with Papillon-Lefevre syndrome. J. Periodontol. **55**:35–46.
53. **Reitamo, S., M. Klockars, M. Adinolfi, and E. F. Osserman.** 1978. Human lysozyme. Ric. Clin. Lab. **8**:211–231.
54. **Rest, R. F., M. H. Cooney, and J. K. Spitznagel.** 1978. Bactericidal activity of specific and azurophil granules from human neutrophils: studies with outer-membrane mutants of *Salmonella typhimurium* LT2. Infect. Immun. **19**:131–137.
55. **Salton, M. R. J.** 1957. The properties of lysozyme and its action on microorganisms. Bacteriol. Rev. **21**:82–100.
56. **Shinitsky, M., E. Katachalski, V. Grisaro, and N. Sharon.** 1966. Inhibition of lysozyme by imidazole and indole derivatives. Arch. Biochem. Biophys. **116**:332–343.
57. **Shockman, G. D., J. S. Thompson, and M. J. Conover.** 1968. The relationship of autolysin to lysozyme sensitivity of *Streptococcus faecalis* 9790, p. 248–260. *In* L. B. Guze (ed.), Microbial protoplasts, spheroplasts, and L-forms. The Williams and Wilkins Co., Baltimore.
58. **Shurin, S. B., S. S. Socransky, E. Sweeney, and T. P. Stossel.** 1979. A neutrophil disorder induced by *Capnocytophaga*, a dental microorganism. N. Engl. J. Med. **301**:849–854.
59. **Slots, J.** 1979. Subgingival microflora and periodontal disease. J. Clin. Periodontol. **6**:351–382.
60. **Sophianopoulos, A. J., C. K. Rhodes, D. N. Holcomb, and K. E. Van Holde.** 1962. Physical studies in lysozyme. I. Characterization. J. Biol. Chem. **237**:1107–1112.
61. **Stevens, R. H., B. F. Hammond, and C. H. Lai.** 1982. Characterization of an inducible bacteriophage from a leukotoxic strain of *Actinobacillus actinomycetemcomitans*. Infect. Immun. **35**:343–349.
62. **Tanner, A. C. R., C. Haffer, G. T. Bratthall, R. A. Visconti, and S. S. Socransky.** 1979. A study of the bacteria associated with advancing periodontitis in man. J. Clin. Periodontol. **6**:278–307.
63. **Tortosa, M., M.-I. Cho, T. J. Wilkens, V. J. Iacono, and J. J. Pollock.** 1981. Bacteriolysis of *Veillonella alcalescens* by lysozyme and inorganic anions present in saliva. Infect. Immun. **32**:1261–1273.
64. **Wecke, J., M. Lahav, I. Ginsburg, and P. Giesbrecht.** 1982. Cell wall degradation of *Staphylococcus aureus* by lysozyme. Arch. Microbiol. **131**:116–123.
65. **Weiss, J., P. E. Elsbach, I. Olsson, and H. Odeberg.** 1978. Purification and characterization of a potent bactericidal and membrane active protein from the granules of human polymorphonuclear leukocytes. J. Biol. Chem. **253**:2664–2672.
66. **Weissmann, G., J. E. Smulen, and H. M. Korchak.** 1980. Release of inflammatory mediators from stimulated neutrophils. N. Engl. J. Med. **303**:27–34.
67. **Westmacott, D., and H. R. Perkins.** 1979. Effects of lysozyme on *Bacillus cereus* 569: rupture of chains of bacteria and sensitivity to autolysins. J. Gen. Microbiol. **115**:1–11.