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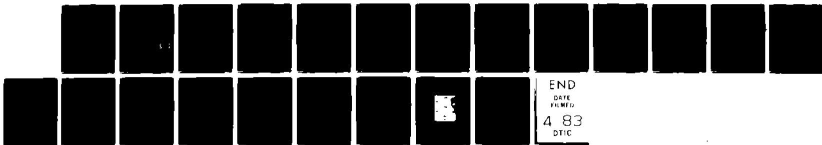

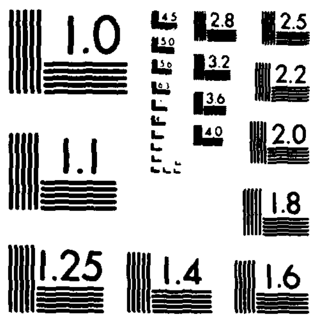

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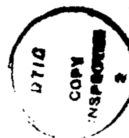

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Eubacterium brachy - Reactivity in *In Vitro*

Bone Resorptive Bioassay

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Eubacterium brachy - Reactivity in *In Vitro*

Bone Resorptive Bioassay

ABSTRACT

Recent studies have demonstrated an association of *Eubacterium* sp. with the subgingival microflora of patients with chronic periodontitis. One species, *Eubacterium brachy*, was evaluated to determine the possible mechanisms by which this microorganism may contribute to this disease process. An extracellular antigen was identified in the culture supernatant which reacted with antibodies in human sera. This antigen was isolated by methanol precipitation and purified by gel filtration. The purified extracellular antigen was reacted *in vitro* with $^{45}CaCl_2$-labeled fetal rat bone in a bone resorptive bioassay. This antigen was shown to have a molecular weight of 170,000, to share a line of identity with a sonicated preparation of *E. brachy* whole cells, and to result in increased $^{45}CaCl_2$ release from fetal rat bones when cultures were exposed to the purified extracellular antigen at concentrations of 10-53 μg/ml.

The role of microorganisms in the etiology of periodontal diseases is well established. This correlation of disease with changes in the oral microflora was described by Löe *et al*[1] in a study in which gingivitis was induced in healthy subjects by withdrawing oral hygiene procedures. The changes in the microflora were determined microscopically as disease became evident. These findings have been followed by extensive studies, both morphological and cultural, of the oral microfloral associated with health and disease. The morphological differences of the microbial flora associated with periodontal health and various clinical disease states have been described by dark field microscopy.[2] These findings describe a substantial increase in the proportion of motile organisms occurring in chronic periodontitis patients in comparison with healthy subjects. The variability of the microbial flora within diseased periodontal sites has also been evaluated morphologically.[3] They concluded that the mean percentages of spirochetes varied directly with increasing disease scores while the mean percentages of coccoid cells followed a reverse pattern. This trend toward increased motility with increasing disease is seen to be reversible by scaling and root planing.[4] Numerous cultural studies have been directed toward identifying specific microorganisms or groups of microorganisms associated with various disease states in man. Newman *et al*[5] characterized the microbial flora associated with periodontosis (juvenile periodontitis) and identified five groups of gram-negative anaerobic rods of which Group II, including the *Capnocytophaga*

species, were isolated more frequently than in healthy controls. Tanner *et al*[6] described the significance of *Actinobacillus actinomycetemcomitans* in lesions of juvenile periodontitis. This organism has been shown to have a direct toxic effect on PMNL, which is felt to be partially responsible for its pathogenicity in this disease process.[7] *Fusobacterium nucleatum* has been shown to increase in numbers with increasing gingival inflammation[8] and in early[9] and advanced periodontitis.[6,10] During the second trimester of pregnancy, *Bacteroides melaninogenicus* ss. *intermedius* levels have been shown to increase proportionately with increasing levels of gingivitis.[11] Recently, three species of *Eubacterium* have been isolated from human periodontitis.[12] These species are isolated in greater numbers in subgingival samples than supragingival samples and are isolated in relatively high frequency (24-70% of samples).[13] In young adult patients with severe generalized periodontitis, these three species can comprise, on the average, 16% of the subgingival flora of diseased sites.

The role of any microorganism in the immunopathology of periodontal diseases is based on the ability of the microorganism, its by-products, or structural components to gain access to the target tissue. The ability of microorganisms to penetrate the junctional epithelium has been shown in ANUG, and in advanced periodontitis in humans.[14-17] The potential of microbial substances to penetrate an intact junctional epithelium has also been demonstrated to include

endotoxin,[18] hyaluronidase and collagenase if preceded by hyaluronidase,[19] horseradish peroxidase,[20] streptococcal polysaccharide following hyaluronidase[21] and ^{14}C-phenytoin and ^{14}C-albumin.[22] The purpose of this study was to describe potentially pathologic mechanisms associated with the gram-positive anaerobic microorganism *Eubacterium brachy*.

MATERIALS AND METHODS

Cultivation and Antigen Preparation

Eubacterium brachy (ATCC 33089)* was grown in a peptone yeast-extract-glucose media (PYG) (per 100 ml) peptone 2 gm, yeast extract 1 gm, cysteine HCl 0.05 gm, glucose 1 gm, resazurin .1 mg and 4.0 ml of VPI salt solution[23] containing /L($CaCl_2$, 0.2 gm; $MgSO_4$, 0.2 gm; K_2HPO_4, 1 gm; and KH_2PO_4, 1 gm) anaerobically in a BBL† gas-pack, anaerobic jar system at 37C. The cells were harvested by centrifugation at 10,000 X g for 10 minutes at 4C, washed 3X in 0.01 M phosphate buffer containing 0.15 M NaCl (PBS), pH 7.2 and resuspended in PBS as a 1:10 dilution of packed whole cells after centrifugation at 2,000 X g for 10 minutes. This whole cell suspension was stored at -20C. To the cell-free broth supernatant was added absolute methanol (1:8) at 4C for 18 hours and the resultant precipitate sedimented at 2,000 X g and resuspended to its original volume in PBS and designated as crude extracellular antigen (ECA). The ECA was reacted by double diffusion in agar (1% agarose in PBS, pH 7.2, 4 ml per glass slide) with human sera previously shown to be reactive with a sonicated preparation of *Eubacterium brachy* whole cells.[24]

*American Type Culture Collection, Rockville, MD
†Baltimore Biological Laboratory, Baltimore, MD

The ECA was isolated by gel filtration on a 2.5 cm X 100 cm column packed with degassed Sephacryl S-200 superfine‡ equilibrated with PBS at a flow rate of 1 ml/minute. Fractions (5 ml) were collected on an Isco Fraction Collector UA-5 monitored with a type 6 optical unit at 280 nm. A P-3 peristolic pump was utilized to insure uniformity of flow. Blue dextran ($2x10^6$ daltons), aldolase (158,000 daltons), ovalbumin (45,000 daltons), chymotrysinogen A (25,000 daltons), and ribonuclease A (13,700 daltons) were used as standards. The fractions containing ECA were pooled and precipitated in absolute methanol, centrifuged, and resuspended in PBS to the original volume and designated purified ECA. Verification of identity was determined by lines of identity by double diffusion in agar.

Bone Resorptive Assay[25]

Briefly, Sprague-Dawley rats in the 18th day of gestation were injected intraperitoneally with 200 µCi of $^{45}CaCl_2$.§ After sixteen hours the rats were sacrificed by CO_2 suffocation and the fetuses removed aseptically. The forelimbs of each fetus were removed and the bony diaphyses of the radial and ulnar bones were dissected free of muscle, connective tissue, and cartilagenous epiphyses. The four bones from each fetus were placed separately in four wells of a 24-well tissue culture plate containing 250 µl/well BGJ_b¶ media supplemented with 1 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 1 mg/ml bovine serum albumin fraction IV.¶ After a

‡Pharmacia Fine Chemicals, Piscataway, NJ
§New England Nuclear, Boston, MA
¶Grand Island Biological, Grand Island, NY

24 hour equilibration period at 37C in 5% CO_2 and 100% humidity, the culture medium from each well was removed by aspiration and replaced with 125 µl of BGJ_b and 125 µl of the appropriate test or control substance so that one pair of bones from each fetus was experimental and the other pair was the corresponding control. The bones from at least two fetuses were used for each assay of the various concentrations of ECA. All tests were repeated to verify results. Protein concentration of ECA was determined by absorbance at 280 nM utilizing BSA Fraction V as a standard. After 120 hours of incubation, the culture medium from each well was removed and the amount of ^{45}Ca released from each bone was determined by liquid scintillation spectrophotometry.** The bones were decalcified with 5% trichloroacetic acid and the amount of ^{45}Ca in each bone was also determined. The per cent ^{45}Ca release from the bones was computed and a test/control (T/C) per cent release ratio determined. T/C ratios greater than 1 indicate osteolytic activity.

RESULTS

Immunodiffusion

Both the crude ECA and the purified ECA shared lines of identity with the sonicated preparation of *Eubacterium brachy*[26] and with the PYG-supernatant. Uninoculated PYG showed no reactivity by double diffusion in agar (Figure 1). This antigen preparation of sonicated whole cells of *Eubacterium brachy* has previously been shown to be distinct from other sonicated preparations of *Eubacterium*

**Packard LCC Model 460C

nodatum and other gram-positive oral microorganisms.[27] The molecular weight of the purified ECA as determined by gel filtration was approximately 170,000 daltons. (Figure 2)

Bone Resorptive Assay

The results of the bone resorptive assay demonstrate an increased release of ^{45}Ca from fetal rat bones when exposed to the purified ECA (Table I). In concentrations exceeding approximately 100 µg/ml of protein in the purified ECA, the ratio of test over control approaches one indicating no difference in ^{45}Ca release between test and control. It is of interest to note that Hausmann *et al*[28] found that bacterial endotoxin resulted in increased ^{45}Ca release but this activity decreased at a concentration of 100 µg/ml and the lipoteichoic acid derived from cell membranes of *Lactobacillus fermenti* resulted in increased release of ^{45}Ca from fetal rat bones when used in a dose range of 10 to 100 µg/ml. The per cent release of ^{45}Ca results closely paralleled the results obtained when comparing the test over control ratios of the culture supernatant (Table II).

DISCUSSION

The microbial flora found in a periodontal lesion is extremely complex. It has been estimated that as many as 264 morphologically and biochemically distinct bacterial groups can be isolated.[29] This complex flora creates further confusion by the fact that many of these organisms remain unidentified. When dealing with such a complex ecosystem, one must bear in mind that the presence of

increased numbers of a given microorganism in a disease state may represent a significant role for this organism in the disease process or may simply demonstrate that the changing environment is more conducive to growth of this species in which case the association is entirely casual.

It seems reasonable to approach this problem by considering more than just the presence of a given species in higher numbers in a disease state. One should consider the spatial association of this organism in relation to the advancing front of the disease state. An organism isolated from subgingival plaque which is also present in high numbers in supragingival plaque may indicate contamination in the sampling technique. *Eubacterium* species have been isolated from diseased subgingival sites but are not isolated from healthy subgingival sites or from supragingival plaque.[12,28] These findings suggest that there may be some relationship between the presence of these microorganisms and the etiology of the disease process.

Actual invasion of the connective tissue of the periodontium by microorganisms is not a consistent histological finding and appears to occur primarily only when there is extensive microulceration of the sulcular and junctional epithelium.[15-17,30] The action of hyaluronidase appears to facilitate the passage of other microbial substances into the underlying connective tissue.[19,21] The collagen-poor characteristic of the gingival tissue could thus be the result of collagenase, known to be produced by certain members of the genera *Bacteroides*, *Clostridium*, and *Bacillus*, all of which have

been isolated from subgingival plaque in areas of active disease. It is conceivable that the activity of hyaluronidase may allow other microbial substances to penetrate the epithelial barrier resulting in the characteristic osseous resorption of alveolar bone associated with chronic periodontitis. Holt *et al*[31] demonstrated the ability of certain gram negative microorganisms to produce LPS membrane vesicles free in the external environment. This characteristic is taken to describe a pathogenic mechanism for these microorganisms based on the known ability of LPS to cause enhanced bone resorption in tissue culture. Hausmann *et al*[32] describes the osteolytic activity of LPS, lipoteichoic acid, the amphipathic macromolecule from *Actinomyces viscosus* and muramyl dipeptide when tested in tissue culture.

The findings presented suggest a mechanism to explain the significance of *Eubacterium brachy* in the etiology and progression of periodontal disease. The ability of this organism to secrete a substance, shown to react with antibody in human sera, and of a molecular weight range consistent with other substances known to penetrate pocket epithelium suggests the possibility of inducing a host response which may contribute to the immunopathology of periodontal diseases. The demonstrated ability of this same substance to cause enhanced bone resorption in tissue culture only serves to underline the potential significance of *Eubacterium brachy* in the etiology and progression of chronic periodontitis.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care, of the Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council.

* * * * *

MILITARY DISCLAIMER

Commercial materials and equipment are identified in this report to specify the investigative procedures. Such identification does not imply recommendation or endorsement or that the materials and equipment are necessarily the best available for the purpose. Furthermore, the opinions expressed herein are those of the author and are not to be construed as those of the U. S. Army Medical Department.

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TABLE I

Bone resorptive assay demonstrating enhanced release of ^{45}Ca into culture supernatant when exposed to purified ECA at concentrations of from 10 to 53 μg/ml

Dilution of Purified ECA	μg/ml Protein	Test/Control ^{45}Ca Release
1:2	160	1.01* **SE 0.02
1:6	53.3	1.56 SE 0.11
1:20	16	1.29 SE 0.03
1:30	10.7	1.23 SE 0.09
1:40	8	1.08 SE 0.06
1.80	4	1.10 SE 0.04
1:160	2	1.09 SE 0.01

*Arithmetic mean of counts per minute of four test wells over mean of CPM of four control wells.

**Standard Error

TABLE II

Bone resorptive assay results showing per cent release of ^{45}Ca when cultures were exposed to purified ECA compared to PBS controls

Dilution of Purified ECA	μg/ml Protein	% Release of ^{45}Ca
1:2	160	0.96* **SE 0.10
1:6	53.3	1.55 SE 0.12
1:20	16	1.31 SE 0.03
1:30	10.7	1.22 SE 0.09
1:40	8	1.14 SE 0.06
1:80	4	1.11 SE 0.05
1:160	2	1.16 SE 0.11

*Arithmetic mean of per cent release of ^{45}Ca of four test wells over mean of per cent release of ^{45}Ca of four control wells.

**Standard Error

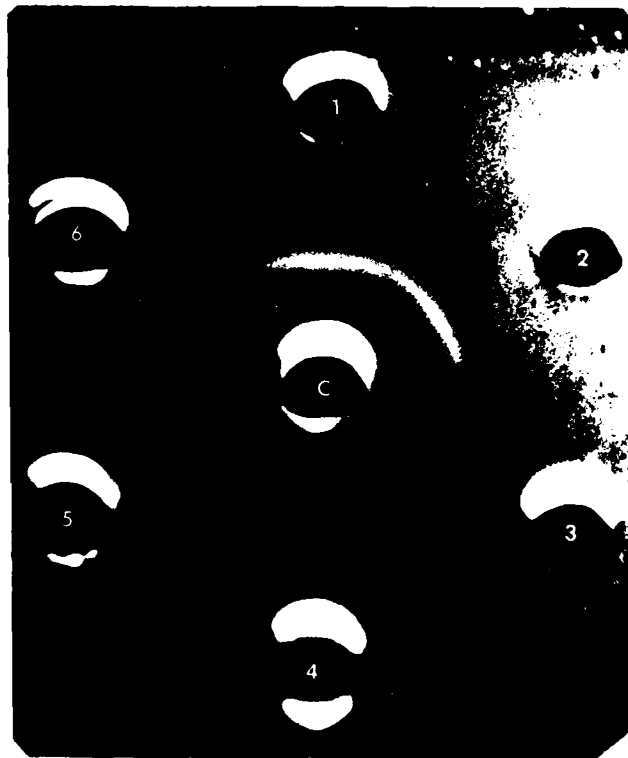

FIGURE 1. Immunodiffusion using human serum reactive to *Eubacterium brachy* (c), showing identity with sonicated preparations of *E. brachy* (1,4) with crude ECA (6) and purified ECA (2). No reactivity was observed with uninoculated broth (3,5) as a control.

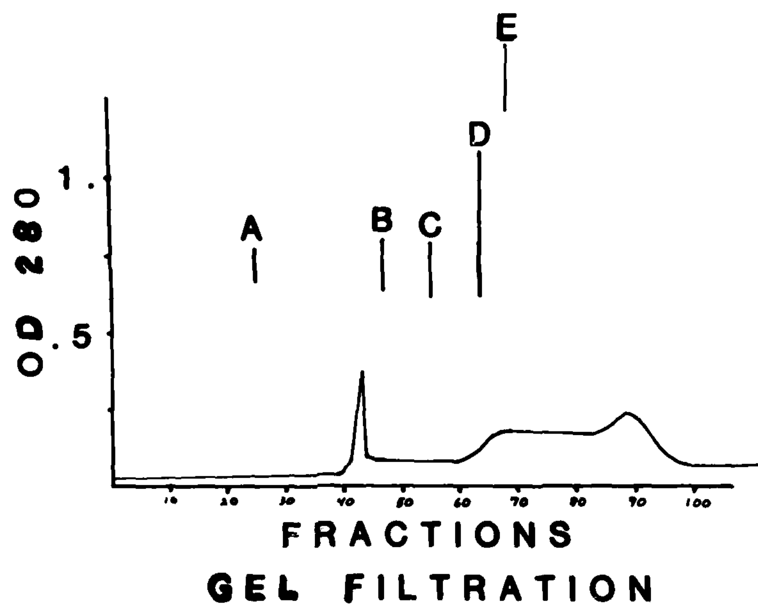

FIGURE 2. Results of gel filtration. Fraction 41-44 contain the purified ECA as determined by immunodiffusion with reactive human sera. Standards (A) blue dextran 2000, (B) aldolase, (C) ovalbumin, (D) chymotrypsinogen A and (E) Ribonuclease A.

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