

AD-A172 202

ACUTE NECROTIZING ULCERATIVE  
GINGIVITIS: MICROBIAL AND IMMUNOLOGIC  
STUDIES

ANNUAL REPORT

William A. Fekier, Jr., Ph.D.

October 1983

Supported by

U. S. Army Medical Research and Development Command

Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD-17-83-C-0181

University of Maryland Dental School

Baltimore, Maryland 21201

Approved for public release;  
distribution unlimited

20030122138

The findings in this report are not to be construed as an official Department of the  
Army position unless as designated by other authorized documents.

Best Available Copy

PHOTOGRAPH THIS SHEET

①

INVENTORY

LEVEL

ACUTE NECROTIZING  
ULCERATIVE GINGIVITIS:  
MICROBIAL AND IMMUNOLOGIC  
STUDIES

ANNUAL REPORT

DOCUMENT IDENTIFICATION

DTIC ACCESSION NUMBER

ADA172202

DISTRIBUTION STATEMENT A

Approved for public release;  
Distribution Unlimited

DISTRIBUTION STATEMENT

ACCESSION FOR

NTIS GRA&I ☒

DTIC TAB ☐

UNANNOUNCED ☐

JUSTIFICATION

BY

DISTRIBUTION /

AVAILABILITY CODES

DIST

AVAIL AND/OR SPECIAL

A-1

DISTRIBUTION STAM.

QUALITY  
INSPECTED

DTIC  
ELECTE  
SEP 25 1986  
S D

DATE ACCESSIONED

DATE RETURNED

DATE RECEIVED IN DTIC

REGISTERED OR CERTIFIED NO.

PHOTOGRAPH THIS SHEET AND RETURN TO DTIC-DDAC

| REPORT DOCUMENTATION PAGE                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |                       | READ INSTRUCTIONS<br>BEFORE COMPLETING FORM                    |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|----------------------------------------------------------------|
| 1. REPORT NUMBER                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 2. GOVT ACCESSION NO. | 3. RECIPIENT'S CATALOG NUMBER                                  |
| 4. TITLE (and Subtitle)<br>Acute Necrotizing Ulcerative Gingivitis:<br>Microbial and Immunologic Studies                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                       | 5. TYPE OF REPORT & PERIOD COVERED<br>Annual Report            |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                       | 6. PERFORMING ORG. REPORT NUMBER                               |
| 7. AUTHOR(s)<br>Dr. William A. Falkler, Jr.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                       | 8. CONTRACT OR GRANT NUMBER(s)<br>DAMD17-80-C-0181             |
| 9. PERFORMING ORGANIZATION NAME AND ADDRESS<br>University of Maryland Dental School<br>Baltimore, Maryland 21201                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                       | 10. PROGRAM ELEMENT, PROJECT, TASK<br>AREA & WORK UNIT NUMBERS |
| 11. CONTROLLING OFFICE NAME AND ADDRESS<br>SGRD-RMS<br>Technical Review Group<br>U.S. Army Medical Research & Development Command<br>Fort Detrick, MD 21761                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                       | 12. REPORT DATE<br>October 1983                                |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                       | 13. NUMBER OF PAGES<br>95                                      |
| 14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                       | 15. SECURITY CLASS. (of this report)                           |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                       | 15a. DECLASSIFICATION/DOWNGRADING<br>SCHEDULE                  |
| 16. DISTRIBUTION STATEMENT (of this Report)<br><br>Approved for public release;<br>distribution unlimited                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |                       |                                                                |
| 17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)<br><br>DOD Distribution Statement B (Proprietary Info.)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                       |                                                                |
| 18. SUPPLEMENTARY NOTES                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |                       |                                                                |
| 19. KEY WORDS (Continue on reverse side if necessary and identify by block number)<br>Periodontal Disease      Spirochetes<br>Microbiology              Fusobacteria<br>Immunology                Bacteroides                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                       |                                                                |
| 20. ABSTRACT (Continue on reverse side if necessary and identify by block number)<br><br>This is an annual report on an ongoing research project aimed at obtaining new information as to the microbial etiology and immunopathology of acute necrotizing ulcerative gingivitis (ANUG). Thirty one patients have been studied as of the date of this report. Completed patient history forms have showed individuals with the disease display a typical and similar life style. They do not have sound employment, they are not financially stable, they smoke and do not display normal living patterns. Subgingival plaque samples taken from the patients revealed the presence of large numbers of spirochetes and |                       |                                                                |

Gram - rods and cultural studies have demonstrated the presence of 8-12 different microorganisms in the lesion with members of the genera Bacteroides and Fusobacterium in the highest numbers. Tests were performed which displayed the characteristic hemagglutination activity of the F. nucleatum and B. gingivalis isolates. Both type I and II colonial variants of F. nucleatum were isolated from the ANUG lesion and serological studies with the F. nucleatum isolates from patients with ANUG, chronic periodontitis, juvenile periodontitis, and adults and children with healthy gingiva suggest that irregardless of which disease they were isolated from the microorganisms share antigenic determinants when reacted with human serum and rabbit antiserum. The reaction of sera from ANUG patients and age and sex matched healthy individuals with microbial isolates from the ANUG patients revealed no differences in the levels of IgG, IgA and IgM or the IgG antibody activity. The results also suggest that antigenic determinants are shared by the Fusobacterium nucleatum isolates and the Bacteroides gingivalis isolates. Electron microscopic observation of the types of spirochetes present in the ANUG plaque samples with regards to the axial filament arrangement suggested the predominant type displayed the "2-4-2" axial filament arrangement however "6-12-6", "8-16-8" and "12-24-12" arrangements were observed. Scanning electron microscopy of an extracted tooth from an ANUG lesion showed the predominant flora to be of spirochetes. Ten strains of spirochetes have been isolated representing "2-4-2" axial arrangement. Rabbit antisera are being prepared to these isolates for serologic characterization. Sodium dodecyl sulfate polyacrylamide gel electrophoretic analysis of the ANUG isolates as compared to known spirochetal strains suggested the ANUG isolates displayed distinct different gel patterns from other T. denticola strains. Studies were undertaken to determine if microorganisms isolated from ANUG lesions have specific binding receptors for corticosteroids. The results suggested that B. gingivalis has specific receptors for dexamethasone and cortisol whereas F. nucleatum and B. intermedius do not. Biopsies of the diseased tissues from two of the patients were obtained and the histopathologic studies suggest that a PMN infiltration is seen early after onset whereas the gingival tissue is infiltrated with lymphocytes as time after onset of the lesion increases.

ACUTE NECROTIZING ULCERATIVE  
GINGIVITIS: MICROBIAL AND IMMUNOLOGIC  
STUDIES

ANNUAL REPORT

William A. Falkler, Jr., Ph.D.

October 1983

Supported by

U. S. Army Medical Research and Development Command  
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD-17-80-C-0181

University of Maryland Dental School

Baltimore, Maryland 21201

Approved for public release;  
distribution unlimited

The findings in this report are not to be construed as an official Department of the  
Army position unless as designated by other authorized documents.

## TABLE OF CONTENTS

|                                                                                                        | <u>Page No.</u> |
|--------------------------------------------------------------------------------------------------------|-----------------|
| Acute Necrotizing Ulcerative Gingivitis: A Brief Literature Review                                     | 1-4             |
| Summary of Research to Date                                                                            | 5, 6            |
| Clinical Evaluation and History                                                                        | 7-12            |
| Culturing Subgingival Plaque and Tissue Surface Scrapings for Microorganisms                           | 13-29           |
| Hemagglutination Activity of ANUG Isolates                                                             | 30-32           |
| Serologic Characterization of <u>F. nucleatum</u> ANUG Isolates with other <u>F. nucleatum</u> Strains | 33-42           |
| Reaction of Serum from ANUG Patients with <u>Fusobacterium</u> and <u>Bacteroides</u> Strains          | 43-53           |
| Studies on Spirochetes from ANUG Lesions                                                               | 54-66           |
| Interactions of Microorganisms of ANUG and Corticosteroids                                             | 67-75           |
| Histopathological Studies                                                                              | 76-78           |
| Bibliography                                                                                           | 79-81           |
| Appendix A: Papers and Abstracts Resulting from Research                                               | 82              |
| Distribution List                                                                                      | 83              |

## ACUTE NECROTIZING ULCERATIVE GINGIVITIS

### A Brief Literature Review

In 1896 Vincent described an ulcerative infection of the gingival tissue which he believed to be caused by fusiform bacilli and spirochetal organisms (1). Orban (2) utilized the term acute necrotizing ulcerative gingivitis (ANUG) to describe this infection and his description follows. The patient usually presents with painful hemorrhagic gingivae, inability to partake of food or to brush his teeth, and often with general malaise. He may or may not be aware of increased salivation, a noisome odor and a metallic taste. There is ulceration and necrosis of the interdental papillae with possible extension to the marginal gingivae. Craters may be present with the loss of interdental papillae. The crateriform lesions are commonly covered with pseudomembranes and surrounded by erythematous borders. Fever and lymphadenopathy are common.

The etiology of the disease has not followed Koch's postulates in that exudate from an infected lesion has not produced ANUG lesions in humans and animals. Although fusospirochetal abscesses have been produced in experimental animals, the lesions are not similar to those of ANUG (2-5). Clinical lesions have been produced in animals only after first traumatizing the involved tissues (6-8). Other investigators have mentioned the importance of local factors such as erupting teeth, poor oral hygiene, poor margins on restorations, calculus build-up, poor contacts, occlusal factors and systemic factors such as physical exhaustion, emotional tension, nutritional deficiencies and metabolic disturbances (2,3,5,9-13).

Of these, emotional factors often appear before the onset of the disease regardless of the other factors (11,12,14,15). Although there have been epidemics of ANUG, the disease has been proven to be noncommunicable. The epidemics were due to the fact that the individuals affected were under the same adverse conditions, such as mental stress or altered living patterns (2,4,5,16-19). This disease has been well documented in military personnel (14,20,21).

Treatments of the disease have utilized chromic acid, nitrates TCA, H<sub>2</sub>O<sub>2</sub>, sodium permanganate, mercurials, arsenicals and triamcinolene acetonide in an adhesive vehicle (4,9,18,22,23). Antibiotics have been and are still used as adjuncts to complete subgingival curettage, debridement and local corrective procedures (19-33). Also ultrasonic instruments have been used in routine periodontal procedures (34-37).

Early studies on the histopathology of ANUG lesions reported that the pathological process appears to begin on the surface of the epithelium. First, the keratin is destroyed, then the degeneration progressed through the various layers of the epithelium (including the basal layer and continues on into the immediate fibrous tissues). The adjacent tissues become edematous and infiltrated with inflammatory cells. On the surface of the lesion were found spirochetes, fusiform bacilli, cocci, and filamenting types (38-43). Spirochetes of large and intermediate size were shown capable of invading non-necrotic tissue of the ANUG lesion and the majority of these spirochetes appeared different from pure strains of cultivated Borrelia vincentii and Treponema microdentium (42). Electron microscopic observation of ANUG lesions allowed the observation of a bacterial zone containing numerous microorganisms, including various morphological types of spirochetes, a zone rich in neutrophils, a zone of necrosis, a zone where larger spirochetes were observed within the tissues of the host in large numbers and to the exclusion of other organisms (42).

The participation of spirochetes in the etiology of fusospirochetal diseases still remains undetermined. Rosebury et al. (44-46) in a series of experiments using mixtures of 29 bacterial cultures and five spirochetal cultures were unable to elicit infections in guinea pigs different than that observed when using fusospirochetal exudate (46). Sixteen bacterial strains recombined with Treponema microdentium did not produce fusospirochetal abscesses in guinea pigs (47). Typical "fusospirochetal" abscesses have been observed in guinea pigs with a mixture of two strains of Bacteroides, a motile gram-negative rod and diphtheroid (48). In this system neither spirochetes nor fusiform bacteria were essential. Borrelia vincentii, Borrelia buccale and small oral treponemes

produced localized abscesses in rabbits (49). Spirochetal abscesses were also observed in the hamster cheek pouch after injection of Borrelia buccalis and small oral treponemes (50). Intracutaneous lesions which resulted in abscess formation were routinely produced in rabbits with both Fusobacterium nucleatum and Fusobacterium polymorphum alone or in combination with oral spirochetes. The synergistic combinations of fusobacteria and spirochetes in intracutaneous lesions in animals showed definite evidence of invasion of both types of organisms into the surrounding tissues (51). Sections of interdental papilla of Vincent's infection stained by the Warthin-Faulkner method revealed spirochetes and fusiform bacilli penetrating the tissue (52).

Cell-mediated immunity and humoral antibody studies were investigated in patients with acute ulcerative gingivitis using antigens from Actinomyces viscosus, Fusobacterium fusiforme, Veillonella alcalescens and Bacteriodes melaninogenicus. No difference in serum antibody levels reactive to the antigens were observed between patients and controls. The significantly greater cell-mediated immunity to F. fusiforme in ANUG suggested this organism might be involved in the change from the chronic to the acute form of the disease (53).

Immunologic studies of ANUG were undertaken to detect humoral antibody reactive with oral spirochetes and to ascertain if spirochetes in vivo were coated with IgG, IgA, IgM and C3. Sera taken at the acute stage of ANUG revealed low antibody titers to oral spirochetes and ranged from 0 to 80. Also in ANUG, spirochetes in smears from the lesions were coated in vivo with IgG, IgA, IgM and C3. The authors suggested these findings are indicative of a localized antibody production to oral spirochetes. It was suggested that the interaction of these antibodies with spirochetes and subsequent complement activation may contribute to the pathogenesis of ANUG (54).

Jacob et al. (55) isolated anaerobic spirochetes from patients and prepared rabbit antisera. The antisera detected, with the use of an indirect fluorescent antibody technique, oral spirochetes in dental plaque from 10 patients suggesting that a common antigenic determinant was shared by the spirochetes detected. These investigators have

also isolated from oral spirochetes a sodium deoxycholate-ethanol extractable antigen which appears to be shared by several oral isolates and to which human antibody is reactive (56).

Loesche et al. (57) anaerobically cultured plaque samples from 22 ulcerated sites in eight patients with ANUC. They observed a constant flora comprised of a limited number of bacterial types and a variable flora composed of a heterogeneous mixture of bacterial types. The constant flora included Treponema and Selenomonas sp., B. intermedius and Fusobacterium sp. Treatment with metronidazole resulted in a prompt resolution of clinical symptoms with a significant reduction in the numbers of Treponema sp., B. intermedius and Fusobacterium sp. for several months following treatment.

## SUMMARY

This is an annual report on an ongoing research project aimed at obtaining new information as to the microbial etiology and immunopathology of acute necrotizing ulcerative gingivitis (ANUG). Thirty one patients have been studied as of the date of this report. Completed patient history forms have showed individuals with the disease display a typical and similar life style. They do not have sound employment, they are not financially stable, they smoke and do not display normal living patterns. Subgingival plaque samples taken from the patients revealed the presence of large numbers of spirochetes and Gram - rods and cultural studies have demonstrated the presence of 8-12 different microorganisms in the lesion with members of the genera Bacteroides and Fusobacterium in the highest numbers. Tests were performed which displayed the characteristic hemagglutination activity of the F. nucleatum and B. gingivalis isolates. Both type I and II colonial variants of F. nucleatum were isolated from the ANUG lesion and serological studies with the F. nucleatum isolates from patients with ANUG, chronic periodontitis, juvenile periodontitis, and adults and children with healthy gingiva suggest that irregardless of which disease they were isolated from the microorganisms share antigenic determinants when reacted with human serum and rabbit antiserum. The reaction of sera from ANUG patients and age and sex matched healthy individuals with microbial isolates from the ANUG patients revealed no differences in the levels of IgG, IgA and IgM or the IgG antibody activity. The results also suggest that antigenic determinants are shared by the Fusobacterium nucleatum isolates and the Bacteroides gingivalis isolates. Electron microscopic observation of the types of spirochetes present in the ANUG plaque samples with regards to the axial filament arrangement suggested the predominant type displayed the "2-4-2" axial filament arrangement however "6-12-6", "8-16-8" and "12-24-12" arrangements were observed. Scanning electron microscopy of an extracted tooth from an ANUG lesion showed the predominant flora to be of spirochetes. Ten strains of spirochetes have been isolated representing the "2-4-2" axial

arrangement. Rabbit antisera are being prepared to these isolates for serologic characterization. Sodium dodecyl sulfate polyacrylamide gel electrophoretic analysis of the ANUG isolates as compared to known spirochetal strains suggested the ANUG isolates displayed distinct different gel patterns from other T. denticola strains. Studies were undertaken to determine if microorganisms isolated from ANUG lesions have specific binding receptors for corticosteroids. The results suggested that B. gingivalis has specific receptors for dexamethasone and cortisol whereas F. nucleatum and B. intermedius do not. Biopsies of the diseased tissues from two of the patients were obtained and the histopathologic studies suggest that a PMN infiltration is seen early after onset whereas the gingival tissue is infiltrated with lymphocytes as time after onset of the lesion increases.

## ANUG PATIENTS

### Clinical Evaluation and History

As of this time 31 patients with ANUG have been studied. The clinical evaluation of the patients is shown in Table 1.

The patients were asked to fill out an "ANUG History" questionnaire. The answers were as follows:

1. What is your present job:

Thirty two percent of the patients were unemployed at the time of coming to the clinic. The following jobs were listed:

- |                       |                            |
|-----------------------|----------------------------|
| a. food service       | h. service station manager |
| b. hair stylist       | i. bakery worker           |
| c. barmaid            | j. clerk                   |
| d. electronics        | k. dancer                  |
| e. operations manager | l. dental hygienist        |
| f. students           | m. insurance broker        |
| g. file clerk         | n. family counselor        |

2. Are you satisfied with your present job?

Only 35% were satisfied with their present job.

3. Living conditions:

58% lived at home with their family  
16% lived alone  
25% lived in an apartment with a roommate

4. Fifty one percent of the patients were male; forty nine per cent were female

5. The mean age of the patients was 23 with a range of 14 to 50.

6. The average length of time that they lived at their current address was 54 months with a range of 1 week to 27 years.

7. The patients classified their own health status as follows:

22% Excellent  
56% Good  
22% Fair

8. They came to the dental clinic because:

62.5% Painful, bleeding gums  
8.3% Painful gums  
4.1% Painful tongue  
8.3% Other

9. Seventy five percent of the patients had sore gums when they entered the dental clinic.

Table I  
ANUG Patients

| Patient # | Gingival Bleeding | Blunting of Papilla | Loss of Papilla 1/3, 1/3-2/3, 2/3 | Pain | Fetid Odor     | Cratering of Papilla | Pseudo-membrane | Lymphadenopathy | Onset    | Temp.           | Area Affected          |
|-----------|-------------------|---------------------|-----------------------------------|------|----------------|----------------------|-----------------|-----------------|----------|-----------------|------------------------|
| 1         | +                 | +                   | +                                 | +    | +              | +                    | +               | +               | 2 weeks  | 99.2            | Mand. Anterior         |
| 2         | +                 | +                   | +                                 | +    | - <sup>a</sup> | +                    | +               | - <sup>a</sup>  | 1 month  | -- <sup>c</sup> | Mand. Ant. Max. Post   |
| 3         | +                 | +                   | +                                 | +    | +              | +                    | +               | +               | --       | --              | Generalized            |
| 4         | +                 | - <sup>a</sup>      | +                                 | +    | +              | +                    | +               | +               | 5 months | --              | Max. Ant. Mand. Ant.   |
| 6         | +                 | +                   | +                                 | +    | +              | +                    | +               | --              | --       | --              | Mand. Ant.             |
| 7         | +                 | -                   | +                                 | +    | +              | +                    | +               | --              | 3 days   | --              | Mand. Ant.             |
| 8         | +                 | +                   | +                                 | +    | +              | +                    | +               | --              | 3 days   | --              | Mand. Ant.             |
| 9         | +                 | -                   | +                                 | +    | +              | +                    | +               | --              | 1 week   | --              | Max. Ant. Max. Post.   |
| 10        | +                 | -                   | +                                 | +    | +              | +                    | +               | +               | 1 week   | 99.6            | Generalized            |
| 11        | +                 | +                   | +                                 | +    | +              | +                    | +               | --              | 5 days   | --              | Generalized            |
| 12        | +                 | +                   | +                                 | +    | +              | +                    | +               | --              | 1 week   | --              | Generalized            |
| 13        | +                 | -                   | +                                 | +    | +              | +                    | +               | +               | 1 week   | --              | Mand. Ant. Max. Ant.   |
| 14        | +                 | -                   | +                                 | +    | +              | +                    | +               | --              | 4 days   | --              | Mand. Ant. Mand. Post. |
| 15        | +                 | +                   | +                                 | +    | +              | +                    | +               | --              | 4 days   | --              | Mand. Ant.             |
| 16        | +                 | -                   | +                                 | +    | +              | +                    | +               | +               | 3 days   | --              | Mand. Ant.             |

|    |    |   |    |   |   |   |   |   |   |                       |       |                               |
|----|----|---|----|---|---|---|---|---|---|-----------------------|-------|-------------------------------|
| 17 | +  | + | +  | + | + | + | + | + | + | 2 weeks               | --    | Mand. Ant.                    |
| 18 |    | + | -- | + | + | + | + | + | + | 1 week                | --    | Mand. Ant.<br>Mand. Max. Post |
| 19 |    | + | +  | + | + | + | + | + | + | 2 days                | 102   | Mand. Ant.                    |
| 20 | +  | + | +  | + | + | + | + | + | + | 3 days                | 99.6  | Generalized                   |
| 21 | +- | + | +  | + | + | + | + | + | + | 2 weeks               | 99.5  | Mand. Ant.<br>Max. Ant.       |
| 22 |    | + | +  | + | + | + | + | + | + | 4 days                | --    | Max. Ant.                     |
| 23 | +  | + | +  | + | + | + | + | + | + | 1 day                 | 100.1 | Mand. Ant.                    |
| 24 | +  | + | +  | + | + | + | + | + | + | 2 days                | 100.1 | Max. Ant.<br>Entire Mouth     |
| 25 | +  |   |    | + | + | + | + | + | + | 1 day                 | 100.0 | Mand. Ant.<br>Mand. Post      |
| 26 | +  | + | +  | + | + | + | + | + | + | 1 day                 | 99    | Mand. Ant.<br>Mand. Post      |
| 27 | +  | + | +  | + | + | + | + | + | + | 1 day                 | 100   | Max. Ant.                     |
| 29 |    | + | +  | + | + | + | + | + | + | 2 days<br>recurrent   | --    | Mand. Ant.<br>Max. Post       |
| 30 | +  | + | +  | + | + | + | + | + | + | 1 day                 | 99.2  | Entire Mouth                  |
| 31 |    | + | +  | + | + | + | + | + | + | 6 months<br>recurrent | 99.0  | Entire Mouth                  |
| 32 | +  | + | +  | + | + | + | + | + | + | 1-2 days              | --    | Max. Ant.                     |

a no  
b slight  
c normal

10. When asked if the gums of the patients were painful prior to coming to the clinic:

71% painful 5 days or more  
13% painful 4 days  
8% painful 3 days  
8% painful 2 days

11. When asked if their gums had ever been painful before:

61% stated never  
21% stated once  
11% stated more than three times  
7% stated twice

12. When asked when was their last episode of painful gums: mean 30 months with a range of 6 months to 4 years ago.

13. When asked what they were doing at the time of the last episode:

They stated they were either working or that this was the first episode.

14. When asked if their gums bleed when they brushed their teeth:

67% said yes; 33% said no.

15. When asked if their gums every bled by themselves - without brushing:

63% said no; 37% said yes

16. When asked how long their gums had been bleeding prior to their coming to the clinic:

10% one day  
15% two days  
10% three days  
10% four days  
55% five days or more

17. When asked if this spontaneous bleeding ever happened before:

71% stated never  
5% stated once  
24% stated more than 3 times

18. The last spontaneous bleeding episode of the patients was: mean 6 months with a range of one-12 months

19. When asked what they were doing during their last episode:

Working, unemployed or in school

20. When asked if they smoke:

81% did smoke; 19% did not

21. When asked how much they smoked:

73% 0-1 pack/day  
18% 1-2 packs/day  
18% over 2 packs/day

22. When asked how much sleep they had per night the week prior to visiting the clinic:

11% 0-4 hours  
33% 4-6 hours  
41% 6-8 hours  
15% over 8 hours

23. When asked how much sleep they had per night this last month:

-- 0-4 hours  
15% 4-6 hours  
56% 6-8 hours  
29% over 8 hours

24. When asked if they sleep restfully when they do sleep:

74% stated yes; 26% said no

25. When asked if they were working at a job for which they were best suited:

60% stated yes; 40% stated no

26. When asked how many hours per day they were at their job:

10% 0-2 hours  
15% 2-4 hours  
-- 4-6 hours  
30% 6-8 hours  
35% 8-10 hours  
10% over 10 hours

27. When asked how many days per week they were at their job:

9% 0-2 days  
5% 3 days  
14% 4 days  
64% 5 days  
4% 6 days  
4% 7 days

28. When asked how many meals they ate each day:

11% 1 meal  
50% 2 meals  
28% 3 meals  
11% more than 3 meals

29. When asked how many meals they ate in a "fast food" establishment: Of the 50% which ate in fast food restaurants:

54% ate one meal  
31% ate two meals  
15% ate three meals

30. When asked if they felt they ate well:

71% stated yes; 29% stated no

31. When asked if they have time to brush their teeth:

96% answered yes; 4% no

32. When asked how often they brush their teeth each day:

4% less than once per day  
37% once per day  
42% twice per day  
12% three times per day  
5% never

33. When asked how often they floss each day:

11% less than once per day  
18% once per day  
11% twice per day  
3% three times per day  
57% never

34. When asked if the patients felt they had enough time to accomplish their responsibilities:

65% stated yes; 35% no

35. When asked if they enjoyed their current living situation:

69% stated yes; 31% no

36. When asked if they enjoyed their present job:

61% stated yes; 39% no

37. When asked if the present condition of their mouth affected their work:

52% stated yes; 48% no

38. Race

18 caucasian; 13 black

## CULTURING SUBGINGIVAL PLAQUE AND TISSUE

### SURFACE SCRAPINGS FOR MICROORGANISMS

At the present time samples have been taken from 30 ANUG patients. Seven of these samples were used in our initial studies in developing the cultural and identification procedures. Also attempts were made initially to isolate Fusobacterium nucleatum and Bacteroides strains from ANUG patients for use in the serologic studies to be presented. Plaque samples from the patients have revealed the isolation of approximately 8-12 different colony types from each patient. The procedure for culturing and identification of the samples follows:

#### Media

The following media were used: Crystal Violet-Erythromycin (CVE) agar; MM10 agar; and Trypticase Soy Agar (TSA) with hemin and menadione (TSAHK). CVE agar (pH 7.2 contains in g/l the following: trypticase (10.0), yeast extract (5.0), NaCl (5.0), Tryptophan (0.2), agar (15.0) and crystal violet (0.001), erythromycin (0.004), and defibrinated sheep blood (50 ml) which were added after autoclaving. MM10 contained/liter: H<sub>2</sub>O (890 ml), 37.5 ml of 0.6% K<sub>2</sub>HPO<sub>4</sub>, 37.5 ml of a salt solution (NaCl), 1.2 g; NH<sub>4</sub> 2SO<sub>4</sub>, 1.2 g; KH<sub>2</sub>PO<sub>4</sub>, 0.6 g, Mg<sub>2</sub>SO<sub>4</sub>, 0.25 g and 100 ml H<sub>2</sub>O), bacto-agar (15.0 g), trypticase (2.0 g), yeast extract (0.5 g), sucrose (30.0 g), KNO<sub>3</sub>(0.25 g), 0.05% hemin, (2.0 ml) and cysteine (0.12 g), 3% Na<sub>2</sub>CO<sub>3</sub> (5 ml), DL-dithio-threitol (0.1 mg) and sheep blood (20 ml) which were added after autoclaving. TSA contained 40 g/l trypticase soy agar and for the preparation of TSAHK 10 ml of 0.5 mg/ml hemin in 0.1 N NaOH, 1 ml of 0.1% menadione in 95% ETOH and 50 ml of defibrinated sheep blood were added.

#### Microbiological Culturing of ANUG Lesion

Samples of subgingival plaque were collected using a curette under nitrogen gas and immediately placed into 1 ml of reduced transport fluid (RTF) containing an Eh indicator (58). The vial was placed into a Coy anaerobic chamber, vortexed for 30 seconds and the contents of the vial diluted in serial ten-fold dilutions with RTF. Each dilution (0.1 ml) was spread plated onto 3 reduced MM10 agar plates. Also 0.1 ml of the 10<sup>-1</sup> dilution was

spread plated onto CVE agar plates and TSAHK. The plates were allowed to incubate for 3 days, after which time they were inspected and the dilution showing 30-200 colonies/MM10 plate was used for quantitation of the viable count. The average of the colony counts for the 3 plates at that dilution was multiplied by the reciprocal of the dilution times 10. The number of colony types on each plate were observed and the average number of each colony type on the plates determined. The % of each colony type in relation to the total viable count was calculated. Each colony type was Gram stained and subcultured for isolation onto MM10 plates and incubated anaerobically for 2 days. Also each colony type was subcultured onto another MM10 plate and incubated aerobically for oxygen tolerance. Each isolate from MM10 was subcultured to BHI broth and allowed to grow 48 h and then placed into litmus milk and frozen. Anaerobes were identified by the API 20 anaerobe system. Facultative anaerobes and gram positive cocci were identified by the API 20S system. Several streptococci were identified by their colonial morphology on MM10, for example, S. sanguis (transparent, shiny, immovable, rigid colonies), S. salivarius (large, transparent, sticky, mucoid colonies) and S. mutans (colorless, granular, indented, small colonies). Black pigmented colonies were taken from the TSAHK and subcultured for isolation on TSAHK and then identified via the use of the API 20A system. F. nucleatum colonies isolated from the CVE agar were also identified with the API 20A system. Sonicated suspensions of the isolates were also identified via reaction by immunodiffusion with rabbit antiserum to prototype strains.

### RESULTS

The results of individual cultural studies on ten of the ANUG patients are presented in Tables 2-11. The results of the cultural studies of twenty of the patients have been combined and presented in Table 2. As can be observed in Table 12, to date the Gram - rods comprised the highest percentage of the total organisms isolated. Of this group B. gingivalis and F. nucleatum were the most frequently isolated with B. intermedius, other Fusobacterium sp. and Vibrio sp. also being identified. The Gram + cocci comprised 15.5% of all of the isolates, Streptococcus and Staphylococcus sp., Pseudomonas micros,

and Peptostreptococcus sp. being isolated. A smaller number of Gram + rods than expected was observed. These included isolates of C. beijerinckie, L. fermentum and A. israelii. V. parvula was the only identified Gram - coccus isolated from these patients.

## ANUG I

Table 2

White Male - Total counts on MM10 at  $10^{-4}$ ;

Avg. Total Viable Count (using 0.1 ml)

1. 90  
2. 96 $93 \times 10^2$ 

On MM10 (nonselective) medium:

ID

| <u>Organism</u>               | <u>Avg. Viable Count/ml</u> | <u>% Total Viable Count</u> | <u>GS</u> | <u>COL</u> | <u>API</u> | <u>SEL MED</u> |
|-------------------------------|-----------------------------|-----------------------------|-----------|------------|------------|----------------|
| <u>Strep. intermedius</u>     | $42 \times 10^5$            | 45.2                        |           | x          |            | x              |
| <u>Strep. morbillorum</u>     | $19 \times 10^5$            | 20.4                        |           | x          |            | x              |
| <u>Bacteroides gingivalis</u> | $2 \times 10^5$             | 2.2                         |           | x          | x          | x              |
| <u>Fuso. nucleatum</u>        | $2 \times 10^5$             | 2.2                         |           | x          | x          | x              |
| <u>Strep. sanguis</u>         | $7 \times 10^5$             | 7.5                         |           | x          | x          | x              |

Non-identified on MM10: classified according to Gram reaction and morphology:

| <u>Colonial Morph.</u>                     | <u>Gram Stain</u>   | <u>O<sub>2</sub></u> | <u>Avg. Viable Cnt./ml</u> | <u>% Total Viable Cnt.</u> | <u>API 20A/20S</u>                                 |
|--------------------------------------------|---------------------|----------------------|----------------------------|----------------------------|----------------------------------------------------|
| Med. brown with dk. ctr.                   | Gm variable cocci   | (+)                  | $1 \times 10^5$            | 1.1                        | 20S - all neg.                                     |
| Smooth, flat, shiny, greenish-gray         | Gm (+) cocci chains | (+)                  | $1 \times 10^5$            | 1.1                        | ND                                                 |
| Creamy-white, raised with Beta hemolysis   | Gm (-) rods         | (-)                  | $7 \times 10^5$            | 7.9                        | 20A - S <sup>-</sup> E <sup>-</sup> C <sup>+</sup> |
| Tiny, beige, smooth, raised with reg. edge | Gm (-) cocci        | (+)                  | $12 \times 10^5$           | 12.9                       | N/A                                                |

| GS - Gram stain | COL - colonial morphology | API-20A or 20S | SEL MED - selective media |
|-----------------|---------------------------|----------------|---------------------------|
|-----------------|---------------------------|----------------|---------------------------|

# ANUG II

Black Female - Total counts on MM10 at  $10^{-3}$ :

1. 152
2. 164

Table 3

Avg. Total Viable Count (using 0.1 ml)  
 $158 \times 10^4$

On MM10 (nonselective) medium:

ID

| Organism                      | Avg. Viable Count/ml | % Total Viable Count | GS | COL | API | SEL<br>MED |
|-------------------------------|----------------------|----------------------|----|-----|-----|------------|
| <u>Peptostrep. prevotii</u>   | $92 \times 10^4$     | 58.2                 |    | x   | x   |            |
| <u>Veillonella parvula</u>    | $8.5 \times 10^4$    | 5.4                  |    | x   | x   |            |
| <u>Peptostrep.</u>            | $2 \times 10^4$      | 1.3                  |    | x   | x   |            |
| <u>Bacteroides gingivalis</u> | $1 \times 10^4$      | 0.7                  |    | x   | x   | x          |
| <u>Strep. sanguis</u>         | $1 \times 10^4$      | 0.7                  |    | x   | x   | x          |
| <u>Fusobacterium spp.</u>     | $23 \times 10^4$     | 14.61                |    | x   | x   | x          |

Non-identified on MM10: classified according to Gram reaction and morphology:

| Colonial Morph.                                 | Gram Stain                       | O <sub>2</sub> | Avg. Viable Cnt./ml | % Total Viable Cnt. | API 20A/20S                                                   |
|-------------------------------------------------|----------------------------------|----------------|---------------------|---------------------|---------------------------------------------------------------|
| Mucoid, red & white, smooth round, shiny        | Small Gm (-) cocci               | (-)            | $1 \times 10^4$     | 0.7                 | 20A - S <sup>+</sup> E <sup>-</sup> C <sup>-</sup> (Lac, Sac) |
| Flat, round, with dark brown center, white rim  | Gm (-) coccobacilli              | (-)            | $4 \times 10^4$     | 2.6                 | 20A - S <sup>-</sup> E <sup>-</sup> C <sup>-</sup>            |
| Green, raised, irreg. edge                      | Gm (+) sequented rods            | (-)            | $0.5 \times 10^4$   | 0.4                 | 20A - S <sup>-</sup> E <sup>+</sup> C <sup>-</sup> (Ind, Esc) |
| Small, pink, irreg. edge, raised, rough surface | Branching, grainy Gm (+) rods    | (-)            | $3.5 \times 10^4$   | 2.2                 | 20A - S <sup>-</sup> E <sup>-</sup> C <sup>-</sup>            |
| Dark brown, irreg. edge, raised surface         | Gm (+) diptheroid-like rods      | (+)            | $10 \times 10^4$    | 6.3                 | N/A                                                           |
| Shiny, smooth, round, irreg. edge               | Shant, plump Gm (-) coccobacilli | (-)            | $1 \times 10^4$     | 0.7                 | 20A - S <sup>-</sup> E <sup>-</sup> C <sup>-</sup>            |

GS - Gram stain    COL - colonial morphology    API-20A or 20S    SEL MED - selective media

Table 4

## ANUG III

White Male - Total counts on MM10 at  $10^{-3}$ ;

1. 231
2. 274
3. 164

Avg. Total Viable Count (using 0.1 ml)  
 $223 \times 10^4$ 

On MM10 (nonselective) medium:

ID

| Organism                       | Avg. Viable Count/ml | % Total Viable Count | GS | COL | API | SEL<br>MED |
|--------------------------------|----------------------|----------------------|----|-----|-----|------------|
| <u>Fusobacterium nucleatum</u> | $33 \times 10^4$     | 14.8                 |    | x   | x   |            |
| <u>Veillonella parvula</u>     | $4.3 \times 10^4$    | 1.9                  |    | x   | x   |            |
| <u>Strep. morbillorum</u>      | $0.3 \times 10^4$    | 0.1                  |    | x   | x   |            |
| <u>Strep. sanguis</u>          | $0.7 \times 10^4$    | 0.3                  |    | x   | x   |            |
| <u>Veillonella parvula</u>     | $1.3 \times 10^4$    | 0.6                  |    | x   | x   |            |

Non-identified on MM10: classified according to Gram reaction and morphology:

| Colonial Morph.                                           | Gram Stain                          | O <sub>2</sub> | Avg. Viable Cnt./ml | % Total Viable Cnt. | API 20A/20S                                                                 |
|-----------------------------------------------------------|-------------------------------------|----------------|---------------------|---------------------|-----------------------------------------------------------------------------|
| Purple, raised center,<br>pink rim, shiny, irreg.<br>edge | Gm variable<br>coccobacilli         | +              | $4 \times 10^4$     | 1.8                 | 20S - all neg                                                               |
| Dark red with pink rim,<br>irreg. edge                    | Gm (+) rods<br>short, plump         | +              | $13.3 \times 10^4$  | 6.0                 | N/A                                                                         |
| White, shiny, raised<br>center                            | Short, plump<br>Gm (-) rods         | +              | $0.3 \times 10^4$   | 0.1                 | N/A                                                                         |
| Small, dark, reddish-<br>brown, convex                    | Long Gm (-) rods<br>in short chains | -              | $16 \times 10^4$    | 7.2                 | N/A                                                                         |
| Tiny, brown, smooth                                       | Small Gm (-) rods                   | -              | $89 \times 10^4$    | 39.9                | 20A - S <sup>+</sup> E <sup>-</sup> C <sup>-</sup><br>(Lac, Sac, Nial, Rha) |

GS - Gram stain    COL - colonial morphology    API-20A or 20S    SEL MED - selective media

Table 5

ANUG XIV

22 YOBM - Total counts on MM10 at  $10^{-4}$ :

1. 135
2. 99
3. 100

Avg. Total Viable Count (using 0.1 ml)  
 $111.3 \times 10^5/\text{ml}$ 

On MM10 (nonselective) medium:

ID

| Organism                        | Avg. Viable Count/ml | % Total Viable Count | GS | COL | API | SEL<br>MED |
|---------------------------------|----------------------|----------------------|----|-----|-----|------------|
| <u>Bacteroides gingivalis</u>   | $6.0 \times 10^5$    | 5.4                  |    | x   | x   | x          |
| <u>Fusobacterium nucleatum</u>  | $5.0 \times 10^5$    | 4.5                  |    | x   | x   | x          |
| <u>Clostridium beijerinckii</u> | $0.6 \times 10^5$    | 0.6                  |    | x   | x   |            |
| <u>Streptococcus sanguis</u>    | $1.0 \times 10^5$    | 0.9                  |    | x   | x   |            |

Non-identified on MM10: classified according to Gram reaction and morphology:

| Colonial Morph.                                                           | Gram Stain                 | O <sub>2</sub> | Avg. Viable Cnt./ml | % Total Viable Cnt. | API 20A/20S        |
|---------------------------------------------------------------------------|----------------------------|----------------|---------------------|---------------------|--------------------|
| White, raised, shiny smooth with regular edge                             | Gm (-) cocci in and chains | (+)            | $3 \times 10^5$     | 2.7                 | API 20S - all neg. |
| Raised, shiny mucoid smooth with regular edges                            | Gm (+) cocci in clusters   | (+)            | $11 \times 10^5$    | 9.9                 | API 20S - all neg. |
| Black-pigmented raised with irreg. edge. Smooth; lighter brown periphery. | Gm (-) cocco bacillus      | (+)            | $6 \times 10^5$     | 5.4                 | N/A                |
| Light brown smooth center with rough dark brown periphery                 | Gm (-) cocci               | (+)            | $2.3 \times 10^5$   | 2.1                 | API 20S - all neg. |
| Brown, rough, flat colony with irreg. edge. Spreader                      | Gm (-) rods                | (+)            | $1 \times 10^5$     | 0.9                 | N/A                |
| White, smooth, mucoid colony with reg. edges, center raised to peak       | Gm (-) long fusiform rods  | (+)            | $2 \times 10^5$     | 1.8                 | N/A                |

GS - Gram stain    COL - colonial morphology    API-20A or 20S    SEL MED - selective media

Table 6

ANUG XV

White Female - Total counts on MM10 at  $10^{-4}$ ;

- Age 42  
1. 60  
2. 89  
3. 99

Avg. Total Viable Count (using 0.1 ml)  
 $82.6 \times 10^5$ 

On MM10 (nonselective) medium:

ID

| <u>Organism</u>     | <u>Avg. Viable Count/ml</u> | <u>% Total Viable Count</u> | <u>GS</u> | <u>COL</u> | <u>API</u> | <u>SEL</u><br><u>MED</u> |
|---------------------|-----------------------------|-----------------------------|-----------|------------|------------|--------------------------|
| <u>Ps. micros</u>   | $2 \times 10^5$             | 2.42                        |           | x          | x          | x                        |
| <u>F. nucleatum</u> | $12.5 \times 10^5$          | 1.51                        |           | x          | x          | x                        |
| <u>S. sanguis</u>   | $1 \times 10^5$             | 1.21                        |           |            | x          |                          |

Non-identified on MM10: classified according to Gram reaction and morphology:

| <u>Colonial Morph.</u>                                            | <u>Gram Stain</u>                         | <u>O<sub>2</sub></u> | <u>Avg. Viable Cnt./ml</u> | <u>% Total Viable Cnt.</u> | <u>API 20A/20S</u>                                                                                     |
|-------------------------------------------------------------------|-------------------------------------------|----------------------|----------------------------|----------------------------|--------------------------------------------------------------------------------------------------------|
| Raised, shiny, brown<br>light brown periphery,<br>reg. edge       | Gm (+) rods<br>w/terminal spores          | (+)                  | $12 \times 10^5$           | 14.5                       | NI                                                                                                     |
| white, raised, shiny,<br>peaked center, reg. edge                 | Gm (+) cocci                              | (+)                  | $3 \times 10^5$            | 3.63                       | 20S - all neg.                                                                                         |
| White, dull, raised,<br>center peak, irreg. edge                  | Gm (+) cocci                              | (+)                  | $3 \times 10^5$            | 3.63                       | 20S - (+) Leu                                                                                          |
| dark brown, pinpoint,<br>raised, reg. edge                        | Gm (-) short<br>blunt rods                | (-)                  | $13 \times 10^5$           | 15.7                       | 20A - S <sup>+</sup> E <sup>-</sup> C <sup>-</sup> (Ind,<br>Glu, Sac, Gel)                             |
| small, creamy, irreg.<br>edge, light brown center<br>peak, raised | Gm variable<br>segmented branching<br>rod | (+)                  | $3.5 \times 10^5$          | 4.2                        | N/A                                                                                                    |
| small, white, raised,<br>reg. edge, shiny                         | Gm (-) cocci                              | (-)                  | $7.3 \times 10^5$          | 8.8                        | 20A - S <sup>+</sup> E <sup>-</sup> C <sup>-</sup> (Glu,<br>Lac, Sac, Mal, Gly,<br>Cel, Raf, Sor, Trc) |

Table 6 con't.

| On TSA/IK                          |  | 1 Black pigmenting colony | API 20A - S <sup>+</sup> E <sup>-</sup> C <sup>-</sup><br>(Glu, Lac, Sac, Mal) |            |                |
|------------------------------------|--|---------------------------|--------------------------------------------------------------------------------|------------|----------------|
| Organism at 10 <sup>-3</sup><br>NI |  |                           |                                                                                |            |                |
| On CVE at 10 <sup>-3</sup>         |  |                           |                                                                                |            |                |
| Organism                           |  | <u>GS</u>                 | <u>COL</u>                                                                     | <u>API</u> | <u>SEL MED</u> |
| <u>F. nucleatum</u>                |  | x                         | x                                                                              | x          | x              |
| <u>F. nucleatum</u>                |  | x                         | x                                                                              | x          | x              |
| <u>F. nucleatum</u>                |  | x                         | x                                                                              | x          | x              |

GS - Gram stain    COL - colonial morphology    API-20A or 20S    SEL MED - selective media

Table 7

Table 7

ANUG XVII

White Female - Total counts on MM10 at  $10^{-5}$ :

1. 75
2. 72
3. 78

Avg. Total Viable Count (using 0.1 ml)  
 $75 \times 10^6$

On MM10 (nonselective) medium:

ID

| Organism                                                                      | Avg. Viable Count/ml                                        | % Total Viable Count | GS                  | COL                 | API | SEL MED                                                                                          |
|-------------------------------------------------------------------------------|-------------------------------------------------------------|----------------------|---------------------|---------------------|-----|--------------------------------------------------------------------------------------------------|
| <i>F. nucleatum</i>                                                           | $4 \times 10^6$                                             | 5.3                  |                     | x                   | x   | x                                                                                                |
| <i>S. sanguis</i>                                                             | $1 \times 10^6$                                             | 1.3                  |                     |                     | x   |                                                                                                  |
| <i>Ps. anaerobius</i> or <i>micros</i>                                        | $.5 \times 10^6$                                            | .67                  |                     | x                   | x   | x                                                                                                |
| Non-identified on MM10: classified according to Gram reaction and morphology: |                                                             |                      |                     |                     |     |                                                                                                  |
| Colonial Morph.                                                               | Gram Stain                                                  | O <sub>2</sub>       | Avg. Viable Cnt./ml | % Total Viable Cnt. |     |                                                                                                  |
| White, raised, smooth, shiny, reg. edge, large                                | Gm (-) thin fusiform rods                                   | -                    | $2 \times 10^6$     | 2.7                 |     | 20A - S <sup>+</sup> E <sup>-</sup> C <sup>-</sup> , Glu Man, Lac, Sac, Mal, Raf, Sor            |
| Small, raised, tan, reg. edge                                                 | Gm (-) rods, med. length, thick, chains, curved             | -                    | $29 \times 10^6$    | 38.7                |     | 20A - S <sup>+</sup> E <sup>-</sup> C <sup>-</sup> - Glu, Man, Lac, Sac, Mal                     |
| Brown, raised, rough, irreg. edge, raised clear center                        | Gm (+) cocci                                                | +                    | $1 \times 10^6$     | 1.3                 |     | 20S - all neg.                                                                                   |
| Brown, spreader, irreg. edge                                                  | Gm (-) rods, large thick, chains, w/ segmentation & single  | -                    | $1 \times 10^6$     | 1.3                 |     | 20A - S <sup>+</sup> E <sup>-</sup> C <sup>-</sup> Glu, Lac, Sac, Mal, Sal, Cel, Mne, Raf, Tre   |
| Brown, clear edge, raised dark center, reg. edge                              | Gm (-) rods, large, thick, chains, w/ segmentation & single | -                    | $1 \times 10^6$     | 1.3                 |     | 20A - S <sup>+</sup> E <sup>-</sup> C <sup>-</sup> , Glu, Man, Lac, Sac, Mal, Mne, Raf, Sor, Tre |
| Small, white, reg. edge, raised                                               | V. small Gm (-) rods, comina shaped                         | -                    | $3 \times 10^6$     | 4                   |     | 20A - S <sup>+</sup> E <sup>-</sup> C <sup>-</sup> Glu, Man, Lac, Sac, Mal                       |

Table 7 cont't.

On TSAHK

Bacteroides gingivalis

Avg. Viable Count  
2 x 10<sup>5</sup>

% of Total Black-  
Pigmenting Organisms

ID

GS COL API SEL MED  
x x x

On CVE

F. nucleatum

7 x 10<sup>5</sup>

x x x

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

## ANUG IX

Table 8

Total counts on MM10 at  $10^{-4}$ :

1. 102
2. 61
3. 60

Avg. Total Viable Count (using 0.1 ml)  
 $74.3 \times 10^3$ 

On MM10 (nonselective) medium:

ID

| Organism                        | Avg. Viable Count/ml | % Total Viable Count | GS | COL | API | SEL<br>MED |
|---------------------------------|----------------------|----------------------|----|-----|-----|------------|
| <i>Clostridium beijerinckii</i> | $24 \times 10^5$     | 32.3                 |    | x   | x   |            |
| <i>Bacteroides gingivalis</i>   | $10 \times 10^5$     | 13.5                 |    | x   | x   | x          |
| <i>Fusobacterium mortiferum</i> | $2.3 \times 10^5$    | 3.1                  |    | x   | x   |            |
| <i>Lactobacillus fermentum</i>  | $5.0 \times 10^5$    | 6.7                  |    | x   | x   |            |
| <i>Peptostrep. prevotii</i>     | $1.3 \times 10^5$    | 1.7                  |    | x   | x   |            |
| <i>Vibrio</i> sp.               | $1 \times 10^5$      | 1.3                  |    | x   | x   | x          |

Non-identified on MM10; classified according to Gram reaction and morphology:

| Colonial Morph.                                                                                              | Gram Stain                                              | O <sub>2</sub> | Avg. Viable Cnt./ml | % Total Viable Cnt. | API 20A/20S |
|--------------------------------------------------------------------------------------------------------------|---------------------------------------------------------|----------------|---------------------|---------------------|-------------|
| Opaque, raised center,<br>flat periph. center<br>smooth, irreg. wrinkled edge                                | Gm (-) med. length<br>rods                              | (+)            | $4.3 \times 10^5$   | 5.8                 | N/A         |
| Smooth flat colonies with<br>slightly raised center. Center<br>dark brown, periph. lt. brown<br>edge regular | Gm (-) coccobacilli                                     | (+)            | $3.3 \times 10^5$   | 4.4                 | N/A         |
| Rough, raised, opaque<br>Irreg. edge. Immovable                                                              | Thin Gm (-) rods<br>branched & sequented                | (+)            | $2 \times 10^5$     | 2.7                 | N/A         |
| Flat white rough red. edge                                                                                   | Small, thin Gm (-)<br>rods                              | (-)            | $1 \times 10^5$     | 1.4                 | Died        |
| Pinpt. dark brown rough<br>with irreg. edge                                                                  | Gm (-) cocci,<br>somewhat kidney-shaped<br>mostly pairs | (+)            | $0.3 \times 10^5$   | 0.4                 | N/A         |

On CVE (selective for detection of presumptive *F. nucleatum* strains)Two strains of *F. nucleatum* isolated & ID via GS, phase, API & growth (colonial morph)

GS - Gram stain    COL - colonial morphology    API-20A or 20S    SEL MED - selective media

Table 9

ANUG XI  
18 YO WM - Total counts on MM10 at  $10^{-5}$ :  
1. 77  
2. 68  
3. 38

Avg. Total Viable Count (using 0.1 ml)  
 $61 \times 10^6/\text{ml}$

On MM10 (nonselective) medium:

| Organism                                                                             | Avg. Viable Count/ml             | % Total Viable Count | GS                  | COL                 | API | ID                                                                                          |     |
|--------------------------------------------------------------------------------------|----------------------------------|----------------------|---------------------|---------------------|-----|---------------------------------------------------------------------------------------------|-----|
|                                                                                      |                                  |                      |                     |                     |     | SEL                                                                                         | MED |
| <i>Streptococcus sanguis</i>                                                         | $0.3 \times 10^6$                | 0.5                  |                     |                     | x   |                                                                                             | x   |
| <i>Actinomyces israelii</i>                                                          | $3.3 \times 10^6$                | 5.4                  |                     | x                   |     |                                                                                             |     |
| Non-identified on MM10: classified according to Gram reaction and morphology:        |                                  |                      |                     |                     |     |                                                                                             |     |
| Colonial Morph.                                                                      | Gram Stain                       | O <sub>2</sub>       | Avg. Viable Cnt./ml | % Total Viable Cnt. |     | API 20A/20S                                                                                 |     |
| Shiny, tan, slightly raised with clear rim and reg. edge                             | Gm (-) short blunt rods          | (-)                  | $8 \times 10^6$     | 13.1                |     | 20A - S <sup>+</sup> E <sup>-</sup> C <sup>-</sup> - Ind, Gly, Lac, Sac, Mal, Xyl, Raf, Rha |     |
| Brown with slightly white peaked center. Rough, immovable with irregular edge        | Gm (-) shant blunt coccobacillus | (+)                  | $4 \times 10^6$     | 6.6                 |     | 20A - All neg.                                                                              |     |
| Rough star-shaped with white center and brown periphery - Immovable with irreg. edge | Gm (-) cocci                     | (+)                  | $1 \times 10^6$     | 1.6                 |     | 20S - All neg.                                                                              |     |
| Small, translucent, shiny smooth & slightly raised                                   | Gm (-) cocci                     | (+)                  | $5.7 \times 10^6$   | 9.3                 |     | 20S - All neg.                                                                              |     |
| Violet colored smooth slightly raised rigid with regular edge                        | Gm (-) long thin rod             | (-)                  | $0.3 \times 10^6$   | 0.5                 |     | Died                                                                                        |     |

Table 9 con't.

|                                                                                         |                  |     |                   |     |                |
|-----------------------------------------------------------------------------------------|------------------|-----|-------------------|-----|----------------|
| Rough, slightly raised<br>and brown with scalloped edge                                 | Gm (-) cocci     | (+) | $2 \times 10^6$   | 3.3 | 20S - All neg. |
| Raised, translucent center<br>brown periphery. Center<br>smooth; periph rough. Spreader | Large Gm (-) rod | (+) | $0.3 \times 10^6$ | 0.5 | N/A            |

On TSAHK (selective for black-pigmenting Bacteroides)

One strain of B. mel. ss intermedius was isolated & ID via GS, API 20A, colonial morph (pigment)

| GS - Gram stain | COL - colonial morphology | API-20A or 20S | SEL MED - selective media |
|-----------------|---------------------------|----------------|---------------------------|
|-----------------|---------------------------|----------------|---------------------------|

ANUG XII

19 YO BF - Total counts on MM10 at  $10^{-4}$ ;  
1. 35  
2. 34

Table 10

Avg. Total Viable Count (using 0.1 ml)  
 $34.5 \times 10^3/\text{ml}$

On MM10 (nonselective) medium:

ID

| Organism                        | Avg. Viable Count/ml | % Total Viable Count | GS | COL | API | SEL<br>MED |
|---------------------------------|----------------------|----------------------|----|-----|-----|------------|
| <u>Bacteroides gingivalis</u>   | $12 \times 10^5$     | 34.8                 |    | x   | x   | x          |
| <u>Lactobacillus fermentum</u>  | $7 \times 10^5$      | 20.3                 |    | x   | x   |            |
| <u>Fusobacterium varium</u>     | $2.5 \times 10^5$    | 7.2                  |    | x   | x   |            |
| <u>Fusobacterium mortiferum</u> | $2 \times 10^5$      | 5.8                  |    | x   | x   |            |

Non-identified on MM10: classified according to Gram reaction and morphology:

| Colonial Morph.                                                                   | Gram Stain                                 | $O_2$ | Avg. Viable Cnt./ml | % Total Viable Cnt. | API 20A/20S                                        |
|-----------------------------------------------------------------------------------|--------------------------------------------|-------|---------------------|---------------------|----------------------------------------------------|
| Beige mucoid with reg. edge                                                       | Thick Gm (-) rods med. length, blunt ends  | (-)   | $8.5 \times 10^5$   | 24.6                | 20A - S <sup>+</sup> E <sup>-</sup> C <sup>-</sup> |
| Dull fried egg appearance colony with dark brown center and light brown periphery | Branching, long thin segmented Gm (-) rods | (+)   | $4.5 \times 10^5$   | 13.1                | N/A                                                |

On TSATIK (selective for black-pigmenting Bacteroides)

Two strains of Bacteroides gingivalis isolated and ID by GS, API 20A, colonial morph.

On CVE (selective for detection of presumptive F. nucleatum strains)

No F. nucleatum detected

---

GS - Gram stain    COL - colonial morphology    API-20A or 20S    SEL MED - selective media

Table 11

## ANUG XIX

28 YO WM - Total counts on MM10 at  $10^{-3}$ :JP superimposed 1. 43  
ANUG 2. 32  
3. 42Avg. Total Viable Count (using 0.1 ml)  
 $39 \times 10^4$ /ml

On MM10 (nonselective) medium:

ID

| Organism               | Avg. Viable Count/ml | % Total Viable Count | GS | COL | API | SEL<br>MED |
|------------------------|----------------------|----------------------|----|-----|-----|------------|
| <u>B. intermedium</u>  | $15.7 \times 10^4$   | 40.3                 |    | x   | x   | x          |
| <u>B. gingivalis</u>   | $14.7 \times 10^4$   | 37.7                 |    | x   | x   | x          |
| <u>F. necrophorum</u>  | $2.0 \times 10^4$    | 5.1                  |    | x   | x   |            |
| <u>S. constellatus</u> | $0.7 \times 10^4$    | 1.8                  |    | x   | x   |            |

Non-identified on MM10: classified according to Gram reaction and morphology:

| Colonial Morph.                                                              | Gram Stain                                                       | O <sub>2</sub> | Avg. Variable Cnt./ml | % Total Variable Cnt. | API 20A/20S                                        |
|------------------------------------------------------------------------------|------------------------------------------------------------------|----------------|-----------------------|-----------------------|----------------------------------------------------|
| Small, creamy, raised<br>smooth with regular edges                           | Gm (+) branched rods<br>long & thin & exhibiting<br>segmentation | (-)            | $3.7 \times 10^4$     | 9.5                   | 20A - S <sup>+</sup> E <sup>-</sup> C <sup>-</sup> |
| Large, dark brown, dull,<br>flat with regular edge                           | Gm (+) cocci in<br>chains, clusters                              | (+)            | $0.7 \times 10^4$     | 1.8                   | 20S - alpha hemolytic                              |
| Small, light brown center<br>dark brown edge, flat,<br>dull with irreg. edge | Gm (+) cocci in<br>chains, clusters<br>and pairs                 | (+)            | $0.3 \times 10^4$     | 0.8                   | 20S - alpha hemolytic<br>ARL - (+)<br>Rest - (-)   |

On TSA11K (selective for black-pigmenting Bacteroides)

| Organism              | Avg. Viable Count/ml | % Total Viable Count of Black-Pigmenting Org's |
|-----------------------|----------------------|------------------------------------------------|
| <u>B. intermedium</u> | $21.4 \times 10^4$   | 95.5                                           |
| <u>B. gingivalis</u>  | $1.0 \times 10^4$    | 4.5                                            |

On CVE (selective for detection of presumptive F. nucleatum strains)No F. nucleatum detected

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

Table 12

Levels of Suspected Odontopathic Organisms in Predominant Cultivable Flora  
of Plaque Taken from ANUG Sites of 20 Patients

| Species                                      | Total Avg.<br>Count | % of Total<br>Organisms | Species                                              | Total Avg.<br>Count | % of Total<br>Organisms |
|----------------------------------------------|---------------------|-------------------------|------------------------------------------------------|---------------------|-------------------------|
| Gram + rods                                  |                     |                         | Gram - rods                                          |                     |                         |
| <u>Clostridium beijerinckii</u>              | $1.17 \times 10^7$  | 2.66                    | <u>Bacteroides intermedius</u>                       | $3.44 \times 10^8$  | 78.2                    |
| <u>Lactobacillus fermentum</u>               | $2.46 \times 10^6$  | .56                     | <u>Bacteroides gingivalis</u>                        | $2.57 \times 10^5$  | .06                     |
| <u>Actinomyces israelii</u>                  | $1.2 \times 10^6$   | .27                     | <u>Fusobacterium nucleatum</u>                       | $3.43 \times 10^7$  | 7.80                    |
| Unidentified                                 | $3.3 \times 10^6$   | .75                     | <u>Fusobacterium sp.</u>                             | $1.5 \times 10^7$   | 3.41                    |
| S <sup>+</sup> E <sup>-</sup> C <sup>-</sup> | $1.17 \times 10^6$  | .26                     | <u>Vibrio sp.</u>                                    | $4.93 \times 10^6$  | 1.12                    |
| S <sup>+</sup> E <sup>-</sup> C <sup>-</sup> | $7.2 \times 10^4$   | .02                     | Anaerobes (unidentified)                             | $2.5 \times 10^5$   | .06                     |
| S <sup>+</sup> E <sup>-</sup> C <sup>-</sup> | $1.1 \times 10^6$   | .25                     | S <sup>+</sup> E <sup>-</sup> C <sup>-</sup>         | $1.04 \times 10^8$  | 23.6                    |
| S <sup>+</sup> E <sup>-</sup> C <sup>-</sup> | $2.0 \times 10^3$   | .001                    | S <sup>+</sup> E <sup>-</sup> C <sup>-</sup>         | $4.95 \times 10^7$  | 11.25                   |
| Facultative (unidentified)                   | $3.54 \times 10^6$  | .80                     | S <sup>+</sup> E <sup>-</sup> C <sup>+</sup>         | $6.05 \times 10^6$  | 1.38                    |
| all nonhemolytic & ARL <sup>-</sup>          |                     |                         | Not tested                                           | $7 \times 10^5$     | .16                     |
|                                              |                     |                         | Died                                                 | $16 \times 10^4$    | 10.93                   |
|                                              |                     |                         |                                                      | $4.81 \times 10^7$  | 10.93                   |
| Gram + cocci                                 |                     |                         | Facultative all<br>nonhemolytic and ARL <sup>-</sup> | $1.85 \times 10^8$  | 42.0                    |
| Facultative (identified)                     | $6.83 \times 10^7$  | 15.5                    |                                                      |                     |                         |
| <u>Streptococcus sanguis</u>                 | $4.7 \times 10^6$   | 1.17                    | Gram - cocci                                         |                     |                         |
| <u>Streptococcus sp.</u>                     | $5.04 \times 10^6$  | 1.15                    | <u>Veillonella parvula</u>                           | $1.55 \times 10^7$  | 3.52                    |
| <u>Staphylococcus sp.</u>                    | $5.0 \times 10^6$   | 1.14                    | Anaerobes (unidentified)                             | $3.3 \times 10^6$   | .75                     |
| Facultative (unidentified)                   | $4.15 \times 10^7$  | 9.43                    | S <sup>+</sup> E <sup>-</sup> C <sup>-</sup>         | $1.15 \times 10^6$  | .26                     |
| 2 hemolytic, ARL <sup>+</sup>                | $3.14 \times 10^7$  | 7.13                    | Died                                                 | $9.95 \times 10^5$  | .22                     |
| 2 hemolytic, ARL <sup>-</sup>                | $7 \times 10^4$     | .002                    | Facultative all                                      | $1.5 \times 10^5$   | .034                    |
| Beta hemolytic, ARL <sup>-</sup>             | $2.5 \times 10^6$   | .57                     | nonhemolytic & ARL <sup>-</sup>                      | $1.10 \times 10^7$  | 2.5                     |
| Nonhemolytic, ARL <sup>+</sup>               | $2.3 \times 10^6$   | .52                     |                                                      |                     |                         |
| Nonhemolytic, ARL <sup>-</sup>               | $5.17 \times 10^6$  | 1.18                    |                                                      |                     |                         |
| Anaerobic (identified)                       |                     |                         |                                                      |                     |                         |
| <u>Pseudomonas micros</u>                    | $7.0 \times 10^5$   | .16                     |                                                      |                     |                         |
| <u>Peptostreptococcus species</u>            | $1.2 \times 10^6$   | .27                     |                                                      |                     |                         |
| <u>Streptococcus sp.</u>                     | $9.12 \times 10^6$  | 2.07                    |                                                      |                     |                         |
| Anaerobes (unidentified)                     | $9.2 \times 10^5$   | .21                     |                                                      |                     |                         |
| S <sup>+</sup> E <sup>-</sup> C <sup>-</sup> | $9.0 \times 10^5$   | 2.05                    |                                                      |                     |                         |
| died                                         | $6.0 \times 10^5$   |                         |                                                      |                     |                         |

### Hemagglutination Activity of ANUG Isolates

Oral strains of Fusobacterium nucleatum and Bacteroides gingivalis have been shown to attach to and cause hemagglutination (HA) of human and sheep red blood cells (59). This activity may be involved in the colonization and pathogenic mechanisms of these organisms in the gingival crevice (60). Studies were undertaken to see if Fusobacterium nucleatum and Bacteroides gingivalis and intermedius isolates from ANUG patients demonstrated HA activity.

#### Materials and Methods

Cultures and cultural conditions. The strains of Fusobacterium and Bacteroides used can be seen in Tables 13 & 14. The fusobacteria were grown in a modified tryptone medium (59) and the Bacteroides strains in Brain Heart Infusion broth supplemented with 5 µg/ml hemin and 2 µg/ml menadione using the BBL anaerobe jar-Gas Pak system. After 24 to 72 h of growth, the organisms were harvested by centrifugation at 10,000 x g for 10 min and washed three times with 0.15 M NaCl. The organisms were routinely resuspended in 0.01 M phosphate buffer containing 0.15 M NaCl and 0.2% sodium azide (PBS) at a concentration of approximately 0.28 g/ml or in a 10% suspension (packed volume, after centrifugation at 800 x g for 15 min, diluted 1:10 in PBS).

HA test. A modification of the microtiter test described by Crawford et al. (61) was used for HA testing. Twenty five microliters of the whole-cell suspensions were serially diluted twofold with microdiluters in a microtiter tray. To this was added 25 µl of PBS followed by 25 µl of a 3 x PBS-washed 1.25% sheep red blood cell suspension. The microtiter tray was shaken on a micromixer for 1 min and incubated at 37° C for 30 min followed by incubation at room temperature 2 h before reading. HA was recorded as 1 to 4+, 4+ being a smooth blanket of RBC covering the bottom of the well. PBS instead of the HA preparation was used as a control for normal buttoning of the RBC.

#### Results

As can be seen in Tables 13 & 14 the F. nucleatum strains displayed a wide range of HA activity whereas the Bacteroides strains displayed little if any HA activity.

Table 13

Hemagglutination Assay of F. nucleatum Isolates

| <u>F. nucleatum</u> <sup>a</sup><br>strain | Hemagglutination Titer |
|--------------------------------------------|------------------------|
| VIII A <sub>14</sub>                       | 1:2                    |
| XII <sub>3</sub>                           | 1:8                    |
| WAF                                        | 1:1024                 |
| XA <sub>12</sub>                           | 1:4                    |
| VIII A <sub>13</sub>                       | 1:1024                 |
| CD <sub>3</sub>                            | 1:16                   |
| Lai                                        | 1:128                  |
| VI A <sub>2</sub>                          | 1:8                    |
| XVII <sub>3</sub>                          | 1:2                    |
| XIII <sub>2</sub>                          | 1:64                   |
| VI A <sub>1</sub>                          | 1:64                   |
| XVI <sub>2</sub>                           | 1:2                    |
| CD <sub>2</sub>                            | 1:4                    |
| Smoot                                      | 1:8                    |
| 4355                                       | 1:1024                 |
| 10197                                      | 1:64                   |
| 10953                                      | 1:4                    |
| DS <sub>1</sub>                            | 1:1024                 |
| MR <sub>3</sub>                            | 1:8                    |

<sup>a</sup> 5% whole cell suspensions<sup>b</sup> dilution displaying at least 2+ HA

Table 14

Hemagglutination Assay of Bacteroides Isolates

| <u>Bacteroides</u> <sup>a</sup><br>strains | Hemagglutination Titer |
|--------------------------------------------|------------------------|
| XII <sub>8</sub> <u>B. gingivalis</u>      | 1:2                    |
| CS44 <u>B. gingivalis</u>                  | -                      |
| CS43 <u>B. gingivalis</u>                  | -                      |
| CS41 <u>B. gingivalis</u>                  | 1:2                    |
| XV <sub>6</sub> <u>B. gingivalis</u>       | -                      |
| XIX <sub>9</sub> <u>B. intermedius</u>     | 1:2                    |
| 25261 <u>B. intermedius</u>                | -                      |
| 382 <u>B. fragilis</u>                     | -                      |
| 25285 <u>B. fragilis</u>                   | -                      |
| MH 678 <u>B. asaccharolyticus</u>          | -                      |
| 687 <u>B. asaccharolyticus</u>             | -                      |
| <u>B. ovatus</u>                           | -                      |

<sup>a</sup> 5% whole cell suspensions<sup>b</sup> dilution displaying at least 2+ HA

## Serologic Characterization of Fusobacterium nucleatum ANUG

### Isolates with Other F. nucleatum Strains

#### Introduction

A study was undertaken to compare the hemagglutination activity and reaction of human sera and rabbit anti-F. nucleatum sera with F. nucleatum isolates obtained from humans demonstrating clinically healthy gingiva and various gingival and periodontal disease states. Additional isolates were obtained from dogs and from a Macaca mulatta monkey demonstrating spontaneous chronic periodontitis. The serologic reactions of the F. nucleatum isolates were compared with selected reference strains of F. nucleatum.

#### Materials and Methods

##### Cultures and cultural conditions.

F. nucleatum clinical isolates were obtained from the following sources: 1) human adults virtually free of periodontal diseases, 2) human children free of periodontal diseases, 3) human adults with diagnosed chronic periodontitis, 4) human adults with diagnosed acute necrotizing ulcerative gingivitis (ANUG), 5) human adults with diagnosed juvenile periodontitis, 6) two mature dogs, one mixed breed and one golden retriever, with clinical signs of early chronic periodontitis and 7) one Macaca mulatta monkey with spontaneous advanced chronic periodontitis (Table 15). Three typed strains of F. nucleatum (ATCC 10953 and VPI 4355 and 10197) were also utilized. All clinical isolates were obtained from subgingival plaque samples using a sterile curette (MC 17/18 - Hu Friedy) and immediately streaked onto CVE agar (62), a selective medium for the isolation of F. nucleatum. All samples were incubated at 37° C anaerobically using the BBL anaerobic jar-Gas Pak system (BBL, Cockeysville, Md.) or a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, Michigan) with an atmosphere of 85% nitrogen, 10% hydrogen and 5% carbon dioxide. After four days of incubation, isolates were identified by colonial morphology (62) and described as type I, a 2 mm transparent smooth blue colony having an entire edge with a dark blue center or type II, a 1 mm to 2

Table 15

Source of F. nucleatum clinical isolates

| <u>Clinical Description</u>                | <u>Number of Subjects</u> | <u>Number of Isolates</u> |
|--------------------------------------------|---------------------------|---------------------------|
| Human Children free from disease           | 2                         | 3                         |
| Human Adults free from disease             | 6                         | 7                         |
| Human patients with chronic periodontitis  | 3                         | 3                         |
| Human patients with ANUG                   | 5                         | 5                         |
| Human patients with juvenile periodontitis | 3                         | 6                         |
| Canine                                     | 2                         | 8                         |
| <u>Macaca mulatta</u>                      | <u>1</u>                  | <u>11</u>                 |
| TOTAL                                      | 22                        | 43                        |

mm transparent round or irregular blue colony with a speckled appearance. Selected colonies were streaked for isolation on blood agar (BBL) and incubation continued for 48 hr. Each isolate was observed by phase contrast microscopy and gram stained to verify the typical morphology of Fusobacterium. Only isolates which demonstrated Gram negative staining with the morphology of long rods with pointed ends and failing to grow aerobically on blood agar were selected for further study. Biochemical reactions were determined using the API 20A system (Analytab Products, Plainville, N. Y.). Isolates were then transferred to a modified tryptone medium (59) and incubated as before. Cells were harvested by centrifugation at 10,000 x g for 10 min at 4° C, washed three times in 0.01 M phosphate buffer containing 0.15 M NaCl (PBS), pH 7.2 and resuspended in PBS to a 10% suspension (1:10 dilution of packed whole cells after centrifugation at 2,000 x g for 10 min).

Whole cell suspensions of these isolates were sonicated using 8 bursts of 30 sec each in a dry ice-alcohol bath with a Heat Systems Sonicator (Plainville, N. Y.) at a microtip setting of 7. Greater than 95% of the cells were lysed as observed by phase contract microscopy. This was designated as the sonicated preparation (SP).

#### Double diffusion in agar.

SP of selected isolates and typed strains of Fusobacterium nucleatum were reacted by double diffusion (63) in 1% agarose in 0.01 M PBS, pH 7.2 with undiluted rabbit anti-F. nucleatum 10953 or 10197 serum (59). Reactions were incubated at 4° C in a humidior and were observed after 24 hr. Protein content of SP as determined by Lowry et al. (64) was approximately 400 µg/ml).

#### Serological evaluation by ELISA.

Selected isolates were reacted by an enzyme-linked immunosorbent assay (ELISA) with the following sera: 1) rabbit anti-F. nucleatum 10953 or 10197 serum, 2) normal rabbit serum, 3) human sera previously shown to be reactive to 10953, 10197 and 4355 and 4) serum obtained from the Macaca mulatta demonstrating spontaneous chronic

periodontitis. A modification of the enzyme-linked immunosorbent assay (ELISA) was utilized (65). Two hundred microliters of a 1:10 dilution of the previously described whole cell suspensions of F. nucleatum in a 60 mM carbonate buffer, pH 9.6, were added to each well of a microtiter plate (Dynatech Laboratories, Inc., Alexandria, VA) and incubated at 37° C for 3 hr followed by refrigeration overnight. Peripheral rows were not utilized. The plate was washed five times with 0.01 M PBS containing 1% BSA and 0.05% tween 20. This was followed by a 3 hr incubation at 37° C with 200 µl of a 1% BSA solution in 60 mM carbonate buffer in order to assure complete coverage of all binding sites in each well. The plate was then washed five times with PBS/tween 20 with 1% BSA. One hundred microliters of serial two fold dilutions in 0.01 M PBS with 0.05% tween 20, pH 7.2, of the sera were added and incubated at 37° C for 30 min. The plates were washed as before and 100 µl of a 1:200 dilution in PBS of peroxidase labeled IgG fraction of goat anti-rabbit  $\gamma$ ,  $\alpha$  and  $\mu$  heavy chain serum or peroxidase labeled goat anti-Rhesus gamma/globulin serum (Cappel Laboratories, Inc., Cochranville, Pa.) were added and the plate incubated again at 37° C for 30 min. After again washing the plates five times, 100 µl of the enzyme substrate (1 ml of 1% w/v O-phenylene-diamine in methanol plus 99 ml distilled H<sub>2</sub>O and 0.1 ml 3% H<sub>2</sub>O<sub>2</sub>) were added. The plates were incubated in the dark for 30 min at room temperature, the reaction was stopped by adding 20 µl of 8N H<sub>2</sub>SO<sub>4</sub> and reactions were determined colorimetrically at 490 nm with a Microelisa Reader (Dynatech Laboratories, Inc.). To determine the optimal concentration of antigen and peroxidase labeled goat antiserum, a dual titration of doubling dilutions of antigen (0.25% to 10% whole cell suspensions) against serial 1:5 fold dilutions of conjugate was performed. Analysis of the protein content of the 1% whole cell suspensions (1:10 dilution of the 10% suspension) was determined to be 400 µg/ml by the technique described by Lowry et al. (64).

#### Hemagglutination (HA)

The HA assay was performed by the method of Falkler and Hawley (60). Briefly,

serial two-fold dilutions of 50  $\mu$ l of 10% whole cell suspensions of selected isolates of F. nucleatum were made in microtitration multi-well plates (Linbro Scientific, Inc., Hamden, Conn.) with 0.01 M PBS, pH 7.2. This was followed by the addition of 25  $\mu$ l of PBS, pH 7.2 and 25  $\mu$ l of a 1.25% suspension of sheep red blood cells (RBCs). Plates were mixed for 20 sec on a Micro-Shaker (Cooke Laboratory Products, Alexandria, Va.) then incubated for 30 min at 37° C followed by 1 hr at 4° C. Titers were determined visually as the reciprocal of the highest dilution demonstrating a 2+ hemagglutination. Inhibition of HA was determined as above except for the addition of 25  $\mu$ l of 50 mM of D-galactose in place of PBS and incubation for 30 min at 37° C prior to adding the sheep RBCs.

### Results

#### Isolation of F. nucleatum

F. nucleatum isolates were obtained on CVE agar plates from all plaque samples. The human isolates demonstrated both type I and type II colonial morphology as did isolates from the Macaca mulatta monkey. All canine isolates showed only type I colonial morphology. All isolates regardless of colony type showed identical results with the API 20A system (a positive indole response with all other reactions negative).

#### Serological evaluation by immunodiffusion.

SP of 10953, 10197 and 4355 were reacted with rabbit anti-F. nucleatum 10953 serum by double diffusion in agar. Several precipitin lines including lines of identity were observed for all typed strains. SP of sixteen separate isolates from various human oral disease states and the three typed strains of F. nucleatum were also reacted with rabbit anti-F. nucleatum 10953 serum. Lines of identity were evident between all sixteen clinical isolates of F. nucleatum and the three typed strains. The eleven Macaca mulatta monkey isolates, eight canine isolates, three human isolates and strain 10953 were also reacted with the rabbit anti-F. nucleatum 10953 serum. Of all nineteen of the animal isolates tested, only two of the Macaca mulatta strains shared lines of identity with the human isolates and 10953.

Eleven human isolates of F. nucleatum were reacted by ELISA with rabbit anti-F. nucleatum 10197 or 10953 serum and with normal rabbit serum. As can be observed in Table 16, similar titers for all isolates were obtained with both hyperimmune sera which displayed substantially higher titers than those observed with normal rabbit serum.

Twenty-three human clinical isolates of F. nucleatum were reacted by ELISA with a human serum previously shown to be reactive with F. nucleatum. The titers that were obtained are shown in Table 17. Irregardless of the source of the F. nucleatum isolates, a similar range of antibody activity was detected with the human serum.

The results obtained when ten Macaca mulatta isolates, eight canine isolates, four human isolates and 10953 were reacted with 1) human sera previously shown to be reactive with F. nucleatum, 2) serum from the Macaca mulatta with spontaneous periodontal disease and rabbit anti-F. nucleatum can be seen in Table 18. All sera showed antibody reactivity to the human (including 10953) and Macaca mulatta strains but not to the canine isolates.

#### Hemagglutination (HA)

Eleven Macaca mulatta isolates, eight canine isolates, three human isolates and strain 10953 were tested for HA activity and with the addition of 50 mM D-galactose, for inhibition of HA. All canine isolates consistently failed to show any HA of sheep RBCs (Table 19). Some of the Macaca mulatta isolates and all of the human isolates showed varying degrees of HA activity. All isolates which demonstrated HA activity also showed a decrease in HA when preincubated with 50 mM D-galactose prior to HA testing.

Table 16

Elis: titers obtained with human isolates of F. nucleatum  
and anti-F. nucleatum and pre-immune sera.

| Patient Classification                                          | Sera                                                       |                                                            |                                   |
|-----------------------------------------------------------------|------------------------------------------------------------|------------------------------------------------------------|-----------------------------------|
|                                                                 | Rabbit anti-<br><u>F. nucleatum</u><br>T0197 mean<br>titer | Rabbit anti-<br><u>F. nucleatum</u><br>T0953 mean<br>titer | Normal<br>rabbit<br>mean<br>titer |
| Normal adult (6) <sup>a</sup>                                   | 6 <sup>b</sup>                                             | 6                                                          | 3                                 |
| Patients with acute<br>necrotizing Ulcerative<br>Gingivitis (5) | 6                                                          | 6                                                          | 3                                 |

<sup>a</sup> number of patients providing isolates of F. nucleatum

<sup>b</sup> highest dilution giving an OD reading of greater than 0.15 at 490 nm after transforming the results from geometric progressions to arithmetic progressions (1:16=1, 1:32=2, 1:64=3, etc.

Table 17

Elisa titers obtained with human isolates of

F. nucleatum and a human sera

| <u>Patient Classification</u>                 |                | <u>mean titer<sup>b</sup></u> |
|-----------------------------------------------|----------------|-------------------------------|
| Normal adult                                  | 7 <sup>a</sup> | 5.7 (5-6) <sup>c</sup>        |
| Normal child                                  | 3              | 6 <sup>d</sup>                |
| Acute necrotizing<br>ulcerative<br>gingivitis | 5              | 5.6 (4-6)                     |
| Chronic<br>periodontitis                      | 2              | 7 <sup>c</sup>                |
| Juvenile<br>periodontitis                     | 5              | 6.8 (6-8)                     |

<sup>a</sup> number of patients providing isolates of F. nucleatum and number of isolates tested

<sup>b</sup> after transforming the results from geometric progressions to arithmetic progressions (1:10=1, 1:20=2, 1:40=3, etc.)

<sup>c</sup> range of titers

<sup>d</sup> all titers were the same within this group

Table 18

Flisa titers obtained with clinical and typed strain  
isolates of F. nucleatum with selected sera.

| <u>F. nucleatum</u><br><u>isolates</u> |                 | <u>Human</u>                        | <u>Rabbit anti-</u><br><u>Macaca</u><br><u>mulatta</u> | <u>F. nucleatum</u><br><u>10953 Serum</u> |
|----------------------------------------|-----------------|-------------------------------------|--------------------------------------------------------|-------------------------------------------|
| <u>Macaca mulatta</u>                  | 10 <sup>a</sup> | 2.9 <sup>b</sup> (3-4) <sup>c</sup> | 4.7 (4-5)                                              | 5.7 (5-6)                                 |
| Canine                                 | 8               | 1                                   | 1                                                      | 1                                         |
| Human                                  | 4               | 5.5 (5-6)                           | 4 <sup>d</sup>                                         | 7                                         |
| 10593                                  | 1               | 6                                   | 4                                                      | 8                                         |

<sup>a</sup> number of isolates from each source

<sup>b</sup> after transforming the results from geometric progressions to arithmetic progressions  
(1:10=1, 1:20=2, 1:40=3, etc.)

<sup>c</sup> range of titers

<sup>d</sup> all titers were the same

Table 19

Hemagglutination obtained from clinical  
isolates of F. nucleatum and 10953.

| Isolates              |    | Hemagglutination<br>titers           | Hemagglutination<br>titers after<br>galactose inhibition |
|-----------------------|----|--------------------------------------|----------------------------------------------------------|
| <u>Macaca mulatta</u> | 11 | 3.3 <sup>b</sup> ( 1-8) <sup>c</sup> | .91 ( 1-1)                                               |
| Canine                | 8  | d                                    | d                                                        |
| Human                 | 3  | 6.3 (4-8)                            | 4.3 (3-6)                                                |
| Typed strain<br>10953 | 1  | 11 <sup>e</sup>                      | 9 <sup>e</sup>                                           |

<sup>a</sup> number of isolates from each source

<sup>b</sup> highest dilution displaying at least a 2+ HA after transforming the results from geometric progressions to arithmetic progressions (1:2=1, 1:4=2, 1:8=3, etc.)

<sup>c</sup> range of titers

<sup>d</sup> hemagglutination was not observed at the lowest dilution used - 1:2

<sup>e</sup> all titers were the same

## Reaction of Serum from ANUG Patients with Fusobacterium and Bacteroides Strains

In an attempt to detect antibody in the sera of ANUG patients with Bacteroides and Fusobacterium strains enzyme linked immunosorbent assays were established. Sera from ANUG patients and age and sex matched healthy individuals were reacted with Fusobacterium nucleatum and Bacteroides strains isolated from ANUG patients, chronic periodontitis patients, non-oral abscesses and healthy gingival sulci. Experiments were conducted to measure reactive isotypes of IgA, IgM and IgG and in other experiments IgG only

### MATERIALS AND METHODS

#### Micro-Enzyme-Linked Immunosorbent Assay (ELISA)

Briefly, 200  $\mu$ l of a 1% Fusobacterium or Bacteroides whole cell suspensions (grown as previously described in HA methodology) in .06 M carbonate buffer, pH 9.6, were added to each well of a microtiter multi-well plate (outside rows of wells not used) and incubated at 37° C for 3 h followed by refrigeration overnight. The plate was washed three times with PBS containing 0.05% Tween 20 and 1% BSA. Then 200  $\mu$ l of a 1% BSA solution in 0.06 M carbonate buffer, pH 9.6, were added to the wells and incubation allowed to proceed for 3 h at 37° C in order to tie up all non-antigen coated binding sites. The tray was then rinsed five times with PBS/Tween 20 with a 1% BSA. Serial two fold dilutions of the sera (50  $\mu$ l) in PBS were added to each well and the plates incubated at 37° C for 30 min. The plates were washed as before and Fusobacterium and Bacteroides absorbed peroxidase labeled IgG fraction of goat anti-human  $\gamma$ ,  $\alpha$  or  $\mu$  heavy chain serum (Miles Laboratories) diluted 1:200 in PBS, was added to each well (50  $\mu$ l) and incubated for 1.5 h at room temperature. After again washing the plates, 50  $\mu$ l of the enzyme substrate (1 ml of 1% w/v O-phenylene-diamine in methanol + 99 ml distilled H<sub>2</sub>O<sub>2</sub>) were added. After 1.5 h of incubation in darkness at room temperature the reaction was stopped by the addition of 8 N H<sub>2</sub>SO<sub>4</sub> (25  $\mu$ l) and the color was read at 490 nm using a Chromoscan spectrometer.

To determine the amount of Bacteroides and Fusobacterium (antigen) required to coat the wells of the microtiter plate and to determine a dilution at which the peroxidase labeled goat antisera should be used, a dual titration of doubling dilutions of antigen (0.25 to 10% whole cell suspensions) against serial 1:5 fold dilutions of conjugate was performed. An excess of reactive sera (1 in 100 dilution) was added to each well. A 1% suspension of Bacteroides and Fusobacterium strains and a 1:200 dilution of conjugate were found to be concentrations which would give an optimum yellow color and were the standard dilutions used for all subsequent experiments. All serologic tests were performed in duplicate and when more than a 0.15 O.D. difference was observed, testing of the serum was repeated. Controls included the reaction of sensitized wells with saline replacing human serum in the assay and reacting substrate with conjugate to check on enzyme activity.

## RESULTS

Five sera from ANUG patients and age matched sera from individuals with healthy mouths were reacted with 18 strains of F. nucleatum and 12 Bacteroides strains. The microorganisms represented ATCC and VPI strains, clinical isolates and isolates from ANUG lesions.

When the five sera were reacted with the 18 F. nucleatum isolates in an ELISA which would detect IgG, IgA and IgM it was observed that the sera displayed similar degrees of activity irregardless of the F. nucleatum strain used as the antigen source (Table 20). It thus appears all of the F. nucleatum strains tested displayed shared antigenic determinants. It was also observed that there were differences in the activity of the sera, some showing significant differences in the amount of antibody reactive with the F. nucleatum strains. When IgG was detected in the ANUG sera and age matched sera from individuals with healthy oral cavities (Table 21) it was observed that there were no differences in the mean antibody activity to the 18 strains in the two groups and in three of the five sera pairs, a slightly higher level of antibody was detected in the sera from the healthy individuals. Again it appeared that IgG was present in each sera to all of the organisms tested and that similar levels of antibody reactive with each organism was detected.

Although a somewhat higher level of antibody was found reactive with the Bacteroides strains, the data paralleled what was observed with the F. nucleatum testing. It appears that the B. gingivalis strains isolated from the ANUG patients share antigens with other B. gingivalis clinical isolates (Table 22) and that no differences were observed between the reactivities of the patient and age matched control sera (Table 23). It was of interest that the levels of antibody in both groups reactive to the non-oral B. fragilis and B. ovatus strains were lower than those observed for the oral strains (Tables 24 & 25).

Table 20  
ELISA Measuring IgG, IgA & IgM

| <u>F. nucleatum</u><br><u>strain</u> | ANUG Patient Serum Source |            |           |            |            |
|--------------------------------------|---------------------------|------------|-----------|------------|------------|
|                                      | <u>XII</u>                | <u>XIV</u> | <u>XV</u> | <u>XVI</u> | <u>XIX</u> |
| 4355                                 | 528 <sup>a</sup>          | 435        | 861       | 330        | 997        |
| Lai                                  | 716                       | 508        | 1169      | 463        | 954        |
| 10953                                | 1028                      | 725        | 1166      | 560        | 1223       |
| CD <sub>3</sub>                      | 495                       | 243        | 930       | 140        | 720        |
| VIII A <sub>14</sub>                 | 506                       | 271        | 581       | 207        | 681        |
| X A <sub>12</sub>                    | 444                       | 323        | 827       | 105        | 751        |
| XIII <sub>2</sub>                    | 397                       | 206        | 629       | 12         | 568        |
| WAF                                  | 460                       | 247        | 746       | 126        | 690        |
| VI A <sub>1</sub>                    | 678                       | 307        | 1215      | 162        | 872        |
| XVII <sub>8</sub>                    | 396                       | 610        | 1022      | 565        | 910        |
| XVI <sub>2</sub>                     | 705                       | 768        | 983       | 505        | 1059       |
| DS <sub>1</sub>                      | 444                       | 225        | 704       | 111        | 744        |
| XII <sub>3</sub>                     | 520                       | 366        | 839       | 199        | 1010       |
| CD <sub>2</sub>                      | 429                       | 346        | 836       | 66         | 721        |
| MR <sub>3</sub>                      | 462                       | 345        | 888       | 256        | 738        |
| VI A <sub>2</sub>                    | 571                       | 433        | 1131      | 127        | 761        |
| VIII A <sub>13</sub>                 | 289                       | 220        | 574       | 33         | 612        |
| 10197                                | 560                       | 490        | 1029      | 214        | 860        |
| <hr/>                                |                           |            |           |            |            |
| Mean =                               | 535                       | 393        | 896       | 232        | 826        |
| S. E. =                              | 121                       | 89         | 202       | 52         | 136        |

<sup>a</sup> optical density at 490 nm - average of duplicates

Significant difference (by S. D.) between 1) serum XV and XIV, XVI; 2) serum XIX and XIV, XVI

Table 21

## ELISA Measuring IgG

| <u>F. nucleatum</u><br><u>strain</u> | <u>XII</u>       | <u>Control</u> | <u>XIV</u> | <u>Control</u> | <u>XV</u> | <u>Control</u> | <u>XVI</u> | <u>Control</u> | <u>XIX</u> | <u>Control</u> |
|--------------------------------------|------------------|----------------|------------|----------------|-----------|----------------|------------|----------------|------------|----------------|
| 4355                                 | 725 <sup>a</sup> | 1383           | <u>b</u>   | 1645           | 1240      | 746            | 378        | 1999           | 1363       | 1311           |
| Lai                                  | 587              | 410            | 905        | 1635           | 1373      | 1005           | 708        | 773            | 1216       | 762            |
| 10953                                | 588              | 734            | 1153       | 1600           | 1086      | 1003           | 697        | 629            | 1488       | 798            |
| CD 3                                 | 162              | 227            | 589        | 1072           | 634       | 662            | 399        | 453            | 1043       | 538            |
| VIII A14                             | 292              | 493            | --         | 995            | 701       | 1013           | 423        | 707            | 1032       | 360            |
| X A12                                | 221              | 329            | 955        | 480            | 741       | 515            | 654        | 437            | 1122       | 480            |
| XII 2                                | 331              | 284            | 1481       | 392            | 392       | 650            | 450        | 539            | 1078       | 366            |
| WAF                                  | 412              | 221            | 1104       | 547            | 827       | 545            | 788        | 616            | 1192       | 563            |
| VI A                                 | 753              | 308            | 1072       | 732            | 1589      | 557            | 881        | 787            | 1035       | 990            |
| XVII 8                               | 811              | 636            | 1457       | 748            | 1091      | 830            | 1216       | 689            | 1399       | 554            |
| XVI 2                                | 592              | 760            | 1319       | 1053           | 942       | 1294           | --         | 494            | 321        | 667            |
| DS 1                                 | 680              | 351            | 492        | 939            | 795       | 1025           | --         | 498            | 1063       | 559            |
| XII 3                                | 464              | 559            | 529        | 1048           | 1122      | 865            | 1083       | 614            | 1021       | 697            |
| CD 2                                 | 296              | 308            | 383        | 946            | 774       | 600            | 425        | 560            | 1088       | 398            |
| MIR 3                                | 509              | 966            | 486        | 1228           | 1210      | 568            | 843        | 427            | 1085       | 782            |
| VI A2                                | 497              | 879            | --         | 1129           | 1534      | 755            | 792        | 619            | 1253       | 761            |
| XVIII A15                            | 682              | 234            | 478        | 1544           | 1012      | 801            | 746        | 932            | 995        | 389            |
| 10197                                | 574              | 731            | --         | 1375           | 811       | 776            | 1054       | 748            | 1054       | 320            |
| Mean                                 | 509.8            | 545.2          | 689.1      | 1061.6         | 993.0     | 789.4          | 640.9      | 695.6          | 1082.1     | 627.5          |
| S.E.                                 | 120.2            | 128.6          | 162.5      | 250.4          | 234.2     | 186.2          | 151.2      | 164.1          | 255.2      | 148.0          |

a optical density at 490 nm  
b no activity

Table 22  
ELISA Measuring IgG, IgA and IgM

| <u>B. gingivalis</u><br><u>strain</u> | ANUG Patient Serum Source |            |           |            |            | <u>X/SD</u>  |
|---------------------------------------|---------------------------|------------|-----------|------------|------------|--------------|
|                                       | <u>XII</u>                | <u>XIV</u> | <u>XV</u> | <u>XVI</u> | <u>XIX</u> |              |
| XV 6                                  | 738 <sup>a</sup>          | 574        | 1448      | 672        | 1501       | 98.6/449.6   |
| XII 8                                 | 627                       | 709        | 1009      | 738        | 982        | 323.0/167.7  |
| CS41                                  | 723                       | 510        | 1221      | 283        | 1614       | 870.2/541/4  |
| CS43                                  | 1194                      | 921        | 1590      | 609        | 1596       | 1132.0/928.5 |
| CS44                                  | 1093                      | 823        | 1705      | 582        | 1663       | 1161.2/503.4 |
| <hr/>                                 |                           |            |           |            |            |              |
| Mean =                                | 863.0                     | 707.4      | 1394.6    | 586.8      | 1471.2     |              |
| S.D. =                                | 239.5                     | 170.1      | 281.1     | 187.4      | 279.7      |              |
| <hr/>                                 |                           |            |           |            |            |              |

<sup>a</sup> optical density at 490 nm - average of duplicates

Table 23

## ELISA Measuring IgG

| <u>B. gingivalis</u><br><u>strain</u> | <u>XII</u>        | <u>Control</u> | <u>XIV</u>      | <u>Control</u> | <u>XV</u> | <u>Control</u> | <u>XVI</u> | <u>Control</u> | <u>XIX</u> | <u>Control</u> | <u>ANUG</u><br><u>X/SD</u> | <u>Control</u><br><u>X/SD</u> |
|---------------------------------------|-------------------|----------------|-----------------|----------------|-----------|----------------|------------|----------------|------------|----------------|----------------------------|-------------------------------|
| XV6                                   | 1349 <sup>a</sup> | 1730           | 1622            | 1223           | 1690      | 1736           | 1613       | 1745           | 1680       | 1496           | 1590.8/139.4               | 1586.0/223.2                  |
| X118                                  | 406               | 489            | -- <sup>b</sup> | 608            | 675       | 175            | 373        | 861            | 1055       | 399            | 501.8/391.7                | 334.8/244.0                   |
| CS41                                  | 1407              | 1802           | 311             | 1133           | 1390      | 1660           | 1235       | 1738           | 1620       | 406            | 1192.6/511.5               | 1347.8/389.3                  |
| CS43                                  | 1640              | 1538           | 1643            | 1670           | 1551      | 1697           | 1011       | 1805           | 1738       | 419            | 1515.6/290.3               | 1427.8/566.4                  |
| CS44                                  | 1675              | 1679           | 1551            | --             | 1432      | 1660           | 588        | 1941           | 1392       | 710            | 1327.6/428.0               | 1198.0/817.0                  |
| Mean                                  | 1295.4            | 1447.6         | 1025.4          | 926.8          | 1347.6    | 1385.6         | 964.0      | 1618.0         | 1497.0     | 688.0          |                            |                               |
| S.D.                                  | 517.0             | 544.5          | 802.4           | 640.9          | 393.7     | 677.4          | 496.9      | 431.0          | 279.8      | 470.0          |                            |                               |

<sup>a</sup> optical density at 490 nm<sup>b</sup> no activity

Table 2\*

ELISA Measuring IgG, IGA and IgM

| <u>Bacteroides</u><br><u>strain</u>        | <u>XII</u>        | <u>XIV</u> | <u>XV</u> | <u>XVI</u> | <u>XIX</u> | <u>X/D</u>   |
|--------------------------------------------|-------------------|------------|-----------|------------|------------|--------------|
| <u>B. asacch</u>                           | 1557 <sup>a</sup> | 1337       | 1614      | 1042       | 1470       | 1404.4/227.7 |
| XIX <sub>9</sub> ( <u>B. intermedius</u> ) | 566               | 385        | 1804      | 140        | 1582       | 895.4/747.8  |
| <u>B. intermedius</u>                      | 996               | 937        | 1555      | 722        | 1431       | 1123.2/351.0 |
| <u>B. mel</u> CSI                          | 1028              | 822        | 1614      | 1243       | 1580       | 1257.4/344.1 |
| <u>B. frag</u> 382                         | 1060              | 709        | 1200      | 432        | 819        | 844.0/300.8  |
| <u>B. frag</u>                             | 572               | 166        | 584       | 67         | 511        | 380.0/244/6  |
| <u>B. ovatus</u>                           | 462               | 315        | 740       | 109        | 968        | 518.8/340.4  |
| <hr/>                                      |                   |            |           |            |            |              |
| Mean =                                     | 891.6             | 667.3      | 1301.6    | 536.4      | 1194.4     |              |
| S.E. =                                     | 336.4             | 251.8      | 490.9     | 202.4      | 450.5      |              |
| <hr/>                                      |                   |            |           |            |            |              |

<sup>a</sup> optical density at 490 nm - average of duplicates

Table 25

## ELISA Measuring IgG

| Bacteroides strain           | XII   | Control | XIV          | Control | XV     | Control | XVI    | Control | XIX    | Control | ANUG X/SD    | Control X/SD |
|------------------------------|-------|---------|--------------|---------|--------|---------|--------|---------|--------|---------|--------------|--------------|
| <u>B. asacch.</u>            | 772   | 895     | 1320         | 1386    | 1545   | 1095    | 1179   | 684     | 1407   | 831     | 1244.6/295.8 | 978.2/271.6  |
| <u>XIX9 (B. intermedius)</u> | 899   | 1247    | 1296         | 972     | 1705   | 528     | 775    | 708     | 1654   | 1030    | 1265.8/424.2 | 897.0/281.8  |
| <u>B. intermedius</u>        | 1538  | 1824    | 1643         | 1670    | 1744   | 1750    | 1574   | 1582    | 1677   | 1637    | 1635.2/81.9  | 1692.6/95.4  |
| <u>B. mel CS1</u>            | 1609  | 1871    | 1506         | 1790    | 1683   | 1677    | 1763   | 1920    | 1736   | 1567    | 1659.4/104.0 | 1765.0/143.9 |
| <u>B. frag 382</u>           | 892   | 606     | 673          | 1263    | 1470   | 1189    | 495    | 674     | 1050   | 751     | 882.5/424.0  | 896.6/306.2  |
| <u>B. frag</u>               | 813   | 261     | <sup>b</sup> | 700     | 499    | 385     | 332    | 271     | 858    | 398     | 500.4/355.1  | 403.0/177.6  |
| <u>B. ovatus</u>             | 200   | 62      | 1630         | --      | 532    | 751     | 883    | 529     | 1518   | 309     | 952.6/617.8  | 330.2/315.4  |
| Mean                         | 960.4 | 966.6   | 1152.6       | 1111.6  | 1311.1 | 1053.6  | 1000.1 | 909.7   | 1414.3 | 931.9   |              |              |
| S.E.                         | 362.4 | 364.7   | 434.9        | 419.4   | 494.8  | 397.4   | 377.4  | 343.3   | 533.7  | 351.6   |              |              |

<sup>a</sup> optical density at 490 nm<sup>b</sup> no activity

This data suggests that:

- (1) Fusobacterium as well as Bacteroides isolates from ANUG lesions each share antigenic determinants.
- (2) A single serum sample taken during an ANUG episode when reacted with Fusobacterium or Bacteroides strains appears not to be useful in the diagnosis of the disease.

### **Studies on Spirochetes from ANUG Lesions**

Attempts were made to determine the number of rods, cocci and spirochetes in the plaque samples and to cultivate spirochetes from these samples. Plaque debris and extracted teeth were studied by transmission and scanning electron microscopy respectively to evaluate the spirochetes involved. In order to characterize the spirochete isolates serological studies and sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis was performed. The materials and methods and results of these studies to date follows.

### **MATERIALS AND METHODS**

#### **Dark-field microscopic counts**

The RTF plaque samples used in the culture attempts were vortexed for 30 sec using a vortex-genie and then a drop of each plaque suspension was placed in the center of a Petroff-Hausser Counting Chamber which had been covered with a No. 1.5 cover glass. The counting chamber was then allowed to stand 15 min at room temperature and was subsequently examined under dark-field at a magnification of 500 X using a Leitz Dialux microscope. All bacteria observed in 80 squares of the counting chamber were counted and divided into 3 categories: (1) rods, (2) cocci, and (3) spirochetes respectively. All counts were completed within 24 hours of plaque collection.

#### **Isolation of Spirochete Strains**

Subgingival plaque samples were collected by a periodontist from clinic patients. These samples were placed in a tube containing prereduced Medium A Broth (described below) and transported to a Coy anaerobic chamber. This chamber was maintained at a temperature of 35° C and with an atmosphere of 85% nitrogen, 10% hydrogen and 5% carbon dioxide. The samples were treated as described by Leschine and Canale-Parola (66) with the plaque sample serially ten-fold diluted in 13 x 100 screw-capped tubes containing the NOS medium with 1-2 µg/ml of rifampin and 0.3% agar. The tubes were then incubated inside the chamber for 7-14 days at which time the resulting individual isolated spirochetel colonies were picked and examined by dark-field microscopy to

determine the presence of spirochetes. Positive colonies were streaked on the appropriate medium in the chamber to obtain isolated clones or rediluted as before in fresh NOS media. A portion of the sample was also placed on 25 mm-0.45  $\mu$ m pore size Millipore filters on the surface of Medium A containing 1.0% agar. The spirochetes migrated through the filters and the larger contaminating bacteria remained on the filter surface. Spirochetes developed as a veil of growth and the leading edge of the growth was removed by taking a plug of agar with a Pasteur pipette and restreaking on solid medium within the anaerobic chamber to obtain isolated clones.

#### Spirochete Cultivation

The oral spirochete strains which we have require slightly different growth media. The composition of the media used follows:

##### Medium A

| <u>Component</u>                            | <u>Amount</u> |
|---------------------------------------------|---------------|
| Mycoplasma Broth (BBL)                      | 21 g/l        |
| Thiamine Pyrophosphate                      | 10 $\mu$ g/ml |
| Sterile Rabbit Serum                        |               |
| Agar (Oxoid)                                | 10.0 g/l      |
| Heat inactivated (56° C for 30 min) 10% V/V |               |
| pH 7.4                                      |               |

##### NOS Medium

###### NOS-Part A

| <u>Component</u>             | <u>Amount</u> |
|------------------------------|---------------|
| Heart infusion broth (Difco) | 1.25 g        |
| Trypticase (BBL)             | 1.0 g         |
| Yeast Extract (Difco)        | 0.25 g        |
| Sodium thioglycollate        | 0.05 g        |
| L-cysteine-HCl               | 0.1 g         |

|                 |         |
|-----------------|---------|
| L-asparagine    | 0.025 g |
| Agar (Oxoid)    | 0.3 g   |
| Distilled water | 90 ml   |

Final pH 7.0

#### NOS-Part B

| <u>Component</u>                                                                   | <u>Amount</u>        |
|------------------------------------------------------------------------------------|----------------------|
| 2% NaHCO <sub>3</sub> (rifampin for<br>isolation 20 µg/ml) filtered<br>sterilized. | 1.0 ml/9.0 ml Part A |

#### VFA-TPP-Serum

|                                                  |                       |
|--------------------------------------------------|-----------------------|
| 1.5 ml 0.2% thiamine pyrophosphate               |                       |
| 1.0 ml VFA soln                                  |                       |
| 10 ml sterile, heat inactivated<br>rabbit serum. | 0.25 ml/9.0 ml Part A |

#### Volatile Fatty Acid Solution (VFA)

|     |   |                          |
|-----|---|--------------------------|
| 500 | 1 | Valeric Acid             |
| 500 | 1 | Isovaleric Acid          |
| 500 | 1 | Isobutyric Acid          |
| 500 | 1 | DL-2-Methyl Butyric Acid |

#### Scanning electron microscopy (SEM) of extracted teeth

Freshly extracted teeth were immediately immersed in 0.2 M sodium cacodylate buffer, pH 7.4, containing 2% glutaraldehyde (GA) and fixed at 4° C for 16 to 24 h. Following the prefixation in GA, the specimens were washed 3 times with 0.2 M cacodylate buffer and transferred into 0.2 M sodium cacodylate with 1.0% osmium tetroxide (pH 7.2) for 2 h at room temperature and then washed 3 times with buffer. The specimens were then dehydrated in a graded series of ethyl alcohol (30%, 50%, 70%, 90%) for 15 min exchanges, and then into 100% ethanol for two fifteen min exchanges. The

specimens, in 100% ethanol, were then transferred to a Tousimis 810 critical point dryer and critically point dried with liquid CO<sub>2</sub>. The dried tissue was mounted on specimen stubs with a silver conductive paint and sputter coated with gold-palladium with a Technics Hummer VI sputter coater and examined with a JEOL T-200 SEM. Final images were recorded with Polaroid Typd 55 P/N film.

#### Transmission electron microscopy (TEM)

Plaque debris obtained from each patient was dispersed in 1 ml of sodium phosphotungstate, pH 7.3. A drop of the suspension was placed onto a formvar-carbon coated 300 mesh copper grid and examined in a Siemens IA electron microscope at 80 kV. Electron micrographs were recorded on Kodak electron image plates.

Each sample examined in the TEM was evaluated for the types of spirochetes present based on the number of axial filaments originating from each end of the cell. In each case, a minimum of 20 spirochetes was counted per sample and the number of filaments and their arrangement recorded. During this analysis selected electron micrographs were taken to record the types of spirochetes found in the samples.

#### Preparation of Antisera

Specific immune rabbit sera is being prepared in New Zealand White rabbits for each of the spirochete strains listed in Table 29 as well as any future isolates. The protocol used for preparing antiserum for these strains consists of weekly intravenous inoculations of 2.0 ml of a 6 to 7 day old broth culture for 4 weeks. Five days after the last immunization, blood is collected by cardiac puncture on three successive days and the serum separated and the three bleedings pooled. Microscopic agglutination tests (MA) are done to determine the titer of the pooled antiserum. In the past we have obtained MA titers of 1:10,000 or greater using this immunization schedule.

#### Serological Relationships Among Spirochete Isolates

All of the spirochete isolates are grown in the appropriate liquid medium and the cell density adjusted to approximately  $1 \times 10^8$  cells/ml. These cell suspensions are then

used to perform reciprocal adsorption microscopic agglutination studies similar to that described by Kemery et al. (67) for Leptospira for determining the degree of antigenic cross-reactivity. We have previously published the results of similar studies with three of our isolates of T. denticola, strains 11, W and 14 (56). Thus we feel that this is the appropriate way to proceed to determine antigenic relatedness. With this approach, we will be able to determine different serotypes. The definition of a serotype will be based upon the criteria established by the World Health Organization for the classification of Leptospira, which states: "two strains are considered to belong to different serotypes if, after cross-adsorption with adequate amounts of heterologous antigen, 10% or more of the homologous titer regularly remains in each of the two antisera in repeated tests" (68).

#### Sodium Dodecyl sulfate-Polyacrylamide Gel Electrophoretic Analysis of Spirochetal Antigens (SDS-PAGE)

The SDS-PAGE analysis of our isolates was performed in the following manner. The gels and solutions used were modifications of those previously described by Swindlehurst, et al. (69), and Payne (70). The spirochete isolates were grown in 50 ml broth cultures for 6-7 days, harvested by centrifugation at 10,000 x g for 20 min. The pellets suspended in 0.1 M Tris buffer, pH 6.8, to an OD<sub>650</sub> of 1.0 as measured in a Spectronic 20 spectrophotometer. One hundred µl of each sample was added to a tube containing 100 µl of a solution of Tris, SDS, 2-mercaptoethanol, sucrose and Bromophenol blue to bring the sample to a final concentration of 0.0625 M Tris-HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% sucrose and 0.001% Bromophenol blue. The SDS-cell suspension was then heated in a boiling water bath for 2 min, cooled to room temperature and applied directly to the gel. Thirty µl of the SDS-cell suspension were added to the wells of a vertical 10 cm x 14 cm x 1.55 mm discontinuous polyacrylamide gel slab composed of a 5% acrylamide (2.5 cm x 14 cm) stacking gel (0.125 M Tris HCl pH 6.8) and a 12.5% acrylamide separating gel (0.375 M Tris HCl, pH 8.8). Using a Bethesda

Research Laboratories (BRL) vertical gel electrophoresis system (Model V161) and an A. H. Thomas electrophoresis power supply (Model 121) set at 50 mA (constant current), electrophoresis was carried out in a 0.025 M Tris-0.192 M glycerine buffer, pH 8.3, until the Bromophenol blue tracking dye reached the bottom of the gel.

The gel was removed from its glass plate sandwich and fixed in an aqueous solution containing 10% trichloroacetic acid, 5% sulfosalicylic acid (W, W, V) for 1 h. The fixative was then removed and the gel allowed to equilibrate in an aqueous solution of 25% methanol, 5% acetic acid (V, V, V) for 30 min. Following equilibration, the gel was then stained for 6 h in 0.1% Coomassie Brilliant R-250 Blue in an aqueous solution of 25% methanol and 5% acetic acid and then destained in an aqueous solution of 25% methanol, 5% acetic acid until the background was clear. Once the gels were destained, they were photographed with Polaroid 55 P/N film to record the gel patterns.

## Results

### Microscopic Analysis

Dark-field microscopic analysis of ANUG plaque samples revealed an abundance of rods, cocci, and spirochetes. Counts of the numbers of each morphological type in the plaque samples with the Petroff-Hausser counting chamber results in the percent composition of each type as shown in Table 27 for each of the 17 samples evaluated. Permanent video tape records of the morphological types seen under dark-field and phase contrast microscopy were also prepared using television photomicrography for selected patient samples.

The distribution of types of spirochetes bases on axial filament numbers and arrangement by negative staining TEM for selected ANUG samples is shown in Table 28. The predominate spirochetes appear to be the one with the "2-4-2" axial filament arrangement which are presumable Treponema denticola. The next most abundant spirochetes observed were equally divided among the large size spirochete of the "12-24-12", "6-12-6", and "8-16-8" class. Examples of some of these types is shown in Figures 1-3.

Table 27

## Percent Morphological Composition of ANUG Plaque

| Sample<br>Number | % Rods       | % Cocci      | % Spirochetes | Total<br>Organisms |
|------------------|--------------|--------------|---------------|--------------------|
| 1                | 28.60        | 21.31        | 41.80         | 122                |
| 2                | 52.90        | 23.22        | 23.87         | 155                |
| 3                | 48.64        | 30.63        | 20.72         | 111                |
| 4                | 48.14        | 31.85        | 20.00         | 270                |
| 5                | 48.75        | 23.75        | 27.50         | 160                |
| 6                | 38.91        | 24.63        | 36.45         | 609                |
| 7                | 43.94        | 18.94        | 37.12         | 528                |
| 8                | 50.34        | 49.65        | 0.00          | 145                |
| 9                | 35.48        | 17.35        | 47.17         | 513                |
| 10               | 39.55        | 32.88        | 27.61         | 268                |
| 11               | 51.38        | 19.89        | 28.73         | 181                |
| 12               | 37.19        | 38.11        | 24.69         | 328                |
| 13               | 42.86        | 50.00        | 7.14          | 266                |
| 14               | 42.80        | 21.02        | 37.31         | 528                |
| 15               | 38.80        | 26.86        | 34.33         | 335                |
| 16               | 46.39        | 38.14        | 15.46         | 97                 |
| 17               | <u>32.01</u> | <u>25.30</u> | <u>42.68</u>  | <u>328</u>         |
| Avg. %           | 42.75%       | 29.02%       | 29.97%        | 4994               |

Table 28

## Distribution of Spirochetes in ANUG Debris

| Patient #  | Axial Filament Arrangement |       |       |       |        |        |          |          |
|------------|----------------------------|-------|-------|-------|--------|--------|----------|----------|
|            | 1-2-1                      | 2-4-2 | 3-6-3 | 4-8-4 | 6-12-6 | 8-16-8 | 12-24-12 | 16-32-16 |
| 1          | 4                          | 8     | 0     | 1     | 8      | 6      | 9        | 0        |
| 2          | 2                          | 11    | 0     | 0     | 3      | 0      | 6        | 0        |
| 3          | 0                          | 0     | 2     | 3     | 5      | 1      | 7        | 5        |
| 4          | 0                          | 5     | 0     | 0     | 1      | 7      | 11       | 2        |
| 5          | 2                          | 5     | 1     | 2     | 0      | 9      | 2        | 0        |
| 6          | 2                          | 16    | 0     | 2     | 2      | 10     | 4        | 1        |
| 7          | 7                          | 16    | 0     | 3     | 16     | 0      | 8        | 0        |
| 8          | 10                         | 15    | 2     | 1     | 12     | 0      | 10       | 0        |
| 9          | 3                          | 13    | 1     | 3     | 10     | 19     | 1        | 0        |
| Totals     | 30                         | 84    | 6     | 15    | 57     | 52     | 58       | 3        |
| % of Total | 9.7                        | 27.0  | 2.0   | 4.3   | 13.4   | 16.3   | 13.7     | 2.6      |

Scanning electron microscopic analysis was performed on freshly extracted teeth from one patient who was diagnosed as having juvenile periodontitis with super imposed ANUG. Several teeth were extracted and the adherent plaque evaluated by SEM. Figure 4 is an SEM of tooth #15 showing that the predominate flora found was that of spirochetes.

#### Isolation and Cultivation of Spirochetes from ANUG Lesions

Significant progress has been made in isolating and cloning new spirochete strains from ANUG lesions (Fig. 5). We have been successful in isolating 10 new strains (Table 29), 5 of which have been found to be T. denticola and the remaining need to be evaluated morphologically and serologically. These ANUG isolates are currently being compared to other strains of spirochetes which we have isolated from patients with other periodontal problems or have been acquired from other investigators. Table 30 is a list of the known isolates and their axial filament arrangement, currently available in our laboratory. To date, isolated strains of MI, MS, RM, AR and DW appear to be T. denticola. Final confirmation will come for these and the other strains when the serological analysis is completed.

#### Serological analysis of ANUG isolates

Antisera is currently being raised against each of the isolates found in Table 29 as well as those strains obtained from other sources (Table 30). Homologous and heterologous MA analysis as well as reciprocal MA adsorption analysis is currently being performed on each of our ANUG isolates. The serological results obtained should indicate which of the isolates are similar and how those isolates obtained from ANUG patients compare serologically to those isolates obtained from patients with other forms of periodontal diseases.

#### SDS-PAGE analysis of ANUG isolates

Preliminary SDS-PAGE analysis has been performed on some of the ANUG spirochetal isolates. SDS-PAGE patterns obtained for each isolate has been compared

Table 29

## Oral Spirochetes Isolated from ANUG Patients

| <u>Organism</u>      | <u>Strain Designation</u> | <u>Axial filament<br/>Arrangement</u> | <u>Source</u> |
|----------------------|---------------------------|---------------------------------------|---------------|
| <u>T. denticola</u>  | AR                        | 2-4-2                                 | ANUG          |
| <u>T. deneticola</u> | MS                        | 2-4-2                                 | ANUG          |
| <u>T. denticola</u>  | DW                        | 2-4-2                                 | ANUG          |
| <u>T. denticola</u>  | MI                        | 2-4-2                                 | ANUG          |
| <u>T. denticola</u>  | RM                        | 2-4-2                                 | ANUG          |
| ?                    | 22                        | ?                                     | ANUG          |
| ?                    | 23                        | ?                                     | ANUG          |
| ?                    | A10211                    | ?                                     | ANUG          |
| ?                    | A10212                    | ?                                     | ANUG          |
| ?                    | A923                      | ?                                     | ANUG          |

**Table 30**

**Spirochete Strains Available in our Laboratory for Comparative Studies**

| <u>Organism (a)</u>  | <u>Strain Designation (b)</u> | <u>Axial filament arrangement</u> | <u>Source (c)</u>  |
|----------------------|-------------------------------|-----------------------------------|--------------------|
| <u>T. denticola</u>  | T                             | 2-4-2                             | AP                 |
| <u>T. denticola</u>  | W                             | 2-4-2                             | AP                 |
| <u>T. denticola</u>  | 11                            | 2-4-2                             | AP                 |
| <u>T. denticola</u>  | JZ                            | 2-4-2                             | AP                 |
| <u>T. denticola</u>  | MS                            | 2-4-2                             | ANUG               |
| <u>T. denticola</u>  | DW                            | 2-4-2                             | ANUG               |
| <u>T. denticola</u>  | MI                            | 2-4-2                             | ANUG               |
| <u>T. denticola</u>  | JP428                         | 2-4-2                             | GJP                |
| <u>T. denticola</u>  | RM                            | 2-4-2                             | ANUG               |
| <u>T. denticola</u>  | USA                           | 2-4-2                             | AP (Rhesus monkey) |
| <u>T. orale</u>      | P2                            | 1-2-1                             | U. MASS            |
| <u>T. orale</u>      | P3                            | 1-2-1                             | U. MASS            |
| <u>T. orale</u>      | P4                            | 1-2-1                             | U. MASS            |
| <u>T. orale</u>      | P5                            | 1-2-1                             | U. MASS            |
| <u>T. orale</u>      | P8                            | 1-2-1                             | U. MASS            |
| <u>T. vincentii</u>  | N-9                           | 5-10-5                            | JOHNS HOPKINS U.   |
| <u>T. phagedenis</u> | Reiter                        | 5-10-5                            | WVU                |
| <u>T. phagedenis</u> | Kazan 5                       | 5-10-5                            | WVU                |

- a. The organisms labelled T. denticola were identified as such based on axial filament arrangement, dark-field morphology and GLC analysis of metabolic end products.

The organisms labelled T. orale were identified as such based on axial filament arrangement, dark-field morphology and nutritional requirements.

- b. T. denticola strains W and 11 are now ATCC #33520 and #33521 respectfully.
- c. T. denticola, USA was isolated from a Rhesus monkey with periodontal disease.

T. orale strains were obtained from Dr. E. Canale-Parola, U. Mass, who had isolated them from the oral cavity of laboratory workers. The medium used had pectin as the sole source of carbon.

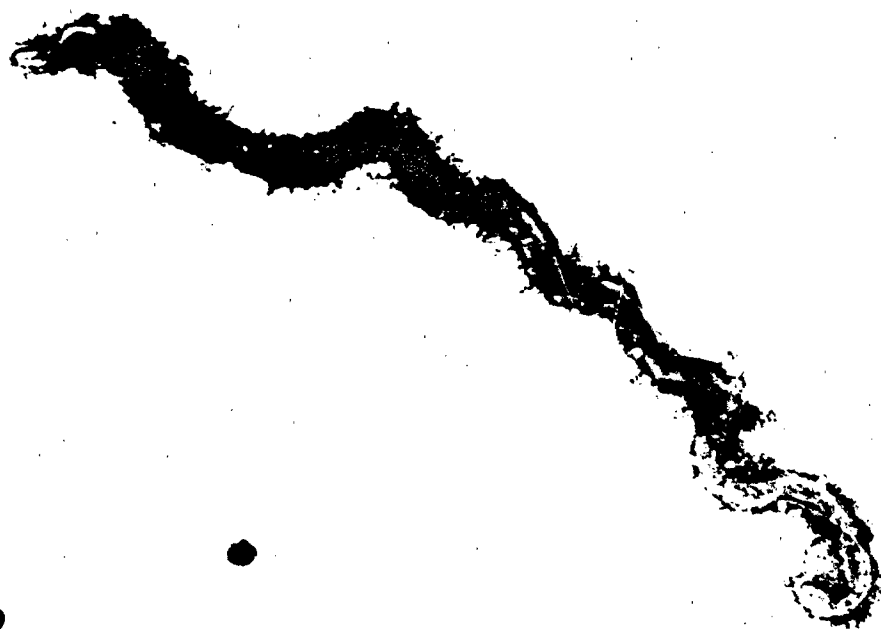
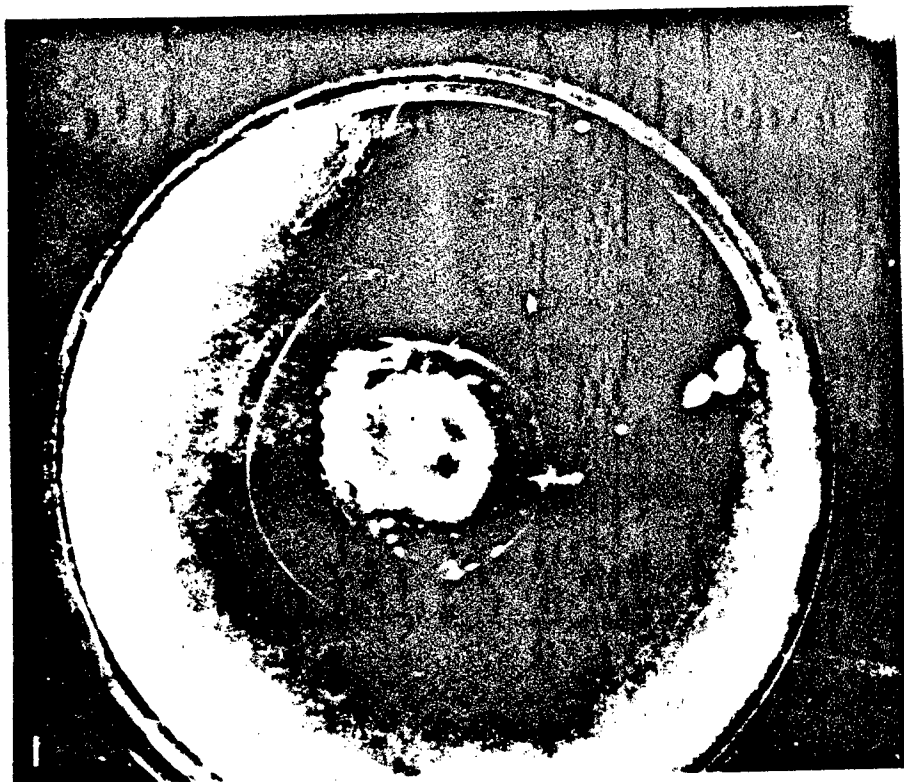
T. vincentii N-9 was obtained from Dr. Paul Hardy, Johns Hopkins U.

T. phagedenis strains were obtained from Dr. Nyles Charon, West Virginia University.

with those of known spirochetal strains. Figure 6 is an example of the typical gel patterns obtained using this technique. A comparison of the major and minor proteins can be made and those organisms that are identical display identical gel patterns. Lanes 1-4 are known T. denticola isolates; lanes 5-7 are unknown ANUG isolate MS, EM and 22; lanes 8-12 represent strains of T. orale; lane 13 contains T. vincentii, N-9 and lanes 14 and 15 contain two non-oral spirochetes, T. phagedenis biotype Reiter and Kazan 5 respectively. As one can see strains that are identical will display identical gel patterns i.e. lanes 1 and 2 and lanes 11 and 12 whereas strains of the same species can display significant differences in protein profiles (lanes 2 and 3). The ANUG isolates chosen for this particular experiment (lanes 5-7) have distinctly different gel patterns and therefore probably represent three different strains. Additional confirmation of this should result from the serological and morphological analysis.

## LEGENDS

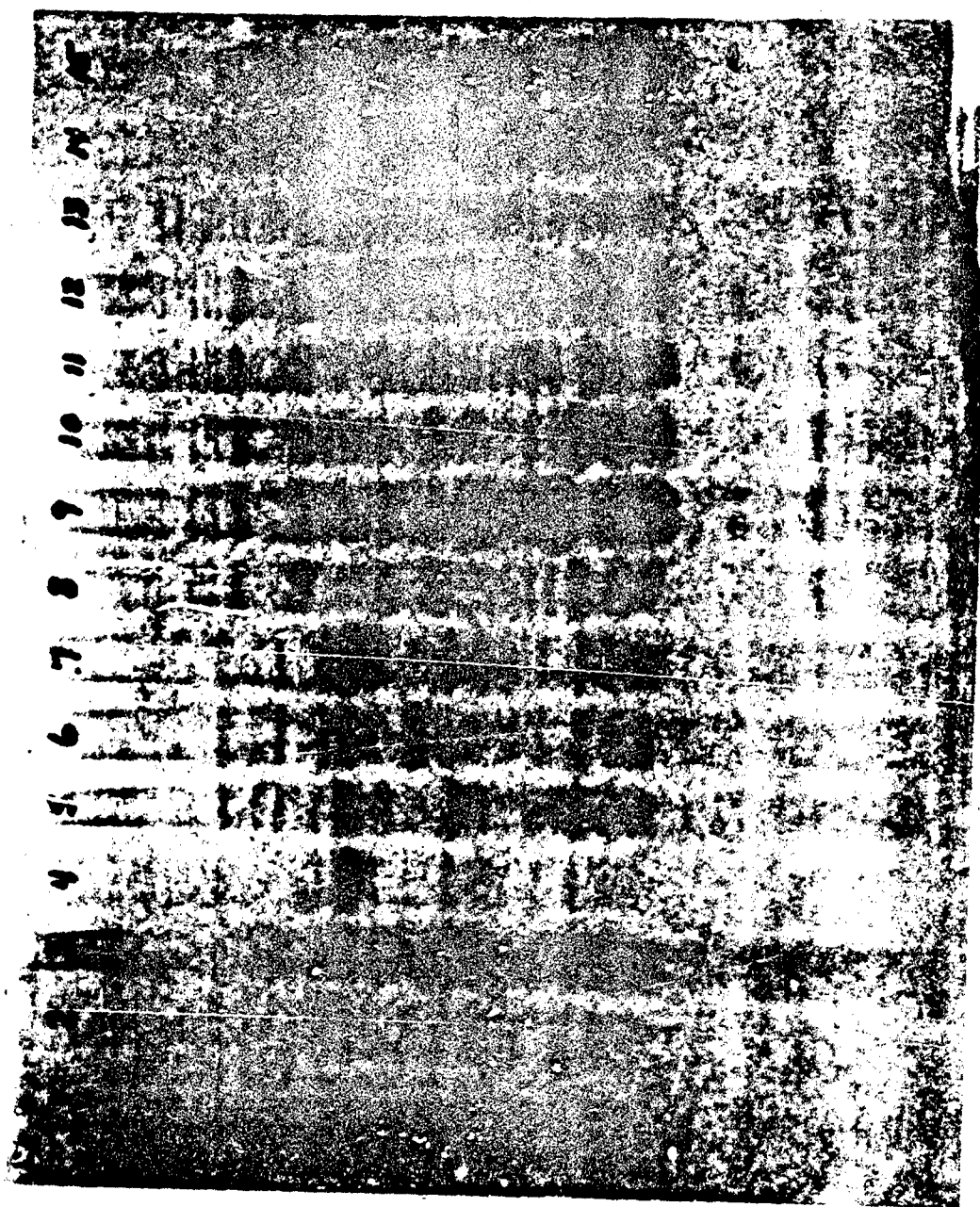
- Figure 1. Spirochetal growth obtained following 7 days incubation on PPLO-serum medium. Note the veil of growth and the holes in the agar where samples were removed for transfer and microscopic observations.
- Figure 2. Electron micrograph of a negatively stained "1-2-1" oral spirochete observed in debris from an ANUG patient. X22,000
- Figure 3. Electron micrograph of a negatively stained "ANUG" spirochete isolate. Note the 2-4-2 axial filament arrangements. X26,000
- Figure 4. Electron micrograph of a negatively stained "8-16-8" oral spirochete observed in debris from an ANUG patient. X55,000
- Figure 5. Scanning electron micrograph of the adherent plaque of an extracted tooth from a patient with ANUG. Bar marker equals 5  $\mu$ m. Note the numerous spirochetes found in the specimen.
- Figure 6. SDS-solubilized whole cell protein profiles of 13 oral anaerobic spirochete isolates, Treponema phagedenis biotype Reiter, and T. phagedenis biotype Kazan 5 after SDS-PAGE and staining with Coomassie Blue dye. Lanes 1-7 represent different isolates of T. denticola; lanes 8-12 represent different isolates of T. orale; lane 14 represents T. vincentii; and lanes 15 and 16 are T. phagedenis biotype Reiter and T. phagedenis biotype Kazan 5.



2







## Interactions of Microorganisms of Acute Necrotizing Ulcerative Gingivitis and Corticosteroids

Individuals under stress and with ANUG have been shown to have higher levels than normal of corticosteroids in their serum and urine. The microorganisms which increase in number in the ANUG lesion appear to be members of the normal oral flora. It is possible that the presence of higher levels of corticosteroids in the serum may allow increased growth and or production of pathogenic factors which may participate in the formation of the ANUG lesion.

Studies were undertaken and are currently being pursued to:

1. Determine if microorganisms associated with acute necrotizing ulcerative gingivitis (ANUG) have surface receptors for human corticosteroids. The organisms to be tested are Fusobacterium nucleatum, Treponema microdentium and Bacteroides gingivalis.
2. If receptors are observed to determine if the growth of the microorganism is increased in the presence of the steroids.

## MATERIALS AND METHODS

### Media

The following media were used: Crystal Violet-Erythromycin (CVE) agar; MM10 agar; and Trypticase Soy Agar (TSA) with hemin and menadione (TSAHK). CVE agar (pH 7.2) contained in g/l the following: trypticase (10.0), yeast extract (5.0), NaCl (5.0), Tryptophan (0.2), agar (15.0) and crystal violet (0.005), erythromycin (0.004), and defibrinated sheep blood (50 ml) which was added after autoclaving. MM10 contained/liter: H<sub>2</sub>O (890 ml), 37.5 ml of 0.6% K<sub>2</sub>HPO<sub>4</sub>, 37.5 ml of a salt solution (NaCl, 1.2 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.2 g; KH<sub>2</sub>PO<sub>4</sub>, 0.6 g, Mg<sub>2</sub>SO<sub>4</sub>, 0.25 g and 100 ml H<sub>2</sub>O), bacto-agar (15.0 g), trypticase (2.0 g), yeast extract (0.5 g), sucrose (30.0 g), KNO<sub>3</sub> (0.25 g), 0.05% hemin (2.0 ml) and cysteine (0.12 g), 8% Na<sub>2</sub>CO<sub>3</sub> (5 ml), DL-dithiothreitol (0.1 mg) and sheep blood (20 ml) which was added after autoclaving. TSA contained 40 g/l trypticase soy agar and for the preparation of TSAHK 10 ml of 0.5 mg/ml hemin in 0.1 N NaOH, 1 ml of 0.1% menadione in 95% ETOH and 50 ml of defibrinated sheep blood was added.

### Cultures and Cultural Conditions

Strains of Bacteroides gingivalis were purchased from ATCC and VPI. They were grown in brain heart infusion (BHI) broth (BBL) supplemented with hemin (5 mg/ml) and menadione (0.2 mg/ml). The Fusobacterium nucleatum strains were grown in a modified tryptone medium. Incubation for all strains was 37° C using the BBL jar Gas-Pak system. All bacterial strains after recovery from the lyophilized state were tested for purity by subculturing on TSA (BBL) with 5% sheep blood. The colonies showing typical morphology were gram stained and the organisms observed by phase microscopy. After designated growth periods in the respective media, each bacterial culture was harvested by centrifugation at 10,000 x g for 20 min and the sedimented organisms washed x 3 in 0.01 M phosphate buffer containing 0.15 M NaCl and 0.2% sodium azide (PBS), pH 7.4. The washed sedimented organisms were resuspended in PBS to a 10% suspension and kept at 4° C until used in steroid binding assays.

### Buffers

All water was deionized followed by glass distillation. All pH values were determined using a Corning No. 476050 combination electrode with Beckman No. 566002 buffer as a standard, and a Corning Model 12 pH meter. TED buffer (.01 M TRIS, .015 EDTA,  $5 \times 10^{-4}$  DTT), TEDG buffer (TED buffer + 10% glycerol) (v/v), and TED buffer + 30% glycerol were adjusted to pH 7.4 at 0 degrees C. The percentage of glycerol in each buffer solution was checked with a Bausch and Lomb refractometer.

### Cytosol Preparation

Bacteria were washed with 10% TEDG, sonicated at maximum speed, six times for 30 sec with 10 sec cooling time in between each time. The homogenate was then centrifuged at 40 K for one hour in a Beckman T50.L Rotor. The supernatant fraction (cytosol) was carefully poured off of the pellet. The protein content of the cytosol was determined according to the method of Lowry (14) using bovine serum albumin as a standard.

### Single Saturation Dose Assay

All assays were performed in duplicate. Cytosol (0.5 ml) was incubated with a final concentration of  $1 \times 10^{-8}$  M labeled steroid to determine total bound steroid. Cytosol (0.5 ml) was also incubated with  $1 \times 10^{-8}$  M labeled steroid in the presence of 100 fold excess unlabeled steroid to determine non-specific bound steroid. Each reaction mixture was then treated with dextran-coated charcoal (DCC) to remove free steroid. Dextran-coated charcoal (0.5 ml) (5 g % Norit A, 125 g % dextran in TED buffer) was pelleted by centrifugation at 2,500 g for 20 min. The reaction mixture was pipetted on to the charcoal pellet and briefly mixed with a vortex mixer. After a 10 min incubation the mixture was again centrifuged at 2,500 g for 10 min. The supernatant fluid (0.3 ml) was layered on gradients and aliquots of 0.1 ml were removed for determination of radioactivity. Specific binding was then determined by subtracting non-specific binding

from total binding.

#### Scatchard Analysis

For Scatchard analysis, cytosol was incubated with steroid solutions containing radioactive steroid (for  $^{17}\beta$ -estradiol ranging from  $9.6 \times 10^{-11}$  -  $2.6 \times 10^{-9}$  M for glucocorticoids using  $^3\text{H}$ -dexamethasone ranging from  $3.8 \times 10^{-10}$  -  $2.7 \times 10^{-9}$  M) for 16-24 h at 0-4 degrees C. At the end of the incubation period, the total radioactivity in the samples were measured using 10  $\mu\text{l}$  samples. The free steroid was then extracted by the addition of 125  $\mu\text{l}$  of a dextran-coated charcoal suspension. (1.25 g of Norit A and .625 g dextran T-40 per 100 ml TED buffer). The charcoal-cytosol mixture was incubated for 20 min at 0-4 degrees C and the charcoal was then sedimented by centrifugation at 3,000 x g for 20 min. The radioactivity in the supernatant was then be measured using 100  $\mu\text{l}$  samples. The plots were corrected for nonsaturable binding.

#### Glycerol Density Gradient Centrifugation

Linear 10-30% glycerol gradients were prepared in 5 ml cellulose nitrate tubes using a locally constructed gradient former and a peristaltic pump (Technicon Instrument Corp., Tarrytown, New York). A portion of the cytosol (0.3 ml) was carefully layered over the gradients. The gradients were either centrifuged in a Beckman Type SW50.1 swinging bucket rotor for 16 h at 149,000 g or a Sorvall type TV865 verticle angle rotor for 2 hours at 365,000 g. Sedimentation values were determined from patterns of known standards (  $^{14}\text{C}$  BSA,  $^{14}\text{C}$  ovalbumin,  $^{14}\text{C}$  globulin, and catalase) run simultaneously with samples in parallel gradients. Radioactive standards were prepared by acetylation of the proteins with  $^{14}\text{C}$  -acetic anhydride. A catalase solution was then prepared by dissolving 100 mg in 0.1 ml of TED buffer and layering 0.1 ml of the solution on the surface of the gradient. After centrifugation the absorbance of each fraction was determined at 405 nm. Fractions were collected by inserting a thin steel tube to the bottom of the gradient and removing contents by peristaltic pump. Three drop fractions (about 0.2 ml ) were collected into scintillation vials using a LKB Ultrorac fraction collector (LKB, Stockholm, Sweden). Scintillation cocktail (4 ml ) was added to each

tube and mixed with a vortex mixer.

## RESULTS

Glycerol density gradient centrifugation profiles of cytosol obtained from Bacteroides gingivalis (CS43) were examined (Figure 7). Cytosols were incubated in  $1 \times 10^{-8}$  M ( $^3\text{H}$ )-dexamethasone for 22 h at  $1-2^\circ\text{C}$  in the presence or absence of a 100-fold excess ( $1 \times 10^{-6}$  M) competing unlabelled dexamethasone or cortisol. It is evidenced by these competition studies that there exists a specific binding protein for dexamethasone in this bacteria. Concentrations of binding protein completed from the suppressible binding of ( $^3\text{H}$ ) dexamethasone in the presence or absence of 100-fold unlabelled competition were determined (19.8 femtomoles/mg protein in 100-fold excess dexamethasone, 38.6 femtomoles/mg protein in 10-fold excess cortisol).

Similar experiments were performed with various bacteria: 10953, 10197, Fusobacterium nucleatum, XIX 9 and VI A (Table 31). No specific binding was observed in these bacteria.

## DISCUSSION

Hormones are chemical signals that interact with specific target cells to promote a particular response. Steroid hormones regulate gene expression in eukaryotic cells which elicits a specific response from the cell. Target cells for a particular hormone contain specialized molecules, receptors, that find the hormone and subsequently mediate its metabolic activities. A receptor has 2 roles, the first is to distinguish a particular signal from the variety of hormones and other molecules infringing on the cell, and the second is to relay this signal in such a way that the appropriate cellular response follows.

In studies with plaque flora associated with periodontal diseases, certain bacterial species are present in greater proportion than during gingival health. If periodontal disease involves increases in specific components of the indigenous population, the determination of factors which might allow or initiate this change is essential to an understanding of the etiology of the disease.

Emotional factors such as stress are associated with higher than normal levels of corticosteroids in blood serum and urine. The microorganism Bacteroides gingivalis (CS43) has been shown to possess a specific binding protein for cortisol. It is possible that the presence of higher levels of corticosteroids in the serum may allow for increased growth of these bacteria. Extensive work on the eukaryotic cell has conclusively demonstrated that specific receptors mediate metabolic processes. We have observed a high specificity glucocorticoid binding protein in these bacteria and speculate that this "receptor-like" protein is instrumental in the normal development and activity of these cells. Further studies are necessary to refine our gross characterization of this protein and qualify its functional role in the bacteria. Several variables exist in this scheme including the effect of glucocorticoid concentration on the growth of the bacteria population, rates of protein and RNA synthesis, and DNA replication. An understanding of these processes will provide an insight into the true nature of this hormone-bacteria interaction. These bacteria allow an ideal system to work with due to their high rate of turnover and general specimen availability.

Figure 7. Glycerol density gradient sedimentation patterns of Bacteroides gingivalis cytosol incubated overnight at 1-2° C with  $1 \times 10^{-8}$  (3H) dexamethasone in the presence or absence of 100-fold excess unlabeled competing steroids. ( ▲ ) in the absence, ( ● ) in the presence of dexamethasone, ( ■ ) in the presence of cortisol.

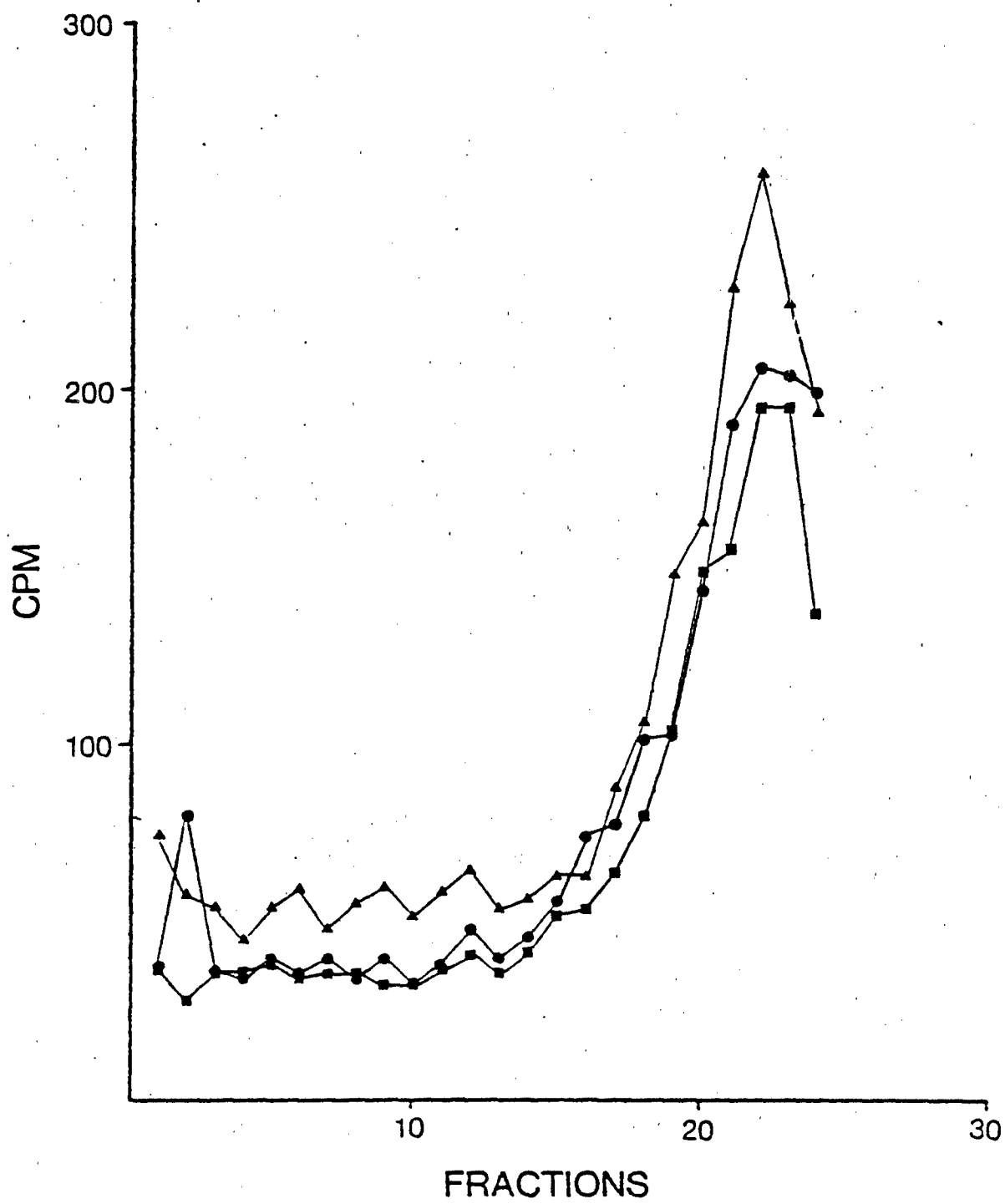


Table 31  
Specifically Bound Steroid  
Fentomoles/Mg Protein

| <u>Bacteria</u>      | <u>*Gluc x 10<sup>-6</sup> M</u> |                          |                          | <u>*Prog x 10<sup>-6</sup> M</u> |                           |                         | <u>*E<sub>2</sub> x 10<sup>-6</sup> M</u> |                       |                         |
|----------------------|----------------------------------|--------------------------|--------------------------|----------------------------------|---------------------------|-------------------------|-------------------------------------------|-----------------------|-------------------------|
|                      | <u>DEX<sup>-4</sup></u>          | <u>Cort<sup>-4</sup></u> | <u>Prog<sup>-4</sup></u> | <u>Prog<sup>-4</sup></u>         | <u>R5020<sup>-4</sup></u> | <u>DHT<sup>-4</sup></u> | <u>DES<sup>-4</sup></u>                   | <u>P<sup>-4</sup></u> | <u>DHT<sup>-4</sup></u> |
| CS43                 | ++                               | ++++                     | -                        | -                                | -                         | -                       | +                                         | -                     |                         |
| 10953                | +                                | -                        | -                        |                                  |                           |                         |                                           |                       |                         |
| 10197                | -                                | -                        |                          |                                  | -                         |                         | +                                         |                       |                         |
| CS44                 | -                                | -                        | -                        | -                                | +                         | -                       | -                                         | -                     |                         |
| <u>F. nuc.</u>       | -                                | -                        |                          |                                  | -                         |                         | +                                         |                       |                         |
| XIX-9 <u>B. Int.</u> | -                                | -                        |                          |                                  | -                         |                         | +                                         |                       |                         |
| XII-8 <u>B.G.</u>    | -                                |                          |                          |                                  | -                         |                         | -                                         |                       |                         |
| IV A <u>Fuso.</u>    | -                                | -                        | -                        | -                                | +                         | -                       | +                                         | -                     | +                       |

### Histopathological Studies

There is a problem obtaining biopsy tissue from ANUG patients as they will not allow removal of their gingival tissue. The biopsy specimens that we have studied (Tables 32 and 33) suggest that the type of inflammatory infiltrate is dependent upon when the biopsy was taken with respect to time after onset. As one would expect the early lesion is primarily PMNs (Table 33) and the later lesion (Table 32) shifts towards a lymphocytic infiltration. We hope to get more tissues for study and to use the tissues we have obtained for immunohistologic studies to determine the types of lymphocytes present.

Table 32

16 Y O B F

\*\*Onset: 7 days prior

ANUG 81-1498

Overall: Predominantly lymphocytes making up cellular infiltrate with PMN's confined mostly to blood vessel (capillary) areas.

Counts: 10 random fields along ulcerated area.

|       | <u>Lymph's</u> | <u>PMN's</u> | <u>Mac's/Mono</u> | <u>Plasma</u> |       |
|-------|----------------|--------------|-------------------|---------------|-------|
| 1     | 49             | 19           | 0                 | 0             |       |
| 2     | 39             | 1            | 2                 | 0             |       |
| 3     | 26             | 2            | 1                 | 10            |       |
| 4     | 33             | 9            | 7                 | 1             |       |
| 5     | 20             | 18           | 1                 | 0             |       |
| 6     | 9              | 9            | 0                 | 0             |       |
| 7     | 29             | 12           | 4                 | 0             |       |
| 8     | 12             | 35           | 1                 | 0             |       |
| 9     | 81             | 9            | 0                 | 1             |       |
| 10    | <u>94</u>      | <u>8</u>     | <u>1</u>          | <u>2</u>      | (1)   |
| Total | 392            | 122          | 17                | 14            | (545) |
| %     | 72             | 22           | 3                 | 3             |       |

Table 33  
18 Y O B F

\*\*Onset: 3 days prior

ANUG XVI

Overall: Predominantly PMN's making up cellular infiltrate seen primarily in ulcerated regions and overlying plaque and blood. Lymphocytes also present to less extent and interspersed among PMN's.

Counts: 10 random fields throughout ulcerated tissue areas.

|              | <u>Lymph's</u> | <u>PMN's</u> | <u>Mac's/Mono</u> | <u>Plasma</u> | <u>Mast Cell</u> |
|--------------|----------------|--------------|-------------------|---------------|------------------|
| 1            | 11             | 41           | 0                 | 0             | 0                |
| 2            | 8              | 58           | 1                 | 0             | 1                |
| 3            | 1              | 32           | 0                 | 0             | 0                |
| 4            | 7              | 63           | 0                 | 0             | 0                |
| 5            | 29             | 199          | 1                 | 2             | 0                |
| 6            | 11             | 48           | 0                 | 0             | 0                |
| 7            | 10             | 74           | 2                 | 1             | 0                |
| 8            | 0              | 26           | 0                 | 0             | 0                |
| 9            | 9              | 39           | 1                 | 0             | 0                |
| 10           | 14             | 51           | 2                 | 0             | 0                |
| Total: (742) | <u>100</u>     | <u>631</u>   | <u>7</u>          | <u>3</u>      | <u>1</u>         |
| %            | 13.5           | 85.1         | 0.9               | 0.4           | 0.1              |

## BIBLIOGRAPHY

1. Vincent, H. (1966) Ann. Inst. Pasteur 10:488.
2. Orban, B. (1958) Periodontics, Mosby, St. Louis.
3. Tiecke, R. W., Stuteville, O. H. and Calandra, J. C. (1959) Pathologic Physiology of Oral Disease, Mosby, St. Louis.
4. Glickman, I. (1958) Clinical Periodontology, Saunders, Phila. and London.
5. Wilson, J. P. (1952) Am. Dent. A. J. 44:671.
6. Rosebury, T. and Foley, G. (1939) Am. Dent. A. J. 26:1800.
7. Smith, D. T. (1936) J. Infect. Dis. 46:304.
8. Kline, B. S. (1923) J. Infect. Dis. 32:481.
9. Miller, S. C. (1950) Textbook of Periodontia, Blakiston, Philadelphia.
10. Bernier, J. L. (1955) Management of Oral Disease. Mosby, St. Louis.
11. Moulton, R., Ewen, S., Thieman, W. (1952) Oral Surg., Oral Med. and Oral Path. 5:833.
12. Grupe, H. E. and Wilder, L. S. (1956) J. Periodont. 27:255.
13. Enwonwu, C. O. (1972) Arch. Oral Biol. 17:1357.
14. Goldhaber, P. and Giddon, D. B. (1964) Int. Dent. J. 14:468.
15. Wirthlin, M. R. and Devine, L. (1978) J. Periodontol. 49:449.
16. Rosebury, T. (1952) Oral Surg., Oral Med. and Oral Path. 5:363.
17. Bernier, J. L. (1944) Am. Dent. A. J. 31:1610.
18. Carter, J. J. and Bell, D. M. (1953) J. Periodont. 24:187.
19. Schulger, S. (1949) Am. Dent. A. J. 38:174.
20. Pindborg, J. J. (1951) J. Amer. Dent. Asso. 42:517.
21. Smitt, P. A. (1965) Dent. Pract. 15:231.
22. Hogan, W. J. (1951) Dent. Med. 931.
23. Zegarelli, E. V., Kutscher, A. H. and Silvers, H. F. (1959) J. Periodont. 30:63.
24. Stout, W. C. (1957) J. Periodont. 28:314.
25. Klock, J. H. (1944) The Military Surgeon 95:43.

26. Kozol, S. M. and Schuster, H. V. (1952) Oral Surg., Oral Med. and Oral Path. 5:717.
27. Cofield, K. R., Ferguson, E. W. and Toye, A. E. (1945) Am. Dent. A. J. 32:529.
28. Hoppe, W. E. and Meyer, G. E. (1946) J. Oral. Surg. 4:304.
29. Shountoff, H. and Shountoff, W. (1954) Am. Dent. J. 48:169.
30. Alling, C. C. and Pulaski, E. J. (1959) Oral Surg., Oral Med. and Oral Path. 12:743.
31. Shinn, D. L. (1957) Excerpta Medica (S. M. Finegold, et.) pp. 334.
32. Emslie, R. D. (1967) Br. Dent. J. 122:304.
33. Duckworth, R., Waterhouse, J. P., Britton, D. E. R., Nuki, K., Sheiham, A., Winter, R and Blake, G. C. (1966) Br. Dent. J. 120:599.
34. Gilson, T. D. (1955) U. S. Armed Forces Med. J. 6:346.
35. Johnson, W. N. and Wilson, J. P. (1957).
36. Ewen, S. J. and Tascher, P. J. (1958) J. Periodont. 29:45.
37. Ewen, S. J. and Tascher, P. J. (1959) J. Periodont. 30:67.
38. Tunnicliff, R., Fink, E. B. and Hammong, C. (1936) JADA 23:1959.
39. Cahn, L. P. (1929) J. D. Res. 9:695.
40. Stammers, A. F. (1946) Brit. D. J. 81:4.
41. Schaffer, E. M. (1953) J. Periodont. Res. 24:22.
42. Listgarten, M. A. and Lewis, D. W. (1967) J. Periodont. 38:379.
43. Graykowski, E. A. and Holroyd, S. V. (1970) Dent. Clin. of N. Amer. 14:721.
44. Rosebury, T., Clark, A. R., Engel, S. G. and Tergis, F. (1950) J. Infect. Dis. 87:217.
45. Rosebury, T., Clark, A. R., Tergis, F. and Engel, S. F. (1950) J. Infect. Dis. 87:226.
46. Rosebury, T., Clark, A. R., Macdonald, J. B. and O'Connell, D. G. (1950) J. Infect. Dis. 87:234.
47. Macdonald, J. B., Sutton, R. M. and Knoll, M. L. (1954) J. Infect. Dis. 95:275.
48. Macdonald, J. B., Sutton, R. M. and Knoll, M. L. (1954) J. Infect. Dis. 95:275.
49. Hampp, E. G. and Mergenhagen, S. E. (1959) Bact. Proc. Soc. Amer. Bact. 59th General Meeting, p. 101.
50. Rizzon, H. A., Hampp, E. G. and Mergenhagen, S. E. (1961) Arch. Oral. Biol. 5:63.
51. Hampp, E. G. and Mergenhagen, S. E. (1963) J. Infect. Dis. 112:84.

52. Blake, G. C. (1968) Proc. Roy. Soc. Med. 61:131.
53. Wilton, J. M. A., Ivanyi, L. and Lehner, T. (1971) J. Periodont. Res. 6:9.
54. Nisengard, R. J., Myers, D., Fischman, S. and Socransky, S. (1976) J. Dent. Res. Spec. Issue BpB206 #576, 1976.
55. Jacob, E., Allen, A. L. and Nauman, R. K. (1979) J. Clin. Micro. 10:934.
56. Jacob, E., Carter, T. B. and Nauman, R. K. (1980) J. Clin. Micro. 12:610.
57. Loesche, W. J., Syed, S. S., Laughon, B. E. and Stoll, J. (1981) J. Periodontol. 53:223.
58. Spiegel, C. A., Hayduk, S. E., Minah, G. E. and Krywolak, G. N. (1979) J. Perio. Res. 14:376.
59. Falkler, Jr., W. A. and Hawley, C. E. (1976) Infect. and Immun. 15:230.
60. Falkler, Jr., W. A., Smoot, C. N. and Mongiello, J. R. (1982) Archs. oral Biol. 27:553.
61. Crawford, Y. E., Nalewalk, R. P., Lythe, R. I. and O'Connell, J. L. (1971) Infect. and Immun. 4:212.
62. Walkler, C. B., Ratliff, D., Muller, D., Mandell, R. and Socransky, S. J. (1979) J. Clin. Microbiol. 10:844.
63. Rose, N. R. and Friedman, H. (1980) Manual of Clin. Micro. (2nd ed.) Amer. Soc. for Microbiol., Washington, D. C.
64. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193:265.
65. Poxton, L. R., (1979) J. Clin. Path. 32:294.
66. Leschine, S. R. and Canale-Parola (1980) J. Clin. Microbiol. 12:792.
67. Kemery, E., Galton, M. M. and Sulzer, C. R. (1970) Bull. Wld. Hlth. Org. 42:733.
68. World Health Organization (1967) Wld. Hlth. Org. Tech. Rep. Ser. #380, p. 32.
69. Swindlehurst, C. A., Shah, H. N., Parr, C. W. and Williams, R. A. D. (1977) J. Appld. Bacteriol. 43:319.
70. Payne, J. W. (1976) Chromatographic and Electrophoretic Techniques (Vol. III). Zonal Electrophoresis, Yearbook Medical Publishers, Inc., Chicago, p. 321.

## Papers and Abstracts Resulting from this Study

### PAPERS

J. W. Vincent, W. A. Falkler, Jr. and J. A. Craig. Comparison of Serologic Reactions of Typed Fusobacterium nucleatum Strains with Isolates from Humans, Canines and a Macaca mulatta Monkey. J. Clin. Micro. 1983 (in press)

W. A. Falkler, Jr., E. B. Clayman and D F. Shaefer. Hemolysis of Human Erythrocytes by the Fusobacterium nucleatum Found in Periodontal Diseases. Archs. oral Biol. 1983 (in press)

### ABSTRACTS

Serologic Studies of Fusobacterium nucleatum from Different Oral Lesions. W. A. Falkler, Jr., J. W. Vincent, R. Lai and J. B. Suzuki, 1982 IADR Meeting

Human Precipitating Antibody Reactive with Eubacterium brachy. J. W. Vincent and W. A. Falkler, Jr., 1983 AADR Meeting.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoretic Analysis of oral Spirochetes. B. D. Tall and R. K. Nauman, 1983 ASM Meeting.

Corticosteroid Receptors in Bacteroides gingivalis. W. A. Falkler, Jr., M. Salah and N. Bashirelahi. To be submitted to 1983 IADR Meeting.

## DISTRIBUTION LIST

- 4 copies: Commander  
U. S. Army Medical Research and Development Command  
ATTN: SGRD-SI  
Fort Detrick  
Frederick, MD 21701
- 12 copies: Administrator  
Defense Technical Information Center  
ATTN: DTIC-DDA  
Cameron Station  
Alexandria, VA 22314
- 1 copy: Superintendent  
Academy of Health Sciences, US Army  
ATTN: AHS-COM  
Fort Sam Houston, TX 78234
- 1 copy: Dean, School of Medicine  
Uniformed Services University of the Health Sciences  
4301 Jones Bridge Road  
Bethesda, MD 20014

END

DATE  
FILMED

11 = 86

DTIC