Release of Toxic Microvesicles by *Actinobacillus actinomycetemcomitans*

A. NOWOTNY,* U. H. BEHLING, B. HAMMOND, C.-H. LAI, M. LISTGARTEN, P. H. PHAM, AND F. SANAVI

Center for Oral Health Research, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Oral isolates of *Actinobacillus actinomycetemcomitans* (strain Y4) release spherical microvesicles in large numbers during normal growth. The biological activities of these products were studied, and it was estimated that approximately 1/10 of their dry weight was made up of heat- and proteolysis-resistant endotoxin. The chicken embryo lethality and bone-resorbing activity of the microvesicles were heat stable but proteolysis sensitive. Other laboratories have reported the presence of a heat- and proteolysis-sensitive leukotoxin in similar preparations. Accordingly, the microvesicles released by strain Y4 may contain, in addition to endotoxin, several potent substances which are highly toxic and active in bone resorption, and these may be significant factors in the pathogenesis of periodontal diseases.

The release of membrane and cell wall-bound components or both from some gram-negative bacteria under nutritionally restricted conditions has been described previously (21). Investigators in our laboratories have demonstrated the release of endotoxins from ethyl methane sulfonate-induced mutants of *Serratia marcescens* (12, 20) as well as from some common gram-negative strains.

Recent electron microscopic studies have revealed that in some oral bacteria numerous microvesicles are released into the medium through a budding process of the outer cell membrane. This process has been reported in certain strains of *Capnocytophaga* (14), *Bacteroides gingivalis* (8), and more recently in *Actinobacillus actinomycetemcomitans* strains (6). These strains were all isolated from the periodontal pockets of patients with adult or juvenile periodontitis. One of these strains, identified as Y4 and originally isolated by Newman and Socransky (11) from a patient with juvenile periodontitis, caused severe alveolar bone resorption in monoinfected gnotobiotic rats (5). Baehni and associates (1) presented the taxonomic evidence that this strain has most of the major characteristics of *A. actinomycetemcomitans*. Detailed morphological studies were published recently (6).

Based on our earlier observations and on results obtained by others (2, 10, 17), we assumed that Y4 microvesicles should contain endotoxins as one of the components. The aims of the research reported here were (i) to estimate the endotoxin content in the microvesicles by biological assays of endotoxicity, (ii) to determine whether the microvesicles contain other biologically active components, and (iii) to investigate whether such substances might be involved in the pathomechanism of destructive periodontitis.

MATERIALS AND METHODS

The isolation of vesicles was based on differential and sucrose density centrifugation of broth culture supernatants as outlined previously (B. F. Hammond, M. Darkes, C.-H. Lai, and C. C. Tsai, J. Dent. Res. **60**:A333, abstr. no. 89, 1981). The extraction and purification of lipopolysaccharide (LPS) of Y4 were carried out by the phenol-water procedure of Westphal and Lüderitz.

Preparations to be tested for heat resistance were kept at 100°C for 3 h at pH 7. A fungal proteinase K (Beckman Instruments, Inc.) was added to suspensions of Y4 microvesicles or endotoxin solution. The concentration of the substrates was adjusted to 1 μg (dry weight) per ml. Proteinase (0.1 μg/ml) was added, and the preparation was thoroughly mixed and incubated at 37°C for 1 h. It was then heated at 100°C to inactivate the enzyme. As controls, heat-inactivated enzyme, heated Y4 endotoxin, or heated suspensions of microvesicles were used. Enzyme-treated Y4-LPS solution was also included as a control.

Endotoxicity assays were used to estimate the endotoxin content of the microvesicles. *Limulus* lysate clotting, local Shwartzman test, and pyrogenicity measurements were carried out by the routine procedures reported previously (13). Chicken embryo lethality was determined by injecting 11-day-old chicken embryos intravenously with various amounts of Y4 endotoxin or microvesicles and observing mortality 24 h later. The method used here has been described by Smith and Thomas (19). The Spearman-Karber equation was used to determine the 50% lethal dose (13).

The bone resorption assay developed by Raisz and

Niemann (15) was also utilized in this study. Details of the assay have been described elsewhere (3). Briefly, 18-day-pregnant Sprague-Dawley rats were injected with 360 µCi of ^{45}Ca. The rats were sacrificed the next day, and the fetal radii and ulnae were removed. The calcified shafts of the bones were dissected and precultured for 24 h in BGJ medium (GIBCO Laboratories). The bones were then transferred to BGJ medium supplemented with 1 mg of bovine serum albumin per ml. The treatment group received various concentrations of test substances in addition to the medium. Bones were kept in culture for 5 days in a 95% air–5% CO_2 atmosphere at 37°C with changes of the medium on days 2 and 4. The activity of ^{45}Ca in bone and medium was measured by liquid scintillation. Bone resorption was measured as the percentage of ^{45}Ca released from the bone into the medium during the culture period. The mean value, standard deviation, and significance of differences were calculated.

RESULTS

The measurements of endotoxin-like activities in the vesicle preparation were compared with phenol-water-extracted Y4 endotoxins (Table 1). In three assays, both heat-treated or proteolyzed and untreated samples were used. These assays were chicken embryo lethality, bone resorption, and local Shwartzman tests. In pyrogenicity and Limulus lysate clotting assays, only untreated microvesicles were tested. All assays indicated that the microvesicles contained endotoxin-like activities. The local Shwartzman test (Table 1) and pyrogenicity assays (data not shown), which are characteristic reactions of endotoxicity, indicated that the endotoxin content in the vesicles could be roughly 1/10 of their dry weight. The Limulus lysate clotting assay indicated lower values (1/10 to 1/100). However, this requires further critical experimentation.

The in vitro bone-resorbing activities of Y4 endotoxin and Y4 microvesicles were about equal on a weight basis (Table 2) despite the fact that the endotoxin content of the vesicles was only about 1/10 of their dry weight. The heat resistance of bone-resorbing activity of endotoxin and vesicles was compared. Endotoxic LPS,

as expected, was not affected by heat. The bone-resorbing activity of microvesicles showed only a slight, insignificant, reduction (Fig. 1). Proteolysis did not alter the bone-resorbing activity of the Y4 endotoxin. This observation is in agreement with the known resistance of such preparations to proteolytic enzymes (7). The vesicle preparation, on the other hand, lost approximately 70 to 90% of its bone-resorptive potency (Fig. 1). This finding indicates the presence of a proteolysis-sensitive but heat-resistant bone-resorbing nonendotoxin component in the vesicles.

Unusually high toxicity of the microvesicle preparation was measured by the chicken embryo lethality assay. The vesicles appeared more toxic on a weight basis than did the isolated LPS. The results indicate that a highly toxic substance is associated with these microvesicles (Table 3). The effect of heat and proteolysis on the toxicity of the microvesicles was also studied. As expected, endotoxin toxicity was not reduced by the treatments described above (7). However, after proteolysis, the toxicity of the microvesicles was greatly reduced. Heat treatment of the vesicles slightly enhanced their toxicity (Table 3). According to these findings, the Y4 microvesicles contain a proteolysis-sensitive but heat-resistant toxin(s). The findings allow the assumption that the bone-resorbing and the toxic substance(s) may be identical.

DISCUSSION

Although several papers describe the phenomenon of bacterial cell wall shedding, only a few attempts have been made to analyze these products chemically (21).

As far as the biological activities of such products are concerned, endotoxicity was detected early. It was also shown that some bacteria will release endotoxin from their surfaces, without any observable damage to the cell itself (2, 10). Gram-positive bacteria are reported to release lipoteichoic acid (9), which is known to

TABLE 1. Local Shwartzman reactivity of Y4 LPS preparations and Y4 microvesicles[a]

Intradermal dose (µg)	Area of lesion (cm^2 [%])			
	Y4 microvesicles	Heated Y4 microvesicles	Y4 LPS	Heated Y4 LPS
2.5	0 (0)	0 (0)	0.16 (100)	0.21 (131)
5.0	0.06 (15)	0.08 (20)	0.40 (100)	0.50 (125)
10.0	0.11 (14)	0.14 (18)	0.78 (100)	0.84 (108)
20.0	0.19 (16)	0.20 (16)	1.21 (100)	1.14 (94)
40.0	0.41	0.44	NT	NT

[a] At 24 h after intradermal injection, the rabbit received 20 µg of LPS intravenously. Four rabbits were used for the determination, and each rabbit received all the concentrations from both Y4 LPS and the tested preparation. The percentages in parentheses express the size of the lesions as compared with lesions elicited by nontreated Y4 LPS. NT, Not tested.

TABLE 2. Effect of Y4 microvesicles, Y4 LPS, and parathyroid hormone on the release of ^{45}Ca from fetal rat long bone in vitro[a]

Substance	% ^{45}Ca release
Y4 microvesicles (1 µg/ml)	60.64 ± 12.81[b]
Y4 LPS (1 µg/ml)	74.54 ± 13.18[b]
Parathyroid hormone (200 ng/ml)	95.6 ± 2.31[b]
Control	21.18 ± 3.59

[a] The embryonal bones were incubated in culture medium for 5 days.

[b] Values are the means ± standard error for 8 to 10 bones significantly different from control ($P < 0.005$).

manifest some biological effects similar to those of endotoxins. These examples show clearly that bacterial cell walls shed various components without impairing the viability of the cell.

In view of these reports, we assumed that Y4 microvesicles would contain endotoxin. We did not use chemical assays for the detection of endotoxins because there are no chemical assays which would give a positive reaction exclusively with endotoxins: 2-keto-3-deoxyoctulosonic reaction is not specific; 3-OH fatty acids are present in a few nonendotoxin natural products, as are all other constituents. Therefore, we decided to attempt to quantitate the endotoxin content of vesicles by using the characteristic endotoxicity reactions such as pyrogenicity, local Shwartzman reactivity, and *Limulus* lysate clotting tests in addition to the non-characteristic toxicity assay with 11-day-old chicken embryos (intravenous injection) and measurement of bone resorption in vitro. We estimated that only about 1/10 or less of the dry weight of the microvesicles was made up of endotoxin. By the local Shwartzman reaction, approximately 15% endotoxin content was estimated; the pyrogenicity test indicated approximately 5% endotoxin, and the *Limulus* test showed a range between 1 and 10%. The finding that these microvesicles contain one or more highly toxic components in addition to endotoxins was unexpected. Even if they consisted of 100% endotoxic LPS, they
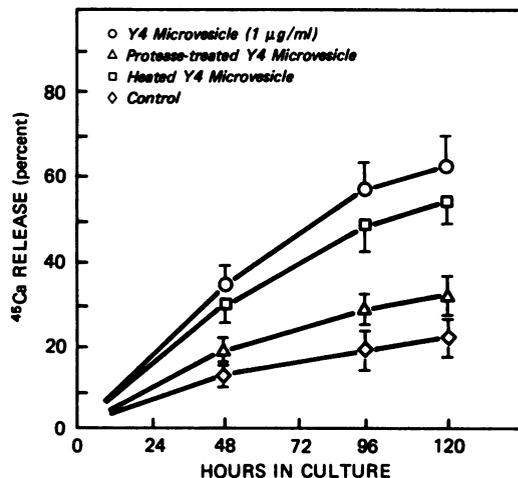

FIG. 1. In vitro bone resorption by heat- or proteolytic enzyme (protease K)-treated microvesicles compared with nontreated preparation.

should be still less toxic than the microvesicles in the chicken embryo assay. Furthermore, we found that this toxin is sensitive to proteolytic enzymes (unlike endotoxins) but resistant to extended heat treatment, similar to the proteolysis and heat sensitivity of a bone-resorbing component present in these microvesicles. It is known that endotoxin is a potent inducer of in vitro bone resorption (3, 4, 16). One of the bone-resorbing substances we found in the microvesicles is different from endotoxin, since it is sensitive to proteolysis, whereas endotoxin is not. Whether the toxic and bone-resorbing components are the same remains to be investigated. It may be important that Y4 microvesicles were also found to be toxic for neutrophils, as reported recently (Hammond et al., J. Dent. Res. 60A:333, abstr. no. 89, 1981). It could be shown that this leukotoxic substance was sensitive to both proteolysis and heat treatment; therefore, it is probably not identical to any of the active components of the microvesicles as discussed above.

TABLE 3. Chicken embryo lethality[a] of heated or proteolytic enzyme-treated Y4 LPS and Y4 microvesicles compared with untreated preparations

Intravenous dose (µg)	Survivors/total no.					
	Y4 microvesicles			Y4 LPS		
	Untreated	Digested	Heated	Untreated	Digested	Heated
1	0/6	3/6	0/6	1/6	0/6	0/6
0.1	1/6	5/6	0/6	3/6	0/6	2/6
0.01	6/6	6/6	5/6	4/6	6/6	5/6
0.001	6/6	6/6	6/6	6/6	6/6	5/6

[a] The 50% lethal doses for Y4 microvesicles were: untreated, 0.046 µg; digested, ~1 µg; heated, 0.021 µg. The 50% lethal doses for Y4 LPS were: untreated, 0.069 µg; digested, 0.032 µg; heated, 0.032 µg.

Accordingly, there may be at least three biologically highly active components in the microvesicles: (i) endotoxins, (ii) another toxin which may be identical to a bone resorption-inducing substance, and (iii) a leukotoxin. Two of these can induce bone resorption, and all three are toxic. Of course, there may be more than three active components in the microvesicles. Only careful fractionation and measurements of additional biological activities will be able to resolve this question.

The potential role of the microvesicles in the etiology of periodontitis should be considered. *A. actinomycetemcomitans* cells have been found in significantly higher numbers in the gingival crevice of juvenile periodontitis patients than in the crevices of normal individuals (18). These cells could be releasing large numbers of microvesicles into the crevicular environment, thereby facilitating the interaction of host cells with the pharmacologically active substances in the vesicles. Since microvesicles have a small diameter, they can pass easily through anatomical barriers which might retain whole bacteria. Finally, when the very significant biological activities concentrated in these small vesicles are considered, it is likely that they have a high pathogenic potential. Conclusive answers, on the other hand, can come only from extensive studies comparing pathogenic and nonpathogenic microvesicle-releasing and non-microvesicle-releasing microorganisms in in vivo and in situ experiments.

LITERATURE CITED

1. Baehni, P., C.-C. Tsai, W. P. McArthur, B. F. Hammond, and N. S. Taichman. 1979. Interaction of inflammatory cells and oral microorganisms. VIII. Detection of leukotoxic activity of a plaque-derived gram-negative microorganism. Infect. Immun. 24:233–243.
2. DeVoe, I. W., and J. E. Gilchrist. 1973. Release of endotoxin in the form of cell wall blebs during in vitro growth of Neisseria meningitidis. J. Exp. Med. 138:1156–1167.
3. Hausmann, E., L. G. Raisz, and W. A. Miller. 1970. Endotoxin: stimulation of bone resorption in tissue culture. Science 168:862–864.
4. Horton, J. E., L. G. Raisz, H. A. Simmons, J. J. Oppenheim, and S. E. Mergenhagen. 1972. Bone resorbing activity in supernatant fluid from cultured human peripheral blood leukocytes. Science 172:793–795.
5. Irving, J. T., M. G. Newman, S. S. Socransky, and J. D. Heeley. 1975. Histological changes in experimental periodontal disease in rats mono-infected with a gram-negative microorganism. Arch. Oral Biol. 20:219–220.
6. 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.
7. Lehrer, S., and A. Nowotny. 1972. Isolation and purification of endotoxin by hydrolytic enzymes. Infect. Immun. 6:928–933.
8. Listgarten, M. A., and C.-H. Lai. 1979. Comparative ultrastructure of Bacteroides melaninogenicus subspecies. J. Periodontal Res. 14:332–340.
9. Markham, J. L., K. W. Knox, A. J. Wicken, and M. J. Hewett. 1975. Formation of extracellular lipoteichoic acid by oral streptococci and lactobacilli. Infect. Immun. 12:378–386.
10. Marsh, D. G., and M. J. Crutchley. 1967. Purification and physicochemical analysis of fractions from the culture supernatant of Escherichia coli O78 K80: free endotoxin and a non-toxic fraction. J. Gen. Microbiol. 47:405–420.
11. Newman, M. G., and S. S. Socransky. 1977. Predominant cultivable microbiota in periodontosis. J. Periodontal Res. 12:120–128.
12. Nowotny, A. 1968. Recent problems of endotoxin research. Ann. Immunol. Hung. 11:12–15.
13. Nowotny, A. 1979. Basic exercises in immunochemistry, 2nd ed. Springer Verlag, New York.
14. 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.
15. Raisz, L. G., and I. Niemann. 1969. Effect of phosphate, calcium and magnesium on bone resorption and hormonal responses in tissue culture. Endocrinology 85:446–452.
16. Robinson, P. J., and I. M. Shapiro. 1975. The effect of endotoxin and human dental plaque on the respiratory rate of bone cells. J. Periodontal Res. 10:305–308.
17. Russel, R. R. B. 1976. Free endotoxin. Microbios Lett. 2:125–135.
18. Slots, J., H. S. Reynolds, and R. J. Genco. 1980. Actinobacillus actinomycetemcomitans in human periodontal disease: a cross-sectional microbiological investigation. Infect. Immun. 29:1013–1020.
19. Smith, R. T., and L. Thomas. 1956. The lethal effect of endotoxins on the chick embryo. J. Exp. Med. 104:217–231.
20. Tripodi, D., and A. Nowotny. 1966. Relation of structure to function in bacterial O-antigens. V. Nature of active sites in endotoxic lipopolysaccharides of Serratia marcescens. Ann. N.Y. Acad. Sci. 133:604–621.
21. Work, E., K. W. Knox, and M. Vesk. 1966. The chemistry and electron microscopy of an extracellular lipopolysaccharide from Escherichia coli. Ann. N.Y. Acad. Sci. 133:438–449.