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Detection of Oral Anaerobic Spirochetes in Dental Plaque by the Indirect Fluorescent-Antibody Technique

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The indirect fluorescent-antibody technique was found to be a rapid and sensitive tool for the detection of oral anaerobic spirochetes in dental plaque.

Anaerobic spirochetes are indigenous members of the oral flora of humans as well as other animal species (6). Cultural studies have shown that the appearance of spirochetes in the succession of microorganisms within dental plaque is closely correlated with the time of onset of experimental gingivitis (9). Light microscopic studies on subgingival plaque have revealed that there is a dramatic increase in the number of spirochetes from 0.6% in patients without periodontal disease to as much as 17% of the total microbial population in patients with chronic severe periodontitis (7). Although a specific role for spirochetes in periodontal disease has not yet been established, based on numerical evidence alone, there is reason to believe that they contribute to the periodontal disease state. The purpose of the present investigation was to determine the applicability of the indirect fluorescent-antibody (IFA) technique for the detection of immunologically distinct spirochetes within subgingival dental plaque.

Our laboratory has been successful in isolating several strains of oral anaerobic spirochetes from subgingival plaque obtained from patients with chronic periodontitis. Three of these strains were designated W, 11, and 14, respectively. For the initial isolation, the membrane filter technique of Loesche and Socransky (5) was utilized, employing the media described by Socransky et al. (8) consisting of PPLO broth (BBL Microbiology Systems) plus 5 μg of cocarboxylase per ml, 10% sterile pooled rabbit serum, and 0.7% ion agar. Subgingival plaque was obtained from the area of periodontal involvement and was immediately transferred to individual vials containing 0.2 ml of PPLO broth. The plaque was blended vigorously in a Vortex mixer for 1 min to disperse the organisms. One drop of this plaque was carefully placed on top of a membrane filter with a diameter of 25 mm and a pore size of 0.45 μm (Schleicher and Schuell, Keene, N. H.) which had been layered on the surface of the PPLO-ion agar medium. All manipulations were carried out in a Coy anaerobic chamber similar to that described by Aranki et al. (2). After 4 to 5 days of incubation at 37°C, the membrane filter was removed and typical spirochetal growth was observed within the agar and then confirmed by dark-field microscopy. A sterile Pasteur pipette was used to remove a plug of agar from the leading edge of spirochetal growth and streaked onto a separate plate of the same media composition for single clone isolation. Once individual clones were obtained, they were transferred to the medium, described above, which lacked ion agar for liquid cultivation. Electron microscopic studies revealed that the three isolates were identical morphologically and possessed the "2-4-2" axial filament arrangement.

Specific immune serum was prepared for each spirochetal isolate in New Zealand White rabbits by inoculating the marginal inner ear vein with 2.5 ml of a 6- to 7-day-old broth culture. The immunization schedule consisted of a single inoculation per week for a total of 4 weeks. One week after the final inoculation, blood was collected by cardiac puncture and the serum was separated. Quantitation of specific spirochetal antibody was carried out by using the microscopic agglutination test as ordinarily used for the quantitation of leptospiral antibody (4). Microscopic agglutination titers obtained were typically 1:10,000 or greater. Microscopic agglutination studies using heterologous rabbit antiserum revealed that strains W and 14 showed some cross-reactivity, indicating that these strains may share common surface antigenic determinants; however, strain 11 appears to be immunologically distinct.

The patients utilized in this study consisted of individuals with chronic, severe periodontitis receiving treatment at the University of Maryland Dental School Clinic. A total of 10 patients—7 males and 3 females—participated in the study. Six of the patients were designated class III, and four were designated as class IV with respect to the severity of periodontal disease (1).

Subgingival plaque was collected from each

patient from both the maxillary and mandibular posterior teeth; the average depth of the periodontal pocket in these regions was 7 to 9 mm. The plaque was transferred to 0.2 ml of PPLO broth and blended for 1 min in a Vortex mixer to disperse the organisms.

The IFA test was performed by the method of Cherry et al. (3) as follows. One drop (0.05 ml) of plaque suspension was placed in each well of microscopic slides containing two rows of 7-mm-diameter circles cut in a Teflon coating (Cell Line Associates). The samples were dried at 37°C for 1 h and fixed in acetone for 5 min. Serial dilutions of specific rabbit antiserum (0.05 ml) to our spirochetal isolates were added to separate plaque samples and incubated at 37°C in a moist chamber. After incubation, the slides were rinsed with two changes of phosphate-buffered saline (pH 7.2) for 30 min. After air drying with a fan, one drop (0.05 ml) of fluorescein isothiocyanate-labeled goat anti-rabbit gamma globulin (Antibodies, Inc.) was applied to the plaque samples and incubated at 37°C for 30 min. After rinsing with phosphate-buffered saline as before, the samples were counterstained with one drop of a 0.1% solution of Evans blue for 5 min. The counterstain was removed with distilled water, and the slides were air dried and covered with buffered glycerol mounting medium and a cover slip. The samples were viewed with the Phaco 100× objective of a Leitz-Dialux microscope equipped with Ploemopak 2 incident fluorescence module containing excitation cube H. The light source was an HBO-50 mercury light with a BG-23 barrier filter. Images were recorded at an ASA rating of 800 with high-speed Kodak Ektachrome 400 film. Typical exposure times were between 1 and 2 min. Positive controls employed consisted of our three spirochetal isolates reacted with the specific homologous antiserum. Negative controls consisted of our spirochetal isolates reacted with nonimmune (prebleed) rabbit serum as well as dental plaque against nonimmune rabbit serum. In addition, plaque was reacted with the conjugate alone to determine whether the commercially prepared conjugate possessed spirochetal antibodies. The fluorescence of the spirochetes was graded on a +4, +3, +2, +1 basis, with +4 indicating the highest intensity of fluorescence.

The results obtained indicate that spirochetes were detected in the subgingival plaque samples of all 10 patients with antisera W and 14 (Table 1). The average number of spirochetes per field was approximately 10 to 20, and the intensity of the fluorescence was +3 to +4 (Fig. 1). The intensity of the fluorescence remained constant when the antiserum was diluted up to a factor of 1:40, but beyond this range all fluorescence was lost and no spirochetes were detected. With antiserum 11, spirochetes were detected in the plaque samples of only two of the patients. The reason for this may be, as pointed out previously, that by microscopic agglutination, strains W and 14 show some degree of cross-reactivity; however, strain 11 appears to be immunologically distinct. This observation was also confirmed by performing the IFA test with our three spirochetal isolates using both homologous as well as heterologous specific immune serum. With the homologous antisera, all three strains showed a +4 fluorescence; however, with the heterologous system strains W and 14 showed some cross-reactivity although the degree of fluorescence was noticeably diminished (+2), whereas with strain 11 no cross-reactivity was observed (Table 2). The striking feature of this test is its marked

TABLE 1. *Intensity of fluorescence of spirochetes observed in subgingival plaque of 10 patients with periodontal disease*

Patient no.	Intensity of fluorescence at antiserum dilution[a]:								
	Anti-W			Anti-14			Anti-11		
	1:10	1:20	1:40	1:10	1:20	1:40	1:10	1:20	1:40
1	+3	+3	+3	+3	+3	+3	–	–	–
2	+3	+3	+3	+3	+3	+3	–	–	–
3	+4	+4	+3	+3	+3	+3	–	–	–
4	+3	+3	+3	+3	+3	+3	–	–	–
5	+3	+3	+3	+3	+3	+3	–	–	–
6	+4	+4	+3	+4	+4	+4	+3	+3	+3
7	+4	+4	+4	+4	+4	+4	–	–	–
8	+4	+4	+4	+4	+4	+3	+4	+4	+4
9	+4	+4	+4	+4	+4	+4	–	–	–
10	+4	+4	+3	+4	+4	+3	–	–	–

[a] +4, Outstanding fluorescence; +3, strong fluorescence; +2, moderate fluorescence; –, no fluorescence. No fluorescence was observed with any of the antisera at 1:80.

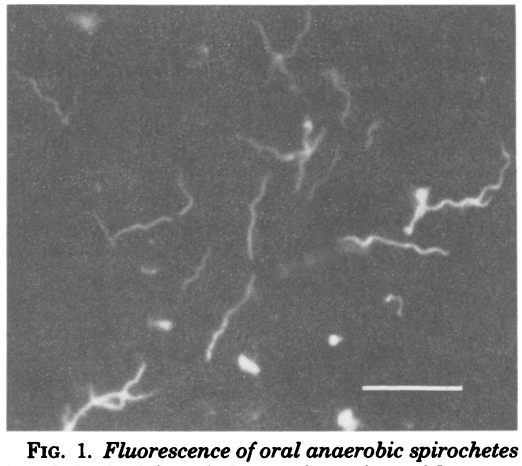

FIG. 1. *Fluorescence of oral anaerobic spirochetes in subgingival dental plaque of a patient with severe, chronic periodontitis, using the IFA technique. Bar represents 10 μm.*

TABLE 2. *Immunological relationships between oral spirochetes, using the IFA test*

Strain	Intensity of fluorescence with the following antisera[a]:		
	Anti-W	Anti-14	Anti-11
W	+4	+2	−
14	+2	+4	−
11	−	−	+4

[a] All dilutions of antisera were 1:20. +4, Outstanding fluorescence; +3, strong fluorescence; +2, moderate fluorescence; +1, minimal fluorescence; −, no fluorescence.

specificity for detecting only spirochetes among the multitude of microorganisms normally present within a sample of subgingival plaque. At no time were other organisms, besides spirochetes, detected by the IFA test, although dark-field microscopy did reveal a multitude of microorganisms within the plaque. At present, efforts are under way in our laboratory to isolate a wide variety of oral anaerobic spirochetes and, by preparing specific immune serum to these isolates, to try to identify their prototype in dental plaque using indirect immunofluorescence. The encouraging results obtained so far lead us to believe that the IFA test will prove to be a rapid and sensitive tool for the detection and eventual identification of oral spirochetes.

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