Comparison of Serological Reactions of Typed *Fusobacterium nucleatum* Strains with Those of Isolates from Humans, Canines, and a *Macaca mulatta* Monkey

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In the present study, we compared typed *F. nucleatum* strains with isolates from various human periodontal diseases, canines, and a *Macaca mulatta* monkey. All isolates displayed biochemical reactions similar to those of the typed strains in the API 20A system (Analytab Products, Plainville, N.Y.). The human and monkey isolates displayed both type I and type II colonial morphologies on crystal violet erythromycin agar, whereas the canine isolates displayed only type I. Antigen preparations of the human isolates, typed strains, and two of the monkey strains shared lines of identity in immunodiffusion testing. An enzyme-linked immunosorbent assay in which rabbit anti-*F. nucleatum* and human sera were used revealed that the human and monkey isolates, when used as antigens, allowed the detection of similar degrees of antibody activity; however, no activity was observed with the canine isolates. None of the canine isolates displayed the characteristic hemagglutination of sheep erythrocytes demonstrated by the human and monkey strains. These results suggest that *F. nucleatum* isolates from various human periodontal diseases share antigenic determinants. Also, differences were observed in *F. nucleatum* isolates from canines when compared with human and monkey strains. These findings are important when considering the immunopathological mechanisms involved in human periodontal diseases and when comparing animal model systems with naturally occurring disease in humans.

Fusobacterium nucleatum is a gram-negative, anaerobic, rod-shaped organism commonly found in human dental plaque. It has been isolated from early (2) and advanced (17, 21) periodontitis, and a significant increase in the numbers of this microorganism has been observed with increasing gingival inflammation (7) in humans. Evaluations of animal model systems have found *F. nucleatum* to be prominent in beagle dogs with advanced periodontitis (20), ligature-induced periodontitis in cynomolgus monkeys (9), and as part of the cultivable flora in the dental plaque of *Macaca mulatta* and *Macaca fascicularis* (10) and *Macaca arctoides* (12) monkeys.

F. nucleatum has been shown to possess a characteristic hemagglutination (HA) activity which can be inhibited by a specific antiserum (4), non-antibody human mucinous salivary and serum glycoproteins (5, 19), D-galactose (14), and adsorption techniques with oral *Streptococcus* and *Bacteroides* strains (3).

The purpose of this study was to compare the HA activity and reaction of human sera and rabbit anti-*F. nucleatum* sera with that of *F. nucleatum* isolates obtained from humans dem-

onstrating clinically healthy gingiva and various gingival and periodontal disease states. Additional isolates were obtained from dogs and an *M. mulatta* monkey demonstrating spontaneous chronic periodontitis. The serological reactions of the *F. nucleatum* isolates were compared with those of selected reference *F. nucleatum* strains.

MATERIALS AND METHODS

Cultures and cultural conditions. *F. nucleatum* clinical isolates were obtained from the following sources: (i) human adults virtually free of periodontal disease, (ii) human children free of periodontal disease, (iii) human adults with diagnosed chronic periodontitis, (iv) human adults with diagnosed acute necrotizing ulcerative gingivitis, (v) human adults with diagnosed juvenile periodontitis, (vi) two mature dogs, one mixed breed and one golden retriever, with clinical signs of early chronic periodontitis, and (vii) one *M. mulatta* monkey with spontaneous advanced chronic periodontitis (Table 1). Three typed *F. nucleatum* strains (ATCC 10953, VPI 4355, and VPI 10197) were also utilized. All clinical isolates were obtained from subgingival plaque samples with a sterile curette (MC 17/18; Hu Friedy Dental Supply, Chicago, Ill.) and immediately streaked onto crystal violet-erythromycin agar (22), a selective medium for the isolation of *F. nucleatum*. All samples were incubated at 37°C anaer-

TABLE 1. Source of *F. nucleatum* clinical isolates

Clinical description	No. of subjects	No. of isolates
Children free from disease	2	3
Adults free from disease	6	7
Patients with chronic periodontitis	3	3
Patients with ANUG ^a	5	5
Patients with juvenile periodontitis	3	6
Canines	2	8
<i>M. mulatta</i> monkey	1	11

^a ANUG, Acute necrotizing ulcerative gingivitis.

obically with the GasPak system (BBL Microbiology Systems, Cockeysville, Md.) or an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) with an atmosphere of 85% nitrogen–10% hydrogen–5% carbon dioxide. After 4 days of incubation, isolates were identified by colonial morphology (22) and described as either type I, a 2-mm, transparent, smooth, blue colony having an entire edge with a dark blue center, or type II, a 1- to 2-mm, transparent, round or irregular, blue colony with a speckled appearance. Selected colonies were streaked for isolation on blood agar (BBL Microbiology Systems) and incubation was continued for 48 h. 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 with the API 20A system (Analytab Products, Plainville, N.Y.). Isolates were then transferred to a modified tryptone medium (4) and incubated as before. Cells were harvested by centrifugation at $10,000 \times g$ for 10 min at 4°C, washed three times in 0.01 M phosphate-buffered saline 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 $2,000 \times g$ for 10 min).

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

Double diffusion in agar. SPs of selected isolates and typed *F. nucleatum* strains were reacted by double diffusion (16) in 1% agarose in 0.01 M PBS (pH 7.2) with undiluted rabbit anti-*F. nucleatum* strain 10953 or 10197 serum (4). Reactions were incubated at 4°C in a humidifier and observed after 24 h. The protein content of the SP as determined by the method of Lowry et al. (11) was approximately 400 µg/ml.

Serological evaluation by ELISA. Selected isolates were reacted by enzyme-linked immunosorbent assay (ELISA) with the following sera: (i) rabbit anti-*F. nucleatum* strain 10953 or 10197 serum; (ii) normal rabbit serum; (iii) human sera previously shown to be reactive to 10953, 10197, and 4355; and (iv) serum obtained from the *M. mulatta* monkey demonstrating

FIG. 1. Immunodiffusion reaction of rabbit anti-*F. nucleatum* 10953 serum (c) with SPs of *F. nucleatum* strains 4355 (1 and 4), 10197 (2 and 3), and 10953 (5 and 6).

noninduced chronic periodontitis. A modification of the ELISA was utilized (15). Two hundred microliters of a 1:10 dilution of the previously described whole cell suspensions of *F. nucleatum* in 60 mM carbonate buffer (pH 9.6) was added to each well of a microtiter plate (Dynatech Laboratories, Inc., Alexandria, Va.) and incubated at 37°C for 3 h, followed by refrigeration overnight. Peripheral rows were not utilized. The plate was washed five times with 0.01 M PBS containing 1% bovine serum albumin and 0.05% Tween 20, followed by 3 h of incubation at 37°C with 200 µl of a 1% bovine serum albumin solution in 60 mM carbonate buffer, to ensure complete coverage of all binding sites in each well. The plate was then washed five times with PBS–Tween 20 with 1% bovine serum albumin. One hundred microliters of serial twofold dilutions of the sera in 0.01 M PBS with 0.05% Tween 20 (pH 7.2) was 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 immunoglobulin G fraction of goat anti-rabbit γ , μ , and α heavy-chain serum or peroxidase-labeled goat anti-Rhesus gamma globulin serum (Cappel Laboratories, Inc., Cochranville, Pa.) were added, and the plate was 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% [wt/vol] *O*-phenylenediamine in methanol plus 99 ml of distilled water and 0.1 ml of 3% H_2O_2) were added. The plates were incubated in the dark for 30 min at room temperature, the reaction was stopped by adding 20 µl of 8 N H_2SO_4 , and reactions were determined colorimetrically at 490 nm with a Microelisa reader (Dynatech Laboratories). 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 dilutions of conjugate had previously been

FIG. 2. Immunodiffusion reaction of rabbit anti-*F. nucleatum* 10953 serum (c) with SPs of *F. nucleatum* strains 4355 (1) and 10953 (4) and *F. nucleatum* isolates from juvenile periodontitis (2), a normal adult (3) and a normal child (6), and chronic periodontitis (5).

done (Falkler et al., unpublished data). Analysis of the protein content of the 1% whole-cell suspensions (1:10 dilution of the 10% suspension) was determined to be 40% $\mu\text{g/ml}$ by the technique of Lowry et al. (11).

HA. The HA assay was performed by the method of Falkler and Hawley (4). Briefly, serial twofold dilutions of 50 μl of 10% whole-cell suspensions of selected *F. nucleatum* isolates were made in microtitration multiwell plates (Linbro Scientific, Inc., Hamden, Conn.) with 0.01 M PBS (pH 7.2), followed by the addition of 25 μl of PBS (pH 7.2) and 25 μl of a 1.25% suspension of sheep erythrocytes (RBCs). Plates were mixed for 20 s on a Micro-Shaker (Dynatech Laboratories) and then incubated for 30 min at 37°C, followed by 1 h at 4°C. Titers were determined visually as the reciprocal of the highest dilution demonstrating a 2+ HA (4). HA inhibition was determined as described above except for the addition of 25 μl of 50 mM D-galactose in place of PBS and incubation for 30 min at 37°C before adding the sheep RBCs.

RESULTS

Isolation of *F. nucleatum* strains. *F. nucleatum* isolates were obtained on crystal violet-erythromycin agar plates from all plaque samples. The human isolates demonstrated both type I and type II colonial morphology, as did isolates from the *M. 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.

FIG. 3. Immunodiffusion reaction of rabbit anti-*F. nucleatum* 10953 serum (c) with SPs of *F. nucleatum* 10953 (1), *F. nucleatum* isolates from chronic periodontitis (4) and *M. mulatta* isolates (2 and 5), which show line of identity, and *F. nucleatum* canine isolates (3 and 6).

SPs of strains 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 (Fig. 1). SPs of 16 separate isolates from various human oral disease states and the three typed *F. nucleatum* strains 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 (Fig. 2). The eleven *M. 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 19 of the animal isolates tested, only two of the *M. mulatta* strains shared lines of identity with the human isolates and strain 10953 (Fig. 3).

Eleven human *F. nucleatum* isolates were reacted by ELISA with rabbit anti-*F. nucleatum* strain 10197 or 10953 serum and with normal rabbit serum. Similar titers for all isolates were obtained with both hyperimmune sera, which displayed substantially higher titers than those observed with normal rabbit serum (Table 2).

Twenty-three human clinical *F. nucleatum* isolates were reacted by ELISA with a human serum previously shown to be reactive with *F. nucleatum* (Table 3). Regardless of the source of the *F. nucleatum* isolates, a similar range of antibody activity was detected with the human serum.

Ten *M. mulatta* isolates, eight canine isolates, four human isolates, and strain 10953 were re-

TABLE 2. ELISA titers obtained with human *F. nucleatum* isolates and anti-*F. nucleatum* and preimmune sera

Patient classification (no. ^a)	Mean titer obtained with following serum ^b :		
	Rabbit anti- <i>F. nucleatum</i> 10197	Rabbit anti- <i>F. nucleatum</i> 10953	Normal rabbit
Normal adults (6)	≥6	≥6	≤3
Patients with ANUG ^c (5)	≥6	≥6	≤3

^a Number of patients providing *F. nucleatum* isolates.

^b Highest dilution giving an optical density at 490 nm reading of greater than 0.15 after transforming the results from geometric progressions to arithmetic progressions (1:16 = 1, 1:32 = 2, 1:64 = 3, etc.).

^c ANUG, Acute necrotizing ulcerative gingivitis.

acted with human sera previously shown to be reactive with *F. nucleatum*, serum from the *M. mulatta* monkey with spontaneous periodontal disease, and rabbit anti-*F. nucleatum* (Table 4). All sera showed antibody activity to the human (including strain 10953) and *M. mulatta* strains, but not to the canine isolates.

HA. Eleven *M. 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 HA inhibition. All canine isolates consistently failed to show any HA of sheep RBCs (Table 5). Some of the *M. 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 before HA testing.

DISCUSSION

F. nucleatum has been implicated in various human oral disease states (2, 17). Recent infor-

TABLE 4. ELISA titers obtained with clinical and typed strain *F. nucleatum* isolates with selected sera

<i>F. nucleatum</i> isolates (no.)	Mean (range) titer obtained with following serum ^a :		
	Human	<i>M. mulatta</i>	Rabbit anti- <i>F. nucleatum</i> 10953
<i>M. mulatta</i> (10)	2.9 (3-4)	4.7 (4-5)	5.7 (5-6)
Canine (8)	<1	<1	<1
Human (4)	5.5 (5-6)	4 ^b	7
10593 (1)	6	4	8

^a Titer obtained after transforming the results from geometric progressions to arithmetic progressions (1:10 = 1, 1:20 = 2, 1:40 = 3, etc.).

^b All titers were the same within this group.

mation suggests genetic heterogeneity between *F. nucleatum* isolates from different sites in the same mouth (Y. Selin and J. L. Johnson, J. Dent. Res. 1981, 60, special issue A, p. 15). The results of this study support the concept that human *F. nucleatum* isolates share antigenic determinants. This concept must be taken into consideration when using immunodiagnostic methodologies or considering the contribution of *F. nucleatum* to the immunopathology of different disease states.

The increase in numbers of *F. nucleatum* which has been reported in isolation studies of oral lesions (2, 7, 9, 17, 20, 21) may reflect an ecological change resulting from an environment more conducive to the colonization and growth of these anaerobic microorganisms. To colonize in the complex ecosystem of the periodontal pocket and resist the natural defense and cleansing mechanisms of the host, it would be advantageous for *F. nucleatum* organisms to have a means of binding to human cell surfaces or to other microorganisms or their products. Oral *F.*

TABLE 3. ELISA titers obtained with human *F. nucleatum* isolates and a human serum

Patient classification (no.) ^a	Mean (range) titer ^b
Normal adult (7)	5.7 (5-6)
Normal child (3)	6 ^c
ANUG ^d (5)	5.6 (4-6)
Chronic periodontitis (2)	7
Juvenile periodontitis (5)	6.8 (6-8)

^a Number of patients providing *F. nucleatum* isolates and number of isolates tested.

^b Titer obtained after transforming the results from geometric progressions to arithmetic progressions (1:10 = 1, 1:20 = 2, 1:40 = 3, etc.).

^c All titers were the same within this group.

^d ANUG, Acute necrotizing ulcerative gingivitis.

TABLE 5. HA obtained with clinical isolates of *F. nucleatum* and strain 10953

Isolates (no.)	Mean (range) HA titer ^a	
	Before galactose inhibition	After galactose inhibition
<i>M. mulatta</i> (11)	3.3 (<1-8)	0.91 (<1-1)
Canine (8)	— ^b	—
Human (3)	6.3 (4-8)	4.3 (3-6)
Typed strain 10953 (1)	11 ^c	9 ^c

^a Titer obtained at the 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.).

^b —, HA not observed at the lowest dilution used (1:2).

^c All titers were the same within this group.

nucleatum strains show a characteristic HA (4) and attach to human oral epithelial cells, gingival fibroblast cells, and leukocytes (6). This involves a glycoprotein on the surface of the bacterium binding to a galactose-containing moiety on the cell surface via a Ca^{2+} -dependent reaction (14). The HA-active moiety of *F. nucleatum* contains or is very near to a major antigenic determinant, as rabbit anti-*F. nucleatum* serum inhibits HA activity, the hypothesis being that antibody, upon reaction with the antigenic determinant, causes steric hindrance of binding (4). It was very interesting in the current study that the canine isolates which did not share antigenic determinants with the human isolates also did not show HA with the sheep RBCs. It would be of interest to determine whether these canine isolates would hemagglutinate dog RBCs or bind to canine buccal epithelial cells. Possibly, canine *F. nucleatum* isolates have adapted to utilize a different means of attachment than the one observed for human *F. nucleatum* strains.

Recently, a new *Fusobacterium* species was isolated from monkey dental plaque and designated *Fusobacterium simiae* (18). This organism was described as being morphologically similar to the *M. mulatta* monkey isolates described here, but they differed biochemically (fermentation of glucose was positive in *F. simiae* monkey isolates and negative in *M. mulatta* monkey isolates).

Efforts have been directed in the past toward developing a suitable animal model system for evaluating the microbiological aspects of the development and progression of periodontal diseases (1, 8, 9, 13). The results presented here suggest that the antigenic characteristics of *F. nucleatum* strains isolated from animals may significantly differ from those isolated from humans. What role these results may play in the significance of *F. nucleatum* in the etiology of periodontal diseases in different host systems is unclear. If, however, an immunological basis of disease is considered, the differences in antigenic determinants in *F. nucleatum* isolates from different species of animals create difficulty in extrapolating results from animal studies to the naturally occurring periodontal diseases in humans.

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LITERATURE CITED

- Adams, R. A., H. A. Zander, and A. M. Polson. 1979. Cell populations in the transeptal fiber region before, during and after experimental periodontitis in squirrel monkeys. *J. Periodont.* 50:7-12.
- Darwish, S., T. Hyppa, and S. S. Socransky. 1978. Studies of the predominant cultivable microbiota of early periodontitis. *J. Periodontal Res.* 13:1-16.
- Falkler, W. A., Jr., and B. W. Burger. 1981. Microbial surface interactions: reduction of the haemagglutination activity of the oral bacterium *Fusobacterium nucleatum* by absorption with *Streptococcus* and *Bacteroides*. *Arch. Oral Biol.* 26:1015-1025.
- Falkler, W. A., Jr., and C. E. Hawley. 1977. Hemagglutinating activity of *Fusobacterium nucleatum*. *Infect. Immun.* 15:230-238.
- Falkler, W. A., Jr., J. R. Mongiello, and B. W. Burger. 1979. Hemagglutination inhibition and aggregation of *Fusobacterium nucleatum* by human salivary mucinous glycoproteins. *Arch. Oral Biol.* 24:483-489.
- Falkler, W. A., Jr., C. N. Smoot, and J. R. Mongiello. 1982. Attachment of cell fragments of *Fusobacterium nucleatum* to oral epithelial cells, gingival fibroblasts and white blood cells. *Arch. Oral Biol.* 27:553-559.
- Helderman, W. H. V. 1976. Total viable count and differential count of *Vibrio (Campylobacter) sputorum*, *Fusobacterium nucleatum*, *Selenomonas sputigena*, *Bacteroides ochraceus*, and *Veillonella* in the inflamed and non-inflamed human gingival crevice. *J. Periodontal Res.* 10:294-305.
- Kennedy, J. E., and A. M. Polson. 1973. Experimental marginal periodontitis in squirrel monkeys. *J. Periodont.* 44:140-144.
- Kornman, K. S., S. C. Holt, and P. B. Robertson. 1981. The microbiology of ligature-induced periodontitis in the cynomolgus monkey. *J. Periodontal Res.* 16:363-371.
- Loftin, K., L. R. Brown, and B. M. Levy. 1980. Comparison of the predominant cultivable microflora in the dental plaque of *Macaca mulatta* (rhesus) and the *Macaca fascicularis* (cynomolgus). *J. Dent. Res.* 59:1606-1612.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Mashimo, P. A., S. A. Ellison, and J. Slots. 1979. Microbial composition of monkey dental plaque (*Macaca arcuoides* and *Macaca fascicularis*). *Scand. J. Dent. Res.* 87:24-33.
- Matsson, L., and R. Attstrom. 1979. Development of experimental gingivitis in the juvenile and adult beagle dog. Test of a model for comparative studies. *J. Clin. Periodont.* 6:186-193.
- Mongiello, J. R., and W. A. Falkler, Jr. 1979. Sugar inhibition of oral *Fusobacterium nucleatum* hemagglutination and cell binding. *Arch. Oral Biol.* 24:539-545.
- Poxton, I. R. 1979. Serological identification of *Bacteroides* species by an enzyme-linked immunosorbent assay. *J. Clin. Pathol.* 32:294-298.
- Rose, N. R., and H. Friedman (ed.). 1980. Manual of clinical immunology, 2nd ed. American Society for Microbiology, Washington, D.C.
- Slots, J. 1977. The predominant cultivable microflora of advanced periodontitis. *Scand. J. Dent. Res.* 85:114-121.
- Slots, J., and T. Potts. 1982. *Fusobacterium simiae*, a new species from monkey dental plaque. *Int. J. Syst. Bacteriol.* 32:191-194.
- Smoot, C. N., and W. A. Falkler, Jr. 1981. Attachment of serum non-antibody glycoproteins to the *Fusobacterium nucleatum* found in periodontal diseases. *Arch. Oral Biol.* 26:859-864.
- Syed, S. A., M. Svanberg, and G. Svanberg. 1981. The predominant cultivable dental plaque flora of beagle dogs with periodontitis. *J. Clin. Periodont.* 8:45-46.
- Tanner, A. C. R., S. C. Haffer, R. A. Bratthall, R. A. Visconti, and S. S. Socransky. 1979. A study of the bacteria associated with advancing periodontitis in man. *J. Clin. Periodontol.* 6:278-307.
- Walker, C. B., D. Ratliff, D. Muller, R. Mandell, and S. S. Socransky. 1979. Medium for selective isolation of *Fusobacterium nucleatum* from human periodontal pockets. *J. Clin. Microbiol.* 10:844-849.