Localization of *Nocardia* in Dental Plaque by Immunofluorescence. (28533)

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Previous investigations(1-3) on the staining of dental plaque sections have been restricted mainly to the use of histochemical techniques designed to obtain information concerning the identity of the organic and inorganic substances found in various areas of plaque. Attempts to study the bacterial composition of these intact sections have been limited to determinations of the Gram reaction of organisms in different locations. It was thought that the fluorescent antibody (FA) technique of Coons(4) would provide a method for detection and localization of the various specific types of organisms found in dental plaque.

This investigation was undertaken in order to develop the fluorescent antibody technique for visualization of *Nocardia* in sections of dental plaque, with the hope that the information obtained might be applied to future research upon other members of the plaque flora.

Materials and methods. The indirect FA technique was used throughout the investigation. Antinocardial serum was obtained from a rabbit after a 4-week injection schedule using a formalin-killed antigenic mixture comprised of 6 oral strains of *Nocardia*. The fluorescein-conjugated sheep antirabbit globulin used in this study was obtained from the Sylvana Chemical Co.

To establish the degree of specificity of the antinocardial serum, it was necessary to test the serum against various oral bacteria. For this purpose, 75 organisms were isolated from plaque samples from 30 different subjects. These organisms were identified as *Nocardia, Bacterionema matruchotii, Corynebacterium, Actinomyces, Lactobacillus,* or *Fusobacterium* on the basis of their micromorphology, their colonial characteristics, and their biochemical characteristics as determined under the following conditions:

1) Catalase production—On trypticase soy (TS) agar plates (Baltimore Biological Laboratories). Tested by covering spot inoculum with 3% H_2O_2.

2) Oxygen tolerance—In TS agar pour tubes. Examined after 5 days growth.

3) Nitrate reduction—In TS broth + 0.1% KNO_3. Tested with reagents as specified in Manual of Microbiological Methods(5).

4) Indole production—In TS broth. Tested with Kovac's reagent(5).

5) Starch hydrolysis—On TS agar containing 0.5% soluble starch. Tested by flooding plates with Gram's iodine.

6) Lactose fermentation—In cysteine trypticase agar (Baltimore Biological Laboratories) + 0.5% lactose.

7) Acetylmethylcarbinol (AMC) production—In TS broth. Detected by the Barritt α-naphthol test(5).

All tests were performed after incubation at 37°C for 1 and 5 days. Tests on organisms suspected of being *Nocardia, B. matruchotii,* or *Corynebacterium* were done on aerobically grown cultures, whereas all tests on the remaining organisms, with the exception of those concerning oxygen tolerance and lactose fermentation, were done on anaerobically grown cultures.

In addition to the oral strains isolated in this laboratory, 3 strains of *Nocardia dentocariosus* were obtained from G. D. Roth (Univ. of Kansas City School of Dentistry), one strain each of *N. rubra* and *N. convoluta* were obtained from R. S. Safferman (Taft Sanitary Engineering Center, Cincinnati), and one strain each of *N. asteroides, N. brasiliensis,* and *N. dentocariosus* were obtained from J. A. Schmitt (Ohio State Univ.).

The antinocardial serum was tested against each of the organisms by the following procedure. A loopful of growth from a 24 to 48 hour TS agar slant culture of each organism was suspended in 1 to 2 ml of 0.5% formal-saline. To obtain homogeneous suspensions, it was necessary to grind several of the bacterial suspensions in a glass homogenizer tube with a motor-driven Teflon pestle for 1 to 2 minutes. The suspension of cells was

TABLE I. Biochemical Reactions of Plaque Isolates.

	Organism					
	Nocardia	*Bacterionema matruchotii*	*Coryne-bacterium*	*Actinomyces*	*Lacto-bacillus*	*Fuso-bacterium*
No. isolated	29	3	14	9	17	3
Catalase production	+	+	+	—	—	—
Oxygen tolerance*	aer.	fac.	fac.	fac., micro.	fac., anaer.	anaer.
Nitrate reduction	+†	+	+	V‡	—	—
Indole production	—	—	—	—	—	+
Starch hydrolysis	—	+	V	V	—	+
Lactose fermentation	—	—	V	V	V	—
AMC production	+	+	V	V	V	—

* aer. = aerobic; fac. = facultatively anaerobic; micro. = microaerophilic; anaer. = anaerobic.

† All strains of *Nocardia* further reduced the nitrite formed in nitrate reduction.

‡ V = variable reaction within group.

then washed 3 times in formal-saline and smears were made from the washed suspension.

Following heat fixation, the smears were stained by the indirect FA technique as described by Cherry *et al.*(6) using a 1:100 dilution of the antinocardial serum and a 1:8 dilution of the fluorescein conjugate. For controls on the specificity of staining, either normal preinjection serum diluted 1:100 or saline was used in place of the specific antinocardial serum in the indirect FA procedure.

Stained smears were examined with a Leitz Labolux microscope using an Osram HBO 200 mercury vapor lamp, a 4 mm BG-12 primary filter between the lamp and the darkfield condenser, and OG-1 secondary filters mounted on binocular eyepieces. The fluorescence intensity of the cells in the smears was measured by placing neutral density filters of 6.3, 12.5, 25, or 50% transmission between the primary filter and the condenser singly or in various combinations.

Plaque samples were obtained from the mouths of 4 human subjects who wore partial dentures by a modification of the procedure used by Meckel(7). The artificial tooth, which in every case was a lower molar, had an open space on the interproximal surface into which could be inserted a gold peg bearing a 3 mm square platform onto which a similar sized square of dental enamel was affixed. A 6.5 μ thick piece of Mylar film was stretched over the enamel square and tied around the gold peg with nylon thread. The peg with the Mylar film attached was then placed into the open space of the tooth and held in place by wax. The denture could then be inserted into the mouth of a subject with the flat surface of the Mylar film opposite an adjacent natural tooth, and left there for varying periods to allow for plaque accumulation.

Dentures containing the films were kept in the mouths of subjects for periods of from 1½ to 3 days. Upon removal of the denture, the peg bearing the Mylar film was placed in 10% formalin in absolute ethanol for 30 minutes, followed by dehydration in absolute ethanol for 30 minutes. After clearing in xylene for 30 minutes, the film was carefully cut away from the sides of the enamel square and embedded in paraffin. (Recently, sections have been prepared from samples embedded in Paraplast [Aloe Scientific Co.], a procedure which has resulted in improved stability of the plaque against disruption and in better contrast between stained and unstained cells.) Five micron sections were cut at right angles to the film, rehydrated, and stained by the indirect FA technique using the same procedures utilized for staining of pure cultures.

Results. The biochemical reactions of the various plaque isolates may be seen in Table I.

The 29 strains of *Nocardia* used in this investigation were obtained from 23 of the 30 subjects after 5 to 6 days of incubation at 37°C on a modification of Czapek's agar containing 0.1% yeast extract (Difco). Al-

though the frequency of isolation (77%) may appear high in view of the paucity of reports concerning oral *Nocardia,* this frequency is paralleled by the isolation by Davis and Freer(8) from saliva samples "from almost all mouths examined" of a strikingly similar organism which they called *Nocardia salivae.*

The morphology of oral Nocardia colonies on primary isolation was quite variable as was the case in the studies by Roth(9) and by Davis and Freer(8). One rather prominent colony type was greyish-white with a central papilla from which wrinkles radiated to the slightly irregular edge of the colony. Some colonies were white, smooth, and entire and others were rough and heaped up in their appearance.

The cellular morphology of the oral *Nocardia* was also somewhat variable. One type, which appears to correspond to the Type A forms of Morris(10) consisted of very small coccobacillary cells and slender filaments of varying lengths. Several of the *Nocardia* isolates, upon primary isolation, consisted entirely of coccobacilli, but after several subcultures on TS agar, filaments were predominant.

A second morphological type, similar to the Type B of Morris(10), consisted of mixtures of thicker filaments and short rods with occasional coccal cells being seen. These coccal cells were larger in size than the above-mentioned coccobacillary forms.

All of the oral *Nocardia* isolates were Gram positive and produced capsular material detectable by India ink negative staining or by specific staining with 0.5% alcian blue.

All 29 of the *Nocardia* isolates from this laboratory as well as the 4 *N. dentocariosus* isolates obtained from other laboratories were stained intensely by the indirect FA technique and could still be seen at 0.1% transmission of the excitation light. Six strains of *Actinomyces,* 4 of *Lactobacillus,* and 2 of *B. matruchotii* showed slight cross-reactions with the antinocardial serum as evidenced by staining intensities sufficiently bright to be visualized at 0.4 or 0.2% transmission. Weak cross-reactions were also noted with the strains of *N. asteroides* and *N. brasiliensis.* However, the fluorescence intensities of all of these cross-reacting organisms were markedly different from those of the oral *Nocardia* isolates when observed without the neutral density filters (Fig. 1).

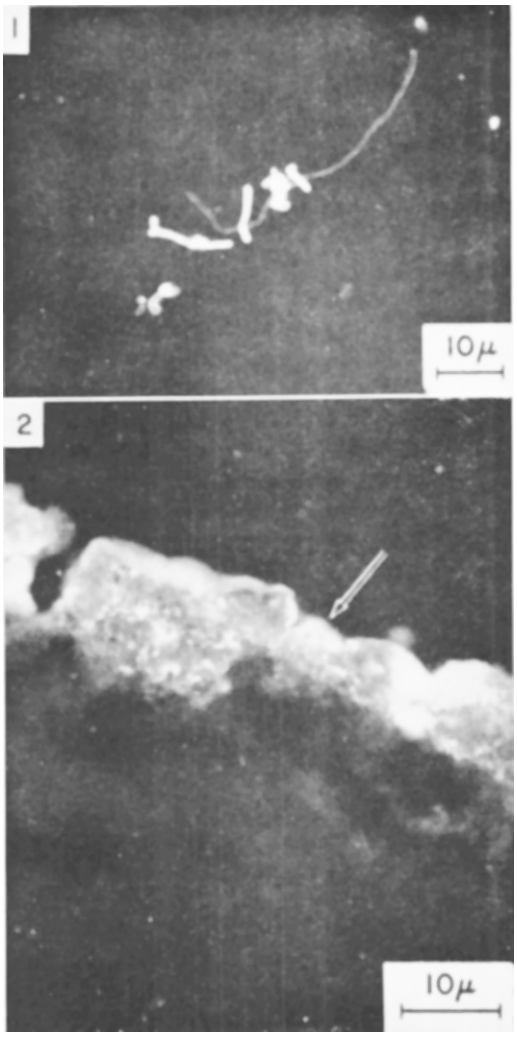

FIG. 1. Appearance of a fluorescent antibody stained smear of a mixture of *Nocardia* and *B. matruchotii* cells under fluorescence microscopy. The long, shadowy filament is *B. matruchotii* and the shorter, bright filaments are *Nocardia* cells.

FIG. 2. Fluorescent antibody stained plaque section (3 day). The fluorescent cells are restricted to the plaque surface originally adjacent to the saliva (arrow). The surface of the plaque which was originally in apposition to the Mylar may be seen as a very faint shadow in lower left-hand corner of illustration.

None of the organisms was stained intensely enough to be seen at per cent transmissions

less than 0.8% when either normal serum or saline was substituted for the antinocardial serum in the indirect FA test.

All plaque samples examined in this study yielded sections which contained brilliantly stained cells when stained with the indirect FA technique using the antinocardial serum. No stained cells were seen in plaque sections when normal serum was substituted for the antinocardial serum.

Clumps of fluorescent cells were restricted to the most superficial areas of plaque where they were found at intervals along the length of the surface (Fig. 2). Occasionally, aggregations comprised of 3 to 5 cells could be found in deeper areas of plaque, but the frequency of their occurrence was rare compared to the frequency of much larger clumps at the outer surface.

Observations on 3 day plaque samples from each of the 4 subjects revealed that one subject formed plaque more rapidly than the other 3 subjects. In addition, fluorescent cells were more numerous and thus easier to find in sections of plaque from the rapid plaque-forming subject.

Several plaque samples of different ages obtained from the rapid plaque-forming subject revealed a difference in the ratio of the numbers of fluorescent *Nocardia* to total cells. In 1½ day plaque samples, which ranged in thickness from 30 to 100 μ, fluorescent cells made up a relatively large portion of the plaque. On the other hand, in 3 day samples, which varied in thickness from 100 to 200 μ, many more nonfluorescent cells were present. The consistency of this effect was revealed by examinations of sections from three 1½ day samples and from four 3 day samples.

Discussion. This study has demonstrated the possibility of the use of FA techniques for the study *in situ* of specific dental plaque microorganisms. The results suggest that the strictly aerobic *Nocardia* may have a role in initiation and subsequent rate of plaque development.

Dental plaque has been previously shown by many investigators to contain a variety of organisms ranging from strict aerobes to strict anaerobes. It is not inconceivable that, for rapid plaque formation to occur, aerobic growth must first occur at the tooth surface until the plaque is thick enough for anaerobic conditions to prevail in the deeper layers, and strictly anaerobic organisms such as the fusobacteria, veillonellae, and spirochaetes can then begin to metabolize and reproduce.

It would also be reasonable to expect that other strictly aerobic plaque microorganisms would contribute to the formation of plaque in a similar fashion. In connection with this, Richardson and Jones(11), in a census of a variety of oral organisms, stated that "the demand of this genus *(Neisseria)* for an aerobic atmosphere might lead one to speculate that subjects with clean mouths would have more aerobes, hence high *Neisseria* counts. Actually, the reverse was true." The possible correlation between the presence of strictly aerobic organisms in dental plaque and rate of formation of that plaque deserves further consideration.

Summary. Seventy-five organisms were isolated from dental plaque samples from 30 subjects and identified on the basis of their colonial morphology, micromorphology, and biochemical reactions. When stained by the indirect technique using an antinocardial serum, all 29 oral *Nocardia* strains isolated in this laboratory and 4 obtained from other laboratories stained brilliantly, whereas heterologous organisms showed much lower intensities of staining. The staining characteristics of paraffin sections of plaque by this technique were suggestive of the possible influence of the aerobic *Nocardia* on the rate of plaque formation.

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Splenectomy and Thymectomy in Human Renal Homotransplantation.* (28534)

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Renal homotransplantation has been given a limited clinical trial in several centers using either cytotoxic drugs or irradiation to weaken the host immunologic potential. The results of these efforts have been discouraging, with a high early failure rate due to rejection. In a recent world survey of experience with renal homotransplantation, Goodwin and Martin found that less than 10% of the 176 known cases had survived for as long as 3 months(1). Only 6 cases had lived for as long as a year.

During the past 6½ months, 5 patients have received kidney transplants, using a somewhat different approach to the problem of host preparation. The thymus and spleen of the recipient patients were removed surgically, either prior to or on the day of renal homografting for the purpose of reducing the antibody producing tissue of the host. In addition, the patients were provided with standard anti-rejection therapy, using total body irradiation or cytotoxic drugs.

Methods. The underlying disease was chronic glomerulonephritis in 4 cases, and polycystic kidney in the fifth (Table I). In each instance, the disease had progressed to a terminal phase, and multiple hemodialyses were required in the weeks or months before transplantation. Two patients had bilateral nephrectomy before transplantation to control severe hypertension. A third patient had one kidney removed before and the other at the time of transplantation (Table I). One of the living patients still retains one of his own kidneys.

Thymectomy was performed 13 to 31 days before transplantation (Table I) through a midline sternum splitting incision, with a transverse cervical collar extension. Care was taken to remove the entire thymus, particularly the inferior bilobed portion and the superior portion which often extends to the thyroid gland. In 2 patients, splenectomy was performed at the same time as thymectomy by another surgical team. In the other 3, splenectomy was performed 19 days before, 7 days before, and on the same day as transplantation.

In addition to thymectomy and splenectomy, standard techniques to prevent rejection were used (Table II). In principle, all patients except the first one were treated in the same way. The first patient was treated with 300 R total body irradiation preoperatively, delivered with a 250 KV unit. Fifty R were given on the 17th, 14th, 11th and 5th days before transplantation, culminating in a final dose of 100 R on the day before operation. On the 4th and 6th days after transplantation, additional doses of 50 R were given, shielding the renal transplant on the second of these occasions. Despite the resultant suppression of the white count to

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