INFECTION AND IMMUNITY, May 1983, p. 773–784
0019-9567/83/050773-12$02.00/0

Vol. 40, No. 2

Lytic Sensitivity of *Actinobacillus actinomycetemcomitans* Y4 to Lysozyme

VINCENT J. IACONO,[1]* PAUL R. BOLDT,[1] BRUCE J. MACKAY,[2] MOON-IL CHO,[2] AND JERRY J. POLLOCK[2]

Department of Periodontics[1] and Department of Oral Biology and Pathology,[2] State University of New York at Stony Brook, Stony Brook, New York 11794

Received 2 December 1982/Accepted 24 February 1983

The ability of both human and hen egg white lysozymes to lyse *Actinobacillus actinomycetemcomitans* Y4 was investigated. Lysis was followed optically at 540 nm by measuring the percent reduction in turbidity of freshly harvested log-phase cells suspended in Tris-maleate buffers within a wide range of pH (5.2 to 8.5) and molarity (0.01 to 0.2 M) and containing various amounts of enzyme and EDTA. In several instances, treated microorganisms were subsequently examined in thin sections by electron microscopy. Reductions in turbidity and clearing of suspensions occurred with small amounts of lysozyme (less than 1 μg) under relatively alkaline conditions and at low ionic strength and in the presence of small amounts of EDTA (greater than 0.01 mM). Under the most alkaline conditions, EDTA alone effected turbidity reductions similar to those observed in the presence of lysozyme, which suggested that EDTA not only increased outer membrane permeability but also caused cell lysis. Ultrastructural analysis did not always correspond to turbidimetric observations. Cell lysis was virtually complete in suspensions containing both lysozyme and EDTA. However, in contrast to turbidimetric findings, a significant percentage of cells (greater than 25%) was lysed in the presence of lysozyme alone. Furthermore, significant damage occurred in the presence of EDTA alone. Spheroplast-like cell ghosts were present which surrounded condensed cytoplasm or relatively clear spaces. These findings further support the concept of the requirement for electron microscopy to assess lytic damage in addition to turbidimetric and biochemical methods. Our results are the first to demonstrate the remarkable sensitivity of *A. actinomycetemcomitans* Y4 to lysozyme and to show that EDTA not only affects outer membrane permeability but effects cell lysis, possibly through activation of autolytic enzymes at the cytoplasmic membrane. The exquisite sensitivity of *A. actinomycetemcomitans* Y4 to lysis could be an important mechanism by which lysozyme participates in the regulation of this suspected periodontal pathogen.

The microbial component in the etiology of periodontal diseases has received intensive investigation during recent years (51, 56). Several bacterial species have been isolated from the complex gingival microflora and have been found to be associated with particular disease states (51, 56). One such microorganism, which appears to be an important periodontal pathogen, is *Actinobacillus actinomycetemcomitans*. This nonmotile, gram-negative capnophilic coccobacillus is frequently found in the periodontal pockets of patients with localized juvenile periodontitis (38, 52, 56), and certain strains have been isolated from individuals with potentially life-threatening clinical conditions, such as endocarditis (59) and brain abscesses (36). Investigations of the effects of host defense factors on *A. actinomycetemcomitans* may shed information on its potential to colonize and grow in the gingival sulcus and at nonoral sites and could perhaps help in the development of an effective means for its control. A host defense factor which may be of importance in the regulation of infections with *A. actinomycetemcomitans* and other periodontal pathogens is lysozyme. Lysozyme is a cationic protein which is found in both the azurophil and specific granules of neutrophils (6, 31) and is a predominant secretory product of macrophages (11). Lysozyme is likely to come in contact with *A. actinomycetemcomitans* in the subgingival microflora since the enzyme is a major gingival crevicular fluid component and its concentration increases with the severity of periodontal inflammation (5). In addition, it has recently been reported that crevicular fluid levels of lysozyme are significantly

higher in localized juvenile periodontitis as compared with gingivitis and adult disease (S. Freidman, M. Herrera, and I. D. Mandel. J. Dent. Res. **61**:318, 1982). However, the role of this cationic protein in the gingival sulcus or periodontal pocket remains unclear. It is generally thought that the major function of the enzyme is hydrolysis of the peptidoglycan of the bacterial cell wall, resulting in cell lysis (47), but lysozyme has also been shown to inhibit bacterial growth (23) and mediate bacterial aggregation (24, 43). The sensitivity of microorganisms to lysis is dependent upon the susceptibility as well as the accessibility of the cell wall peptidoglycan to the enzyme (48). The intact outer membrane of most gram-negative bacteria has been shown to serve as a permeability barrier to lysozyme and other large molecules (39, 48). This barrier can be disrupted in vivo by antibody and complement (1) and by neutrophil components (16, 61) and in vitro by several standard techniques, including pretreatment of cells with EDTA (34, 44). It is of interest, however, that unlike most gram-negative microorganisms, oral *Veillonella* species are lysed in the presence of lysozyme alone (4, 57). This may be due, in part, to an ineffective outer membrane barrier or an accessible system of endogenous autolytic hydrolases (57).

The aim of the present study was to determine the lytic sensitivity of *A. actinomycetemcomitans* Y4, a strain which was originally isolated from a periodontal pocket of a patient with localized juvenile periodontitis (26), to both human and hen lysozymes and to determine the extent of cellular damage by electron microscopy. Our results indicate that, similar to *Veillonella* species, *A. actinomycetemcomitans* Y4 is sensitive to lysozyme from either source and that spheroplast-like cell ghosts are formed in the presence of EDTA alone, possibly through activation of autolytic enzymes.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *A. actinomycetemcomitans* Y4 was obtained originally from R. Stevens (University of Pennsylvania, Philadelphia, Pa.) and was maintained in the lyophilized state in the Stony Brook culture collection. Cultures were reconstituted and grown in a modified fluid thioglycolate broth containing 0.05% sodium thioglycolate, 0.5% yeast extract, 1.5% trypticase peptone, 0.75% glucose, 0.25% sodium chloride, 0.75% L-cysteine, and 0.4% sodium bicarbonate, as previously described (54). For assays of cell lysis, 0.2 ml of a stationary-phase culture (optical density of 1.0 at 700 nm) was transferred to 5 ml of fresh thioglycolate medium and grown to exponential phase (optical density of 0.5 at 700 nm), and the cells were harvested by centrifugation (12,000 × *g*, 10 min, 4°C).

Lysozyme. Hen egg white lysozyme (HEWL; crystallized three times) was obtained from Sigma Chemical Co. (St. Louis, Mo.). The concentration of HEWL in stock solutions was determined spectrophotometrically at 280 nm, using the extinction coefficient of $E_{1cm}^{1\%} = 26.4$ (53). Human lysozyme (HuL) was isolated from the urine of chronic monocytic leukemic patients (41) and purified to homogeneity by immunoadsorption affinity chromatography (35a). The concentration of HuL was determined from the extinction coefficient of $E_{1cm}^{1\%} = 25.5$ at 280 nm (8).

Procedure for cell lysis. Lysozyme sensitivity of *A. actinomycetemcomitans* Y4 was analyzed turbidimetrically. Assays consisted of the addition of freshly harvested log-phase cells that were washed once in the appropriate Tris-maleate buffer before resuspension in fresh buffer to an optical density of approximately 0.6 at 540 nm. Samples (1.98 ml) were added to continuously stirred cuvettes at 37°C (Gilford 2400 recording spectrophotometer, spectrostir setting of 6.5) and preincubated for 5 min, and changes in absorbance were monitored at 540 nm after the addition of 20 μl of buffer alone or of buffer containing HEWL or HuL. The resulting recordings are referred to as lysis curves.

The buffers used in the assays were varied in their molarity (0.01 to 0.2 M) and pH (5.2 to 8.5) for optimalization of the lytic system. In certain instances, EDTA was incorporated into the buffer at concentrations ranging from 0.01 to 100 mM.

Electron microscopy. To assess lytic damage of bacterial cells, we previously reported that electron microscopy must be used in conjunction with turbidimetric or biochemical assays (57). Ultrastructural studies were therefore carried out to assist us in the interpretation of the results of the spectrophotometric assay. In these studies, freshly harvested log-phase cells were washed three times in 0.01 M Tris-maleate buffer (pH 7.3) and resuspended in 14 ml of fresh buffer (optical density at 540 nm was 0.6) alone or buffer containing various amounts of EDTA and lysozyme. Incubation was carried out in screw-cap tubes (17 by 100 mm) on a blood tube rotator with end-over-end mixing at 37°C for 1 h. At the end of the reaction period, triplicate suspensions were pooled, centrifuged (12,000 × *g*, 1 h, 4°C), resuspended in 9 ml of fresh buffer in thick-walled glass centrifuge tubes (15 ml), and mixed with glutaraldehyde (1 ml, 50% [vol/vol]) to make a final concentration of 2.5% glutaraldehyde in Tris-maleate buffer. The suspensions were centrifuged (27,500 × *g*, 1 h, 4°C), and the cell pellets obtained were resuspended in fresh fixative for 5 h at room temperature and then sliced into approximately 2-mm^2 sections. After three 5-min washes in 0.1 M sodium cacodylate buffer (pH 7.4), the pellets were postfixed in 1% osmium tetroxide in cacodylate buffer (pH 7.4) for 2 h at room temperature. The pellets were then washed (three times for 5 min each time) with 0.1 M maleate buffer (pH 5.15) and stained enblock for 1.5 h at room temperature with 1% (wt/vol) uranyl acetate in 0.1 M maleate buffer (pH 6.0). The stained cells were washed in maleate buffer (pH 5.15) and dehydrated in a graduated cold ethanol series (50 to 100%). Propylene oxide acted as a transitional solvent for embedding in Epon (35).

Ultrathin sections were cut on a Porter-Blum MT2 ultramicrotome and placed on 200-mesh grids coated

with 0.3% Formvar film for low-magnification observations or on 300-mesh uncoated grids for high-magnification observations. Sections were sequentially stained for 25 min with freshly saturated uranyl acetate in 50% ethanol and for 7 min with lead citrate (46). All thin sections were examined and photographed in a JEOL 100B transmission electron microscope operating at 60 kV.

RESULTS

Effect of pH and buffer molarity on lysozyme sensitivity. Since it has been demonstrated that EDTA alters outer membrane permeability of gram-negative bacteria, thus facilitating lysozyme penetration to its peptidoglycan substrate, studies of lysozyme sensitivity have frequently been done with EDTA-treated cells (34, 45). The effectiveness of EDTA treatment appears to be optimal when done in Tris buffer at a specific molarity and pH which are dependent on the test microorganism (18, 33, 37, 45). We therefore varied these parameters in the evaluation of lysozyme sensitivity of *A. actinomycetemcomitans* Y4 by a turbidimetric assay. A reduction in turbidity, or lysis, did not occur when freshly harvested log-phase cells were suspended in either 0.01 or 0.1 M Tris-maleate buffer at pH 5.5, 7.3, or 8.5 (data not shown). When *A. actinomycetemcomitans* Y4 cells were suspended in 0.1 M Tris-maleate buffer containing 10 mM EDTA, a slight, but variable, reduction in turbidity was obtained when the pH was increased in the range of 6.5 to 8.5 (Fig. 1). However, even at the highest pH tested, clearing of the suspensions did not occur, and only a 17% reduction in turbidity was recorded after 1 h of incubation. The addition of lysozyme (10 μg of HEWL per ml) resulted in a marked reduction in turbidity at alkaline pH (Fig. 1). A 65% reduction in optical density occurred after 1 h of incubation with lysozyme at pH 8.5 (Fig. 1). *A. actinomycetemcomitans* Y4 was therefore sensitive to both lysozyme and EDTA, but apparently under the more alkaline conditions.

The effect of buffer molarity in the lytic system was then studied. In the experiments shown in Fig. 2, the reduction in turbidity for all cell suspensions was found to be inversely proportional to the ionic strength of the Tris-maleate buffer at pH 7.3. However in comparison to those samples containing 10 mM EDTA alone, at low ionic strength the presence of a small amount of lysozyme (10 μg of HEWL per ml) effected a dramatic and almost complete loss of turbidity within the first few minutes of incubation (Fig. 2). The possibility that similar lysis might occur under different conditions of Tris molarity and pH was investigated. Table 1 shows the percent reduction in optical density observed after 1 h of incubation of *A. actinomycetemcomitans* Y4 cells in Tris-maleate buffer at concentrations ranging from 0.01 to 0.2 M and at pH 5.5, 7.3, and 8.5. Lysis always occurred more readily at alkaline pH and low buffer molarity. Note that for most Tris concentrations the reductions in turbidity were similar at both pH 7.3 and 8.5, which suggested that lysozyme effects occurred within a relatively broad range of conditions rather than at a specific pH and ionic strength (Fig. 1 and 2; Table 1). In addition, if cell suspensions were incubated under acidic conditions (e.g., pH 5.5) or if the buffer molarity was high or both, lysis was inhibited (Table 1). Of interest was the finding that in the

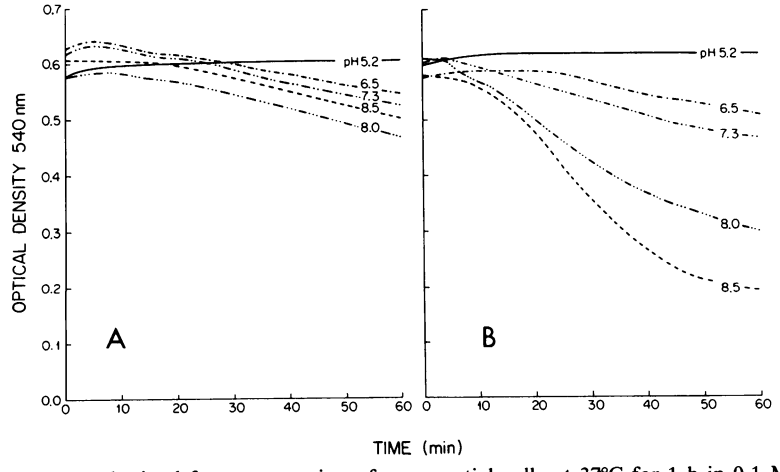

FIG. 1. Lysis curves obtained from suspension of exponential cells at 37°C for 1 h in 0.1 M Tris-maleate buffer containing 10 mM EDTA (A) and in buffer containing 10 mM EDTA and 10 μg of HEWL per ml (B) at pHs ranging from 5.2 to 8.5, as indicated.

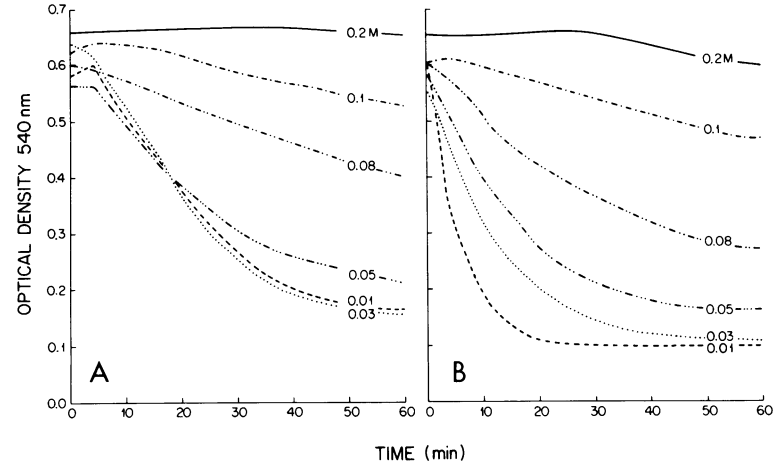

FIG. 2. Lysis curves obtained from suspension of exponential cells at 37°C for 1 h in Tris-maleate buffer (pH 7.3) containing 10 mM EDTA (A) and in buffer containing 10 mM EDTA and 10 μg of HEWL per ml (B) at concentrations of buffer ranging from 0.01 to 0.2 M, as indicated.

absence of lysozyme, the EDTA effect became very pronounced at low buffer molarity and at alkaline pH (Table 1). A 72% reduction in turbidity occurred after a 1-h incubation of *A. actinomycetemcomitans* Y4 cells in 0.01 M Tris-maleate buffer at pH 7.3 containing only 10 mM EDTA (Table 1). The fact that this reduction effected by EDTA approached that which occurred with lysozyme and EDTA (i.e., 84% [Table 1]) suggested that EDTA not only increased outer membrane permeability, but also caused cell lysis.

Effect of EDTA concentration on lysis. The percent reductions in turbidity which occurred after 1 h of incubation of cells in buffer containing from 0.01 to 100 mM EDTA in the presence and absence of lysozyme are shown in Table 2. A minimum amount of EDTA (greater than 0.01 mM) was required for turbidimetric evidence of lysis. Marked reductions in turbidity occurred in the presence of HEWL within a broad range of EDTA concentrations (0.1 to 20 mM). However, at 5, 10, and 20 mM EDTA, reductions in turbidity were not appreciably different in the presence or absence of lysozyme (Table 2). It is also noteworthy that the effects of both EDTA and lysozyme were markedly reduced with large concentrations of EDTA (50 and 100 mM).

Effect of lysozyme concentration on lysis. The extent of lysis was dependent on the amount of lysozyme in the reaction mixtures (Fig. 3). When exponential-phase *A. actinomycetemcomitans* Y4 cells were suspended in 0.01 M

TABLE 1. Effect of Tris buffer molarity and pH on lysis of *A. actinomycetemcomitans* Y4[a]

Tris buffer (M)	% Reduction in optical density					
	EDTA			EDTA and HEWL		
	pH 5.5	pH 7.3	pH 8.5	pH 5.5	pH 7.3	pH 8.5
0.01	32	72	64	49	84	82
0.03	10	73	61	44	80	73
0.05	0	63	68	10	69	70
0.08	0	33	52	0	55	67
0.10	0	15	17	0	24	65
0.20	0	0	0	0	9	8

[a] Lysis is the percent reduction in optical density at 540 nm after 1 h of incubation in Tris-maleate buffer containing either 10 mM EDTA or 10 mM EDTA and 10 μg of HEWL per ml.

TABLE 2. Effect of EDTA concentration on lysis of *A. actinomycetemcomitans* Y4[a]

EDTA (mM)	% Reduction in optical density	
	EDTA	EDTA and HEWL
0.01	0	0
0.1	46	85
0.5	41	81
1.0	32	80
5.0	81	87
10.0	72	79
20.0	78	82
50.0	31	30
100.0	0	0

[a] Lysis is the percent reduction in optical density at 540 nm after 1 h of incubation in 0.01 M Tris-maleate buffer (pH 7.3) containing either EDTA or EDTA and 10 μg of HEWL per ml. In the absence of EDTA, reductions in turbidity did not occur during the 1-h period.

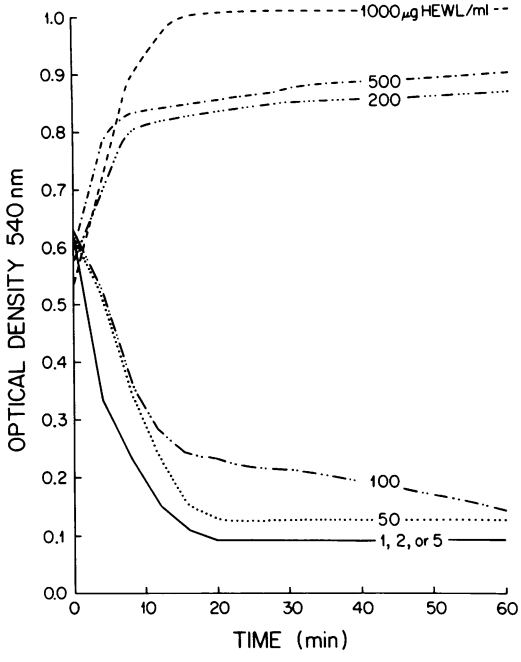

FIG. 3. Lysis curves obtained from suspension of exponential cells at 37°C for 1 h in 0.01 M Tris-maleate buffer (pH 7.3) containing 10 mM EDTA and HEWL at concentrations ranging from 1 to 1,000 μg/ml, as indicated.

Tris-maleate buffer (pH 7.3) containing 10 mM EDTA and from 1 to 1,000 μg of HEWL per ml, lysis occurred more readily at the lower enzyme concentrations (Fig. 3). A marked reduction in optical density was effected by 1, 2, and 5 μg of HEWL per ml (Fig. 3). Although reductions in turbidity were recorded for 50 and 100 μg of HEWL, upon visual examination aggregated material was noted at the bottom of the cuvettes.

With the higher enzyme concentrations tested (greater than 200 μg of HEWL per ml), both turbidimetric and visible evidence of aggregation were obtained (Fig. 3). This tendency toward aggregation occurred at all three pHs tested (Table 3). There was an upper limit for lysozyme concentration below which lysis of the cells occurred (Fig. 3; Table 3). Above that value, even under alkaline conditions and in the presence of EDTA, aggregation rather than lysis was evident (Fig. 3; Table 3). When human enzyme was tested in the turbidimetric assay, marked lysis was noted with very small amounts of lysozyme (Fig. 4). Less than 1 μg of HuL per ml effected greater than 80% reduction in optical density after 1 h of incubation of *A. actinomycetemcomitans* Y4 in 0.01 M Tris-maleate buffer (pH 7.3) containing 10 mM EDTA (Fig. 4). However, even in those suspensions containing as little as 1 μg of HuL per ml, some degree of

TABLE 3. Effect of lysozyme concentration and pH on lysis of *A. actinomycetemcomitans* Y4

HEWL (μg/ml)	% Change in optical density at pH:[a]		
	5.5	7.3	8.5
1	ND[b]	−82	−86
2	−36	−82	ND
5	ND	−82	−86
10	−49	−84	−86
50	ND	−80	−82
100	+10	−79	−76
200	+60	+50	+9
500	+60	+77	+92
1,000	+60	+87	ND

[a] Percent change in optical density at 540 nm as a reduction (−) or increase (+) in turbidity after 1 h of incubation in 0.01 M Tris-maleate buffer containing 10 mM EDTA and HEWL.

[b] ND, Not determined.

aggregation was noted upon visual examination, despite the dramatic reduction in turbidity (Fig. 4).

Electron microscopy. When exponential cells of *A. actinomycetemcomitans* Y4 were incubated for 1 h in buffer containing 1 μg of human enzyme per ml, greater than 25% of the cells were lysed (Fig. 5). Dense granular cytoplasmic material was observed in many of the lysing organisms, and extensive damage to the outer membrane-cell wall complex was readily discernible (Fig. 5). The remainder of the cells were uniform in shape, appeared similar to control

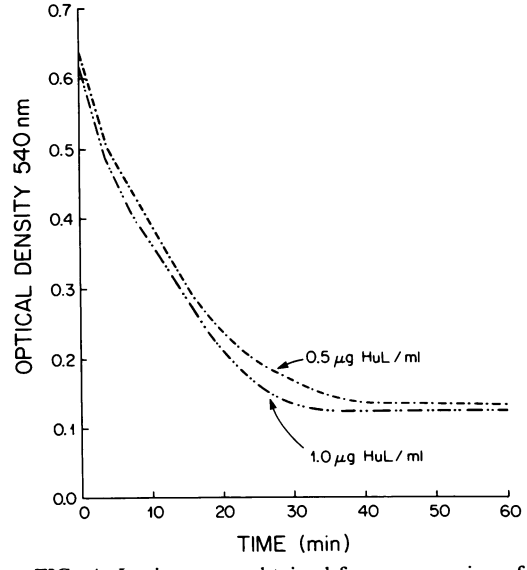

FIG. 4. Lysis curves obtained from suspension of exponential cells at 37°C for 1 h in 0.01 M Tris-maleate buffer (pH 7.3) containing 10 mM EDTA and HuL at 0.5 and 1 μg/ml, as indicated.

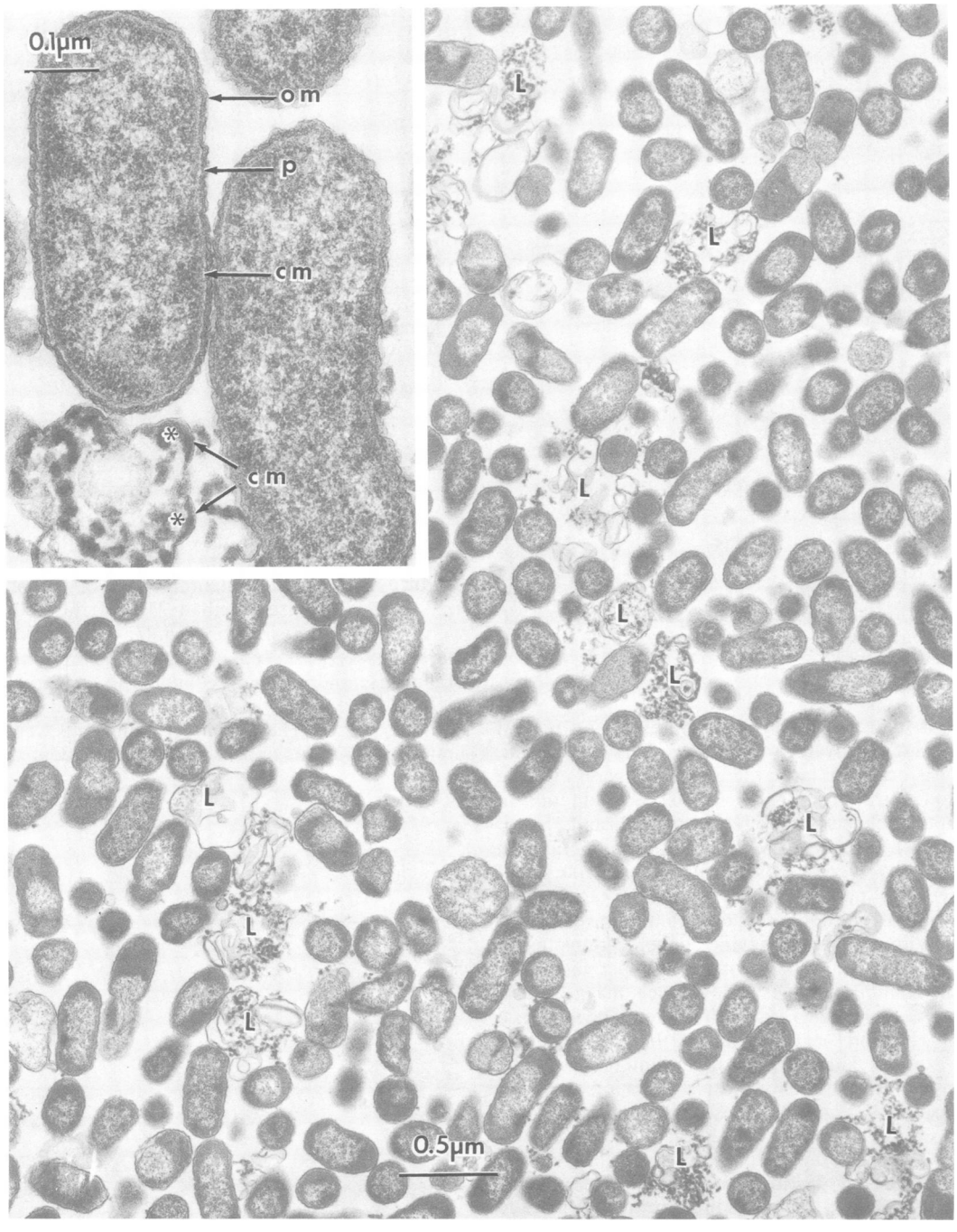

FIG. 5. Electron micrographs of *A. actinomycetemcomitans* Y4 cells treated with human enzyme alone. Cells were incubated for 1 h in 0.01 M Tris-maleate buffer (pH 7.3) containing 1 μg of HuL per ml. Low magnification (×26,400) demonstrates both lysed (L) and unlysed cells. Insert, High magnification (×64,000) of intact cells and a lysed cell. The lysed cell exhibits extensive damage to the outer membrane-cell wall complex, with dissolution of portions of the outer membrane, peptidoglycan, and cytoplasmic membrane. cm, Cytoplasmic membrane; om, outer membrane; p, periplasmic space; *, cytoplasmic blebs.

cells, and were without detectable damage (Fig. 5, insert). Intact cells appeared as homogeneous coccobacilli, as reported by others (29) and had features characteristic of gram-negative bacteria (4, 22). They possessed a three-layered cell wall-outer unit membrane complex composed of an outer membrane, a periplasmic space of variable electron opacity with a thin peptidoglycan layer, and an inner cytoplasmic membrane (Fig. 5, insert). Fibrillar nucleoid was well defined and was surrounded by granular cytoplasm (Fig. 5, insert). When cells were incubated in buffer containing both 1 μg of HuL per ml and 10 mM EDTA, all cells were either lysed or undergoing lysis (Fig. 6). Extensive damage to the outer membrane, to peptidoglycan, and to the inner membrane was readily seen. Cellular debris was scattered throughout the field and was both granular and stringy in appearance (Fig. 6). Although lysis was complete for most cells, there were significant numbers of cell ghosts composed of outer membrane components but lacking other cellular constituents (Fig. 6). This pattern of lysis also occurred in suspensions of cells treated with EDTA and HEWL (micrographs not shown). When *A. actinomycetemcomitans* Y4 cells were incubated for 1 h in buffer containing only 10 mM EDTA, significant damage was noted (Fig. 7). Although damage to the outer membrane was in evidence for some cells, clearing of the periplasmic space and of the inner cytoplasmic membrane was often seen (Fig. 7). In most instances, outer membranes were present in the shape of intact cells, but they surrounded either a condensed granular cytoplasm or a relatively clear space (Fig. 7).

DISCUSSION

These experiments clearly demonstrate the lytic sensitivity of *A. actinomycetemcomitans* Y4 cells to both HEWL and HuL. Turbidimetric evidence of lysis was dependent on several parameters, including concentrations of lysozyme, Tris, and EDTA, in addition to the pH of the suspension buffer. We found that a reduction in turbidity occurred most rapidly and to the greatest extent during the 1-h experimental period when cells were incubated under conditions of dilute Tris buffer and relatively alkaline pH (Fig. 1 and 2; Table 1). This was more likely related to the efficacy of EDTA action on outer membrane permeability rather than to the enzymatic properties of lysozyme (32–34), as the muramidase potential of lysozyme for isolated peptidoglycan substrate is minimally effected by ionic strength and gross electrostatic interactions (12, 13, 27, 37). In addition, it has been reported that lysozyme is quite capable of lysing coliform bacteria within a pH range of 3.5 to 10 (39). It is of interest that very small amounts of lysozyme were capable of lysing the cells under optimal experimental conditions (Fig. 3 and 4; Table 3). As little as 0.5 and 1 μg of human enzyme effected a dramatic reduction in turbidity of *A. actinomycetemcomitans* Y4 cells in the presence of 10 mM EDTA (Fig. 4). The reduction in turbidity was determined by electron microscopy to be due to cell lysis (Fig. 7). Surprisingly, large amounts of lysozyme increased the turbidity of cell suspensions even in the presence of EDTA (Fig. 3; Table 3). This was probably a result of lysozyme-mediated agglutination, which we reported to occur for both *A. actinomycetemcomitans* Y4 and other oral bacteria when they are suspended in buffer in the absence of EDTA (24, 43). Agglutination or aggregation may have been caused by neutralization of cell surface charge, possibly through the binding of lysozyme to outer membrane components such as lipopolysaccharide and lipoproteins (9, 14, 43, 50).

Increased turbidity of cell suspensions does not rule out possible cell damage or lysis. Visible agglutination may have been due to not only aggregation of cells, but also to clumping of cellular debris (4, 57). The occurrence of different degrees of lysis and clumping of cellular debris within the reaction mixtures may account for the variability in rates of reduction of optical density observed at the different enzyme concentrations tested (Fig. 3 and 4). Therefore, ultrastructural analysis must be used in conjunction with turbidimetric assays to assess bacteriolysis. In this regard, we observed that a reduction in turbidity did not occur when cells were suspended for 1 h in Tris buffer alone or buffer containing lysozyme but lacking EDTA (Table 2; reference 24). Yet, when examined by electron microscopy, although no lysis was evident when cells were suspended in Tris buffer alone, more than 25% of the cells were lysed in the presence of just 1 μg of human enzyme (Fig. 5) or 10 μg of HEWL (data not shown). This result is not unlike that previously observed with the gram-negative oral bacterium *Veillonella alcalescens* (57). It is possible that because exponentially growing cells were used in the experiments, cells which lysed were at a point in their cell cycle when either autolysins were activated, the outer membrane was permeable to lysozyme, or the peptidoglycan was more sensitive to hydrolysis (57). Preliminary studies with stationary-phase *A. actinomycetemcomitans* Y4 have revealed that these cells are more difficult to lyse (24). This is similar to other studies of lysozyme sensitivity, when it was observed that stationary-phase cells were more resistant than log-phase cells (7, 21). This has been attributed, in part, to an increase in cell wall *O*-acetyl groups and a decrease in *N*-acetyl groups (7, 21)

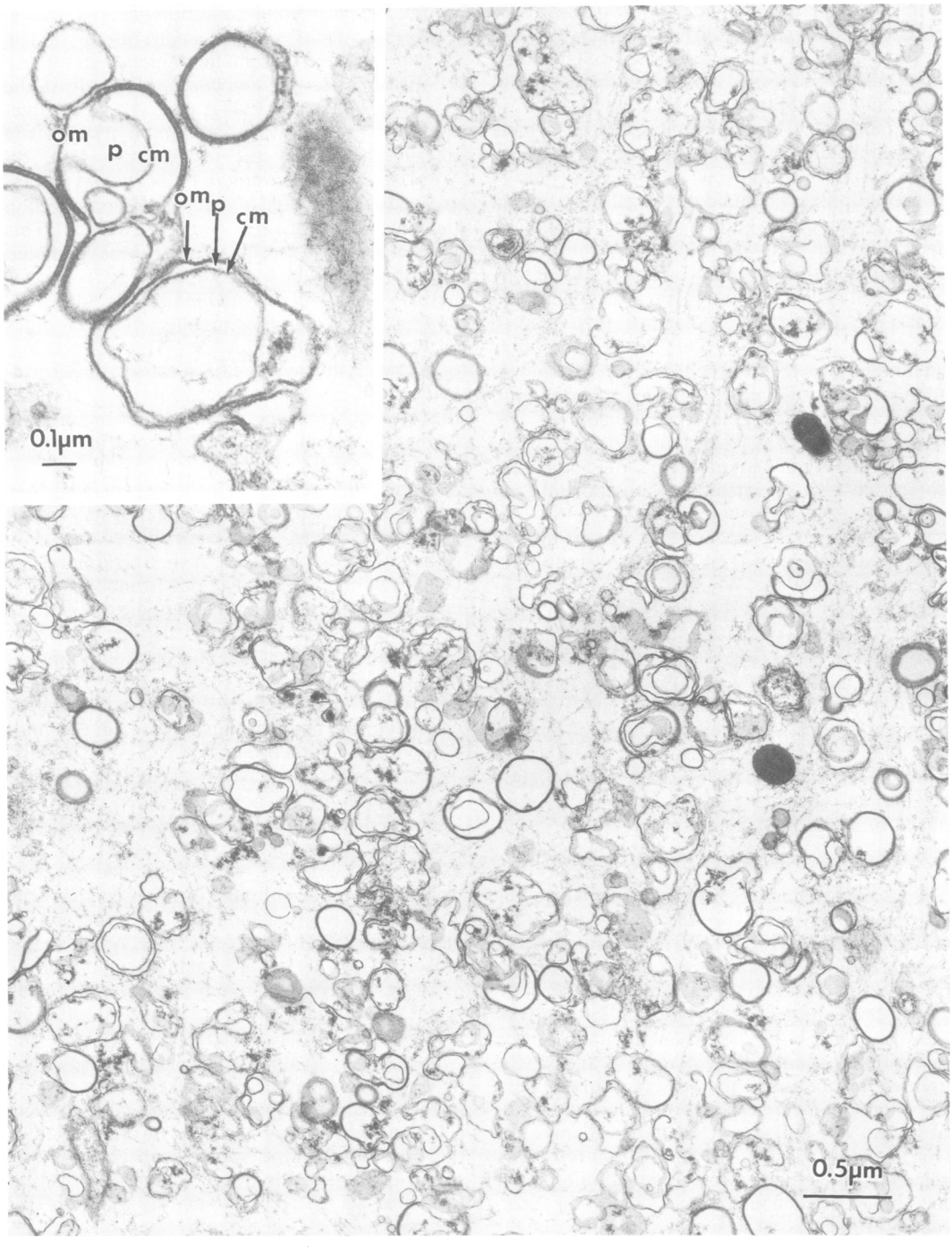

FIG. 6. Electron micrographs of *A. actinomycetemcomitans* Y4 cells treated with human enzyme and EDTA. Cells were incubated for 1 h in 0.01 M Tris-maleate buffer (pH 7.3) containing 10 mM EDTA and 1 μg of HuL per ml. Low magnification (×12,000) demonstrates cellular debris, lysing cells, and cell ghosts. Insert, High magnification (×45,000) illustrating extensive damage to and separation of the membrane-cell wall complex including outer membrane (om), periplasmic space (p), and cytoplasmic membrane (cm). Note clearing of intracellular compartment.

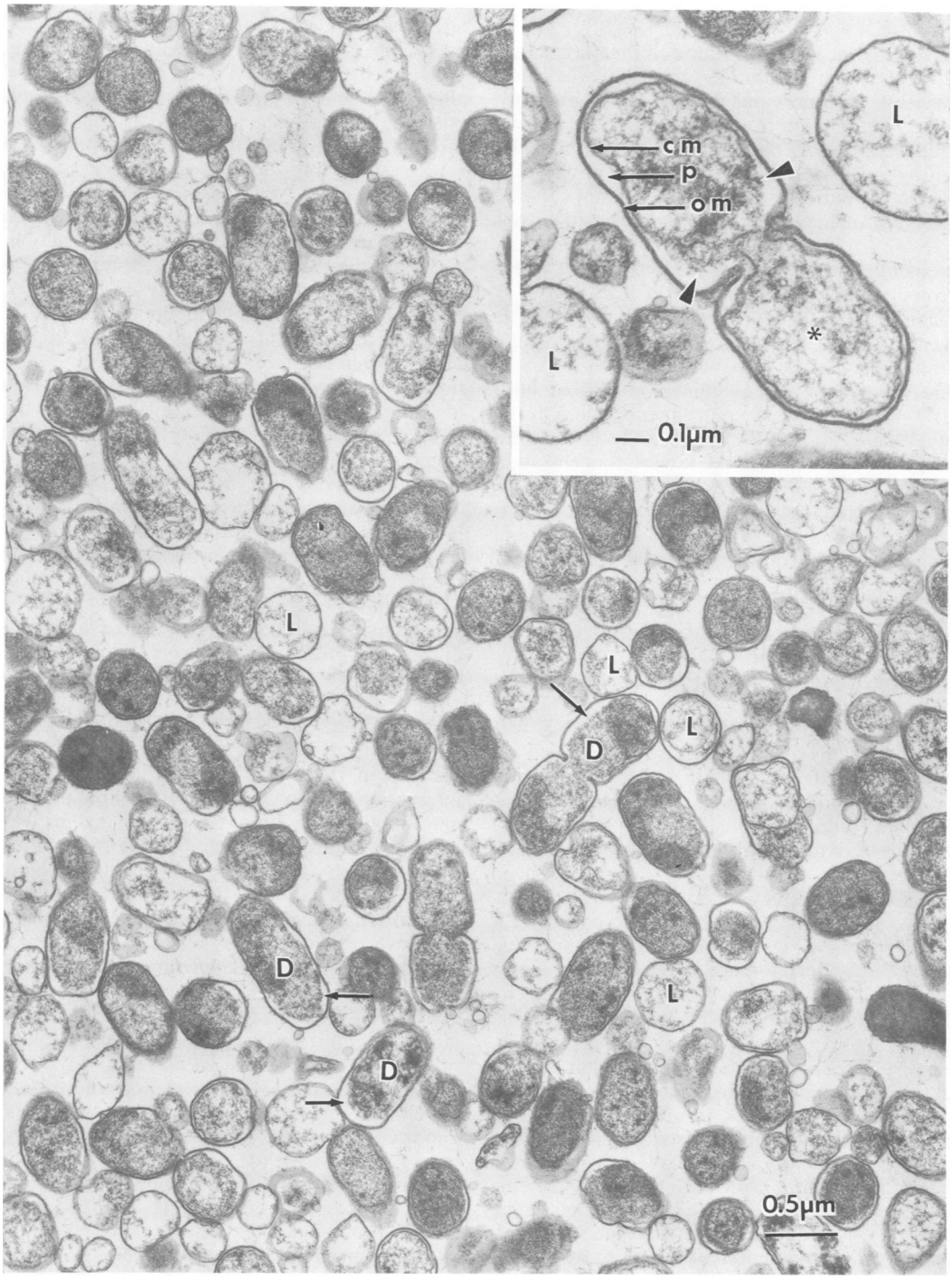

FIG. 7. Electron micrographs of *A. actinomycetemcomitans* Y4 cells treated with EDTA alone. Cells were incubated for 1 h in 0.01 M Tris-maleate buffer (pH 7.2) containing 10 mM EDTA. Low magnification (×20,240) demonstrates spheroplast-like lysing cells (L) and damaged cells (D) with degenerating cytoplasmic membrane and widened periplasmic space (arrow). Insert, High magnification (×50,000) of spheroplast-like lysing cells (L) and damaged cells (*) illustrating extensive damage to cytoplasmic membrane (cm, arrowhead) and periplasmic space (p) and relatively intact outer membrane (om).

and has been documented for resistant *Bacillus* strains (2).

Although it has been reported that Tris alone alters the outer membrane permeability of gram-negative microorganisms (25, 60), possibly through disruption of ionic interactions (49), we found that *A. actinomycetemcomitans* Y4 cells not only did not exhibit a reduction in turbidity (Table 2), but also were normal in appearance after 1 h of incubation in Tris buffer (data not shown). In addition, we have observed that preincubation of cultures in Tris did not affect growth of this microorganism (P. R. Boldt, V. J. Iacono, B. J. MacKay, and J. J. Pollock. J. Dent. Res. **61**:207, 1982). However, our results do not rule out the possibility that cell lysis was due in part to the combined effects of both EDTA and Tris at the outer membrane. On the other hand, the greatest degree of lysis occurred in those buffers having the lowest concentration of Tris (Fig. 2; Table 1). Furthermore, it has been reported that other buffers can be substituted for Tris in the evaluation of EDTA and lysozyme effects on gram-negative bacteria (33, 34). It is apparent, however, that further studies are required to determine the contribution of Tris to possible permeability changes of *A. actinomycetemcomitans* Y4.

The reduction in turbidity observed for those cells suspended in buffer containing EDTA in the absence of lysozyme (Fig. 2; Table 2) was not totally unexpected because it has been reported that EDTA destabilizes the outer membrane and initiates the release of lipopolysaccharides and other components (33, 34), possibly through activation of phospholipase A (15, 19). However, the extent of lysis was significant in that turbidity reductions approached those observed with suspensions containing both EDTA and lysozyme (Fig. 2; Table 2). This suggested that EDTA effected more than a change in outer membrane permeability. When examined by electron microscopy, it became apparent that EDTA alone initiated a lysis of the cells (Fig. 7). The pattern of lysis was unusual in that cellular destruction appeared to first involve a breakdown of the cytoplasmic membrane and disorganization of the periplasmic space (Fig. 7). Only completely damaged cells had a disintegrated outer membrane layer. Thus, many of the EDTA-treated cells appeared as spheroplast-like cell ghosts surrounding empty cytoplasmic compartments. The potential of EDTA to lyse gram-negative microorganisms has received very little attention in the literature (20, 28, 30, 34). It has been suggested that EDTA treatment of *Escherichia coli* cells disrupts a membrane barrier between autolytic enzymes and their cell wall substrate, leading to peptidoglycan hydrolysis and subsequent cell lysis (28). Our results are the first electron microscopic evidence for such an occurrence in a gram-negative periodontal pathogen (Fig. 7). EDTA would therefore not only increase outer membrane permeability and allow lysozyme to gain access to its substrate, but would also enhance cellular degradation through activation of autolysins. In support of a role for autolysins during lysis, we observed that the EDTA effect was markedly dependent on buffer pH and ionic strength (Fig. 1 and 2; Table 1). Furthermore, in a preliminary investigation, we found that heat-killed (60°C, 30-min incubation) log-phase cells were not lysed by EDTA, even under the most ideal conditions (P. R. Boldt et al., J. Dent. Res. **61**:207, 1982). Our observations should facilitate further investigations of the mechanism(s) of autolysin function in *Actinobacilli* spp. and an evaluation of the lytic responses to a variety of antibacterial agents.

The sensitivity of *A. actinomycetemcomitans* Y4 cells to small amounts of lysozyme may be of in vivo significance during the initial establishment of this potential periodontal pathogen in the gingival sulcus. Lysozyme is a major granular component of neutrophils (31), which are the predominant cell types in the gingival sulcus during the initiation and progression of periodontal diseases (3, 42). These polymorphonuclear leukocytes have been shown to release hydrolytic enzymes and bactericidal factors, in addition to lysozyme, when exposed to oral bacteria in vitro (55). The outcome of such an interaction in vivo would depend on the number and functional health of the neutrophils, as well as on the nature of the microbial flora and their sensitivity to the leukocyte granular components. Individuals with localized juvenile periodontitis have been shown to have a systemic defect in neutrophil chemotaxis (10, 17) which would tend to temper the migration of these cells into the gingival sulcus. In support of this concept, we have recently demonstrated that sulcular neutrophils from patients with localized juvenile periodontitis exhibit a delayed and reduced in vivo response to the local application of a chemotactic agent (S. Singh, L. Golub, V. Iacono, G. Nicoll, N. Ramamurthy, and P. Kaslick. J. Dent. Res. **61**:235, 1982). Conceivably, this defect in neutrophil migration could initially result in lower numbers of cells and available granular components in the sulcus, which would allow the normally lysozyme-sensitive *A. actinomycetemcomitans* Y4 to become established in the subgingival microflora. In addition, this microorganism has been shown to produce a component which is cytotoxic for human neutrophils (58). Although this leukotoxin may be of benefit to the initial colonization of the bacterium, the release of such cytotoxins from growing

cells (40) could initiate a negative feedback mechanism, i.e., lysozyme and other granular components from damaged neutrophils would then have access to the susceptible bacteria. In this regard, the lytic sensitivity of *A. actinomycetemcomitans* Y4 could represent a mechanism whereby lysozyme participates in the control of this periodontal pathogen.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service research grant DE-04296 from the National Institute of Dental Research.

LITERATURE CITED

1. **Amano, T., S. Inai, Y. Seki, S. Kashiba, K. Fugikawa, and S. Nishimura.** 1954. Studies on the immune bacteriolysis by lysozyme-like substance of leucocytes and egg-white lysozyme. Med. J. Osaka Univ. **4**:401–418.
2. **Araki, Y., T. Nakatoni, K. Nakayama, and E. Ito.** 1972. Occurrence of *N*-substituted glucosamine residues in peptidoglycan of lysozyme-resistant cell wall from *Bacillus cereus*. J. Biol. Chem. **247**:6312–6322.
3. **Attstrom, R.** 1971. Studies on neutrophil polymorphonuclear leukocytes at the dento-gingival junction in gingival health and disease. J. Periodontal Res. **8**(Suppl.):1–15.
4. **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.
5. **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.
6. **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.
7. **Brumfitt, W. A., A. C. Wardlaw, and J. T. Park.** 1958. Development of lysozyme-resistance in *Micrococcus lysodeikticus* and its association with increased *O*-acetyl content of the cell wall. Nature (London) **181**:1783–1784.
8. **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.
9. **Chopra, I., T. G. B. Howe, and P. R. Ball.** 1977. Lysozyme-promoted association of protein I molecules in the outer membrane of *Escherichia coli*. J. Bacteriol. **132**:411–418.
10. **Cianciola, L. J., R. J. Genco, M. R. Patters, J. McKenna, and C. J. van Oss.** 1977. Defective polymorphonuclear leukocyte function in a human periodontal disease. Nature (London) **265**:445–447.
11. **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.
12. **Davies, R. C., and A. Neuberger.** 1969. Modification of lysine and arginine residues of lysozyme and the effect on enzymatic activity. Biochim. Biophys. Acta **178**:306–317.
13. **Davies, R. C., A. Neuberger, and B. M. Wilson.** 1969. The dependence of lysozyme activity on pH and ionic strength. Biochim. Biophys. Acta **178**:294–303.
14. **Day, D. F., M. L. Marceau-Day, and J. M. Ingram.** 1978. Protein-lipopolysaccharide interactions. I. The reaction of lysozyme with *Pseudomonas aeruginosa* LPS. Can. J. Microbiol. **24**:196–199.
15. **Duckworth, D. H., E. M. Bevers, A. J. Verkleij, J. A. F. op Den Kamp, and L. L. M. Van Deenen.** 1974. Action of phospholipase A_2 and phospholipase C on *Escherichia coli*. Arch. Biochem. Biophys. **165**:379–387.
16. **Elsbach, P.** 1980. Degradation of microorganisms by phagocytic cells. Rev. Infect. Dis. **2**:106–128.
17. **Genco, R. J., and S. E. Mergenhagen.** 1979. Summary of a workshop on leukocyte function in bacterial diseases with an emphasis on periodontal disease. J. Infect. Dis. **139**:604–612.
18. **Goldschmidt, M. C., and O. Wyss.** 1967. The role of Tris in EDTA toxicity and lysozyme lysis. J. Gen. Microbiol. **47**:421–431.
19. **Hardaway, K. L., and C. S. Buller.** 1979. Effect of ethylenediaminetetraacetate on phospholipids and outer membrane function in *Escherichia coli*. J. Bacteriol. **137**:62–68.
20. **Hartmann, R., B. S. Bock-Henning, and U. Schwarz.** 1974. Murein hydrolases in the envelope of *Escherichia coli*. Eur. J. Biochem. **41**:203–208.
21. **Holden, J. T., and J. N. A. van Balgooy.** 1965. Effect of nutritional and physiological factors on the reaction between *Lactobacillus plantarum* and muramidase. Biochem. Biophys. Res. Commun. **19**:401–406.
22. **Holt, S. C., E. R. Leadbetter, and S. S. Socransky.** 1979. *Capnocytophaga*: new genus of gram-negative gliding bacteria. II. Morphology and ultrastructure. Arch. Microbiol. **122**:17–27.
23. **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.
24. **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.
25. **Irvin, R. T., T. J. MacAlister, and J. W. Costerton.** 1981. Tris(hydroxymethyl)aminomethane buffer modification of *Escherichia coli* outer membrane permeability. J. Bacteriol. **145**:1397–1403.
26. **Irving, J. T., M. G. Newman, S. S. Socransky, and J. D. Heeley.** 1975. Histological changes in experimental periodontal disease in rats monoinfected with a gram-negative organism. Arch. Oral Biol. **20**:219–220.
27. **Johnston, L. S., and F. C. Neuhaus.** 1978. Spin-label assay for lysozyme. Anal. Biochem. **85**:56–62.
28. **Kitano, K., and A. Tomasz.** 1979. Triggering of autolytic cell wall degradation in *Escherichia coli* by beta-lactam antibiotics. Antimicrob. Agents Chemother. **16**:838–848.
29. **Lai, C.-H., M. A. Listgarten, and B. F. Hammond.** 1981. Comparative ultrastructure of leukotoxic and non-leukotoxic strains of *Actinobacillus actinomycetemcomitans*. J. Periodontal Res. **16**:379–389.
30. **Leduc, M., R. Kasra, and J. van Heijinoort.** 1982. Induction and control of the autolytic system of *Escherichia coli*. J. Bacteriol. **152**:26–34.
31. **Leffell, M. S., and J. K. Spitznagel.** 1972. Association of lactoferrin with lysozyme in granules of human polymorphonuclear leukocytes. Infect. Immun. **6**:761–765.
32. **Leive, L.** 1965. Release of lipopolysaccharide by EDTA treatment of *E. coli*. Biochem. Biophys. Res. Commun. **21**:290–296.
33. **Leive, L.** 1968. Studies on the permeability change produced in coliform bacteria by ethylenediamine-tetraacetate. J. Biol. Chem. **243**:2373–2380.
34. **Leive, L.** 1974. The barrier function of the gram-negative envelope. Ann. N.Y. Acad. Sci. **235**:109–127.
35. **Luft, J. H.** 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. **9**:409–414.

35a. **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.

36. **Martin, B. F., B. M. Derby, G. N. Budzilovich, and J.**

Ransohoff. 1967. Brain abscess due to *Actinobacillus actinomycetemcomitans*. Neurology **17**:833–837.
37. **Neu, H. C.** 1969. The role of amine buffers in EDTA toxicity and their effect on osmotic shock. J. Gen. Microbiol. **57**:215–220.
38. **Newman, M. G., and S. S. Socransky.** 1977. Predominant cultivable microbiota in periodontosis. J. Periodontal Res. **12**:120–129.
39. **Noller, E. C., and S. E. Hartsell.** 1961. Bacteriolysis of *Enterobacteriaceae*. I. Lysis by four lytic systems utilizing lysozyme. J. Bacteriol. **81**:482–491.
40. **Nowotny, A., U. H. Behling, B. Hammond, C.-H. Lai, M. Listgarten, P. H. Pham, and F. Sanavi.** 1982. Release of toxic microvesicles by *Actinobacillus actinomycetemcomitans*. Infect. Immun. **37**:151–154.
41. **Osserman, E. F., and D. P. Lawlor.** 1966. Serum and urinary lysozyme (muramidase) in monocytic and myelomonocytic leukemia. J. Exp. Med. **124**:921–955.
42. **Page, R. C., and H. C. Schroeder.** 1976. Pathogenesis of inflammatory periodontal disease. A summary of current work. Lab. Invest. **33**:235–249.
43. **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. III. Information Retrieval, Inc., Washington, D.C.
44. **Repaske, R.** 1956. Lysis of gram-negative bacteria by lysozyme. Biochim. Biophys. Acta **22**:189–191.
45. **Repaske, R.** 1958. Lysis of gram-negative bacteria by lysozyme. Biochim. Biophys. Acta **30**:225–232.
46. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. **17**:208–213.
47. **Salton, M. R. J.** 1957. The properties of lysozyme and its action on microorganisms. Bacteriol. Rev. **21**:82–100.
48. **Salton, M. R. J.** 1968. Lytic agents, cell permeability, and monolayer penetrability. J. Gen. Physiol. 52:2275–2525.
49. **Schindler, P. R. G., and M. Teuber.** 1978. Ultrastructural study of *Salmonella typhimurium* treated with membrane-active agents: specific reaction of dansylchloride with cell envelope components. J. Bacteriol. **135**:198–206.
50. **Scibienski, R. J.** 1979. Cellular parameter of the immunological memory induced by lysozyme-LPS mixtures and complexes. Immunol. Commun. **8**:325–336.
51. **Slots, J.** 1977. The predominant cultivable microflora of advanced periodontitis. Scand. J. Dent. Res. **85**:114–121.
52. **Slots, J., H. S. Reynolds, and R. J. Genco.** 1980. *Actinobacillus actinomycetemcomitans* in human periodontal disease: a cross-sectional microbiological investigation. Infect. Immun. **29**:1012–1020.
53. **Sophianopoulos, A. J., C. K. Rhodes, D. N. Holcomb, and K. E. Van Holde.** 1962. Physical studies of lysozyme. I. Characterization. J. Biol. Chem. **237**:1107–1112.
54. **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.
55. **Taichman, N. S., C.-C. Tsai, P. C. Baehni, N. Stoller, and W. P. McArthur.** 1977. Interaction of inflammatory cells and oral microorganisms. IV. In vitro release of lysosomal constituents from polymorphonuclear leukocytes exposed to supragingival and subgingival bacterial plaque. Infect. Immun. **16**:1013–1023.
56. **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.
57. **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.
58. **Tsai, C.-C., W. P. McArthur, P. C. Baehni, B. F. Hammond, and N. S. Taichman.** 1979. Extraction and partial characterization of a leukotoxin from a plaque-derived gram-negative microorganism. Infect. Immun. **25**:427–439.
59. **Vandepitte, J., H. De Geest, and P. Jousten.** 1977. Subacute bacterial endocarditis due to *Actinobacillus actinomycetemcomitans*. Report of a case with a review of the literature. J. Clin. Pathol. **30**:842–846.
60. **Voss, J. G.** 1967. Effects of organic cations on the gram-negative cell wall and their bactericidal activity with ethylene diaminetetraacetate and surface active agents. J. Gen. Microbiol. **48**:391–400.
61. **Weiss, J., P. 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.