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# Rapid Method for Identification and Enumeration of Oral *Actinomyces*

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Serotype-specific antisera prepared against whole cells of *Actinomyces viscosus*, *A. naeslundii*, and *A. israeli* were labeled with fluorescein dye and used to detect and quantitate antigenically related microorganisms in human dental plaque. By relating the DNA content of the dental plaque microflora to the number of *Actinomyces* present in the plaque samples, a reproducible method was developed for specifically enumerating five serotypic representatives of this genus found in human plaque.

The ability to recognize and identify rapidly oral *Actinomyces* has recently acquired a new importance with the demonstration that these microorganisms are capable of producing periodontal lesions and bone loss in germfree rodents (4-6). Although their exact role in the etiology of human periodontal disease has not been defined, a number of investigators have suggested that this group of bacteria contribute to the onset or persistent nature of the disease (3, 15). A number of taxonomically distinct representatives of the genus *Actinomyces* may inhabit the mouths of both normal and diseased individuals simultaneously. The ability to identify quickly specific serotypes and to quantitate their numbers is essential for any longitudinal study designed to determine whether causal relationships exist between certain members of this group and the various forms of periodontal disease.

Studies initiated by Slack and his associates (12-14) have demonstrated that fluorescent-antibody staining provided a rapid and reliable method for identifying and enumerating *Actinomyces* species. Initially, problems with nonspecific cross-reactions were circumvented by simply diluting the antisera sufficiently so that only homologous strains reacted with it; but, as new serotypes were isolated and studied, the extent of heterologous cross-reactivity increased to a degree where dilution of the antisera could no longer effectively compensate for the nonspecific reactions produced by shared antigenic determinants (2). This report describes the preparation of five antisera that react, almost exclusively, with their own serotype of *Actinomyces* and the development of a procedure for identifying and enumerating these organisms in specimens of human dental plaque.

## MATERIALS AND METHODS

**Organisms.** Organisms used in this study are listed in Table 1. Cultures were maintained in a medium containing (in grams per liter): fluid thioglycolate medium (BBL), 36.7; beef infusion (Difco dehydrated beef), 22.5; and $CaCO_3$, 62. The pH was adjusted to 7.0. Cell suspensions to be used for immunization or immune adsorption chromatography were grown in Trypticase soy broth (Difco) for 48 h at 37°C under an atmosphere of 90% air-10% $CO_2$. Only cultures of *A. israelii* required an atmosphere of 95% $N_2$-5% $CO_2$ for optimal growth.

**Preparation of antisera.** *Actinomyces* cell suspensions used for immunization were harvested from 20-ml Trypticase soy broth cultures, washed twice in normal saline (0.85% NaCl), resuspended in 20 ml of normal saline, and killed by heating at 60°C for 15 min. The suspensions were then adjusted to an optical density at 550 nm of 0.5 with normal saline and used to inject white, male New Zealand rabbits. Beginning with a 0.5-ml dose and increasing the dosage in 0.5-ml increments until a volume of 2 ml was reached, injections were administered twice weekly for a month into the marginal ear vein. Upon completion of the regimen, the titers of the antisera were estimated by conventional agglutination tests (7); if a titer of 1:6,000 was not obtained, injections were continued for another 2 weeks in an attempt to raise the titer.

Blood obtained from the central ear vein of the immunized rabbits was treated in the following manner. After clotting, fibrin, leukocytes, and erythrocytes were removed by centrifugation, and the clarified serum was applied to a diethylaminoethyl (DEAE)-Sephadex A-50 column (2.5 by 25 cm) equilibrated with 0.5 M sodium phosphate buffer containing 0.85% NaCl, pH 7.5 (PBS), and having a conductivity of 15,000 mho. Elution with the PBS yielded a purified immunoglobulin G (IgG) preparation. The peak IgG fractions were pooled and reduced in volume in an Amicon ultrafiltration cell to give a final protein concentration of 10 mg/ml.

To reduce cross-reactions between the individual antisera and certain heterologous *Actinomyces* cells,

TABLE 1. *Organisms and sources*

| Organism | Source[a] |
|---|---|
| *A. naeslundii* (serotype 1) | |
| W1096 | ATCC 27040 |
| WVU45 | M. A. Gerencser |
| W826 | ATCC 27040 |
| *A. naeslundii* (serotype 2) | |
| W752 | CDC |
| W1544 | CDC |
| *A. naeslundii* (serotype 3) | |
| N16 | M. A. Gerencser |
| WVU820 | M. A. Gerencser |
| W1527 | CDC |
| *A. naeslundii* (serotype 4) | |
| WVU852 | M. A. Gerencser |
| *A. viscosus* (serotype 1) | |
| A828 | ATCC 15987 |
| X602 | A. Howell |
| *A. viscosus* (serotype 2) | |
| M100 | B. Hammond |
| W859 | ATCC 19246 |
| W1053 | ATCC 27044 |
| W1557 | CDC |
| W1528 | CDC |
| W1628 | CDC |
| *A. israelii* (serotype 1) | |
| W855 | ATCC 12102 |
| X522 | ATCC 10048 |
| *A. israelii* (serotype 2) | |
| W1011 | M. A. Gerencser |
| WVU307 | M. A. Gerencser |
| *A. bovis* (serotype 1) | |
| W827 | ATCC 13683 |
| *A. bovis* (serotype 2) | |
| W1755 | M. A. Gerencser |
| *A. odontolyticus* (serotype 1) | |
| X363 | ATCC 17929 |
| *A. odontolyticus* (serotype 3) | G. Haegage |
| *Arachnia propionica* (serotype 1) | |
| W857 | ATCC 14157 |
| *Arachnia propionica* (serotype 2) | |
| W904 | M. A. Gerencser |
| *Rothia dentocariosa* | |
| X599 | ATCC 17931 |
| *Bacterionema eriksonii* (*Bifidobacterium*) | |
| X407 | CDC |
| W573 | CDC |

[a] ATCC, American Type Culture Collection; CDC, Center for Disease Control, Atlanta, Ga. (S. L. Bragg).

the sera were next passed through triethylaminoethylcellulose (TEAE) immunoabsorbant columns (2.5 by 50 cm) equilibrated with PBS according to the procedure of McKinney and Thacker (8). Highly specific IgG was eluted from the column with PBS. The nontype-specific cross-reacting antibody bound to the bacterial TEAE matrix was subsequently removed by washing the column with 0.5 M NaCl in PBS adjusted to a pH of 2.3. The column was then reconditioned by several washings with 0.5 M borate buffer containing 0.85% NaCl, pH 7, and re-equilibrated with PBS.

Antibody from the immunoabsorbant columns was conjugated with fluorescein isothiocyanate by reacting 50 μg of dye with 1 mg of IgG in 0.1 M $Na_2HPO_4$ for 2.5 h at ambient temperature. This treatment generally produced a ratio of fluorescein to protein of 20 or 30:1 (μg/mg) for the purified conjugate; unbound fluorescein isothiocyanate was separated from dye-IgG conjugate by applying the mixture to a Sephadex G-50 column and eluting the latter with PBS, pH 8.0.

**Whole-plaque experiments.** To test the reproducibility and specificity of the staining and counting procedures for *Actinomyces* associated with or imbedded in a heterogeneous mass of organic material, plaque samples from the gingival crevices of three individuals were collected and treated as follows. Each specimen was dispersed in 4 ml of distilled water by homogenization and divided into 10 0.3-ml samples; the samples were then adjusted to 1.5 ml with distilled water. The remaining 1-ml portions of the three individual specimens were combined to make up a "pooled" plaque sample which was subsequently divided in the same fashion as above. The 1.5-ml samples were subjected to two 10-s treatments with a Branson model 140 Sonifier operating at 70% of its total output. For standardization of plaque samples, the number of *Actinomyces* was related to the DNA content of the plaque sample. Total DNA was determined in a 0.5-ml sample by the ethidium bromide procedure of Drummond and Donkersloot (1). A second 0.5-ml fraction was treated with 150 U of micrococcal deoxyribonuclease (DNase) for 30 min at 37°C and assayed for DNA content. The first determination represented the entire bacterial population as well as leukocytes and other eucaryotic cells, whereas the second determination represented the DNA concentration of intact gram-positive bacteria (10).

As expected, the DNA values were markedly reduced after DNase treatment (Table 2) because the ultrasonic treatment disrupted all leukocytes and most of the gram-negative bacteria present in the plaque sample (10). In plaque samples of normal individuals, the DNA values fell by 40% after DNase treatment while the DNA content of plaque from subjects with chronic periodontitis was reduced by 60%. The greater decrease of DNA content in the latter probably reflects the presence of higher numbers of leukocytes and gram-negative bacteria in the plaque samples from the periodontal pocket. Differences in DNA values of this magnitude are not surprising since human leukocytes, like other eucaryotic cells, contain an amount of DNA

TABLE 2. *Effect of ultrasonic disruption and DNase treatment on plaque DNA recovery*

| Subject | DNA (μg/sample)[a] before treatment (avg) | DNA (μg/sample) after treatment (avg) | Decrease (%) |
|---|---|---|---|
| A | 1.66 ± 0.056 | 0.96 ± 0.038 | 42 |
| B[b] | 2.25 ± 0.084 | 0.8 ± 0.039 | 63 |
| C[b] | 2.76 ± 0.274 | 1.08 ± 0.094 | 63 |

[a] Average of 10 samples.

[b] From clinically diagnosed advanced periodontal lesion.

at least three orders of magnitude greater than most gram-positive bacteria (11).

The remaining 0.5 ml of plaque sample was diluted 1:5, 1:25, and 1:125 and applied to the 5-mm wells of replicate Teflon slides in 10-μl quantities. After drying overnight in moist chambers, the slides were fixed with 95% ethanol and stored at 4°C until stained and counted. Conventional procedures (9) were used to stain material applied to the centers of the Teflon-coated slides. The slides were then fixed with 95% ethanol and examined with incident illumination from a Xenon lamp mounted on a Leitz microscope. Cells exhibiting bright fluorescence in 12 separate fields containing between 5 and 30 cells were counted.

## RESULTS

**Specificity of anti-*Actinomyces* sera.** The DEAE-Sephadex A-50 fractions of IgG prepared against *A. viscosus* M100, *A. naeslundii* W826 and N16, and *A. israelii* W855 and WVU307 invariably exhibited some degree of cross-reactivity with one or more heterologous species. Table 3 exemplifies data obtained with a strongly cross-reactive serum; at dilutions of 1:80, antiserum prepared against *A. viscosus* M100 (serotype 2) reacted with three heterologous serotypes. Passing the serum through a TEAE column containing adsorbed cells of *A. naeslundii* serotypes I through IV removed the nonspecific cross-reacting antibodies. Table 3 shows that only the homologous strain reacts with the adsorbed serum at dilutions as low as 1:20. However, these data raise some question as to the initial serological classification of two strains used in this study because *A. viscosus* W859 was also classified as serotype 2 and exhibits no antigenic similarity to strain M100. It appears that strains M100 and W859 are immunologically distinct.

In contrast to the treatment of the high-titer *A. viscosus* M100 antiserum, the low-titer antiserum produced against *A. naeslundii* N16 required a different treatment. Interspecific cross-reactions were eliminated by passing the serum through a column containing adsorbed cell suspensions of *A. naeslundii* N16. After the antiserum preparation was applied to the column, the weakly binding heterologous antibody was eluted with PBS. Homologous antibody was then eluted with PBS, pH 2.3. The eluate was collected in tubes containing sufficient neutral buffer to raise the pH to 6.8. Antiserum obtained by this procedure was specific for type 3 strains of *A. naeslundii*. The strains of *Actinomyces* used to adsorb the remaining three antisera are summarized in Table 4.

The specificities of the five adsorbed antisera were determined by testing each against cells of the *Actinomyces*, *Rothia*, *Arachnia*, and *Bacterionema* species listed in Table 1. With the exception of anti-*A. viscosus* M100 (see above), all sera reacted only with their respective serotypic strains (Table 4), even when the latter were mixed with cells of one or more immunologically heterologous groups. In such mixtures the enumeration of a specific serotype fell within 10% of a standard determination in which only homologous cells were used.

A polyvalent antiserum was prepared by mixing unabsorbed DEAE-Sephadex A-50-treated sera in the following amounts: anti-*A. viscosus* A828 (type 1), 5.7 mg; anti-*A. viscosus* M100 (type 2), 5.4 mg; anti-*A. naeslundii* W1544 (type 2), 5 mg; anti-*A. naeslundii* N16 (type 3), 5 mg; anti-*A. naeslundii* W826 (type 1), 10 mg; anti-*A. naeslundii* WVU852 (type 4), 5 mg; anti-*A. israelii* W855 (type 1), 5 mg; and anti-*A. israelii* WVU307 (type 2), 2 mg. Because of its high degree of cross-reactivity, the polyvalent antiserum readily detected all of our laboratory strains of *Actinomyces* when they were stained individually or combined at a cell dilution of 1:10. Even though the pooled antisera did not react with strains of *Rothia* sp., *Arachnia* sp., or *Bacterionema* sp., cell size and morphology were carefully monitored during cell counting procedures to be certain that the stained mate-

TABLE 3. *Cross-reactivity of anti-A. viscosus M100 sera before and after column adsorption*[a]

| Strain tested[b] | Cross-reactivity (before/after) at antiserum dilution of: | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1,280 |
| *A. viscosus* (2) M100 | 4+/4+ | 4+/3+ | 4+/3+ | 4+/3+ | 3+/2+ | 3+/+ | 2+/ |
| *A. viscosus* (1) A828 | 2+/− | +/− | +/− | −/− | −/− | −/− | −/− |
| *A. viscosus* (2) W859 | 3+/− | 3+/− | 2+/− | 2+/− | +/− | −/− | −/− |
| *A. naeslundii* (1) W816 | 4+/− | 3+/− | 3+/− | 2+/− | +/− | −/− | −/− |
| *A. naeslundii* (2) W752 | 4+/− | 3+/− | 3+/− | 3+/− | 2+/− | +/− | −/− |
| *A. naeslundii* (3) WVU820 (N16) | 3+/− | 2+/− | +/− | −/− | /− | /− | /− |
| *A. naeslundii* (4) WVU852 | 3+/− | 3+/− | 2+/− | +/− | −/− | −/− | −/− |
| *A. israelii* (1) W855 | +2/− | +2/− | +/− | −/− | −/− | −/− | −/− |
| *A. israelii* (2) W1011 | +/− | +/− | −/− | −/− | −/− | −/− | −/− |

[a] Column matrix of TEAE to which *A. naeslundii* W826, W752, WVU820, and WVU852 were adsorbed.
[b] Numbers within parentheses indicate serotypes.

TABLE 4. *Specificity and working dilutions of the five adsorbed anti-Actinomyces species sera*

| Antiserum against: | Strains fixed to adsorption column | Strains reacting with adsorbed antiserum | Antibody dilution used in stains |
|---|---|---|---|
| *A. viscosus* M100 | *A. naeslundii* W826, W752, WVU820, and WVU852 | *A. viscosus* M100 | 1:40 |
| *A. naeslundii* WVU820 | Homologous adsorption | *A. naeslundii* WVU820, W1527, and N16 | 1:10 |
| *A. naeslundii* W826 | *A. viscosus* A828 and M100; *A. naeslundii* W1544, WVU820, and WVU852; *A. israelii* W855 | *A. naeslundii* W826, W1096, and WVU45 | 1:10 |
| *A. israelii* W855 | *A. naeslundii* W1544; *A. viscosus* W859; *A. israelii* W307 | *A. israelii* W855 and X552 | 1:40 |
| *A. israelii* WVU307 | *A. israelii* W855 and W826 | *A. israelii* W307 and W1011 | 1:320 |

rial corresponded to an *Actinomyces* species.

**Test of anti-*Actinomyces* sera in a "plaque" model system.** The 10 replicate samples obtained from each of the subjects (A, B, and C) were analyzed for DNA content, and the *Actinomyces* population was enumerated by using the polyvalent and five specific antisera. The reproducibility of the procedure is apparent from data with the adsorbed *A. viscosus* M100 antiserum (Table 5). In this instance, the counts of antigenically similar *Actinomyces* vary by 15% between the highest and lowest samples. The uniformity of plaque dispersion and sampling technique is also evident from the DNA determination of the respective plaque samples (Table 5) because DNA varied by no more than 10% between the highest and lowest values of the samples. Results from studies of the two other subjects and with the four remaining adsorbed antisera were similar to those cited above and are therefore not shown.

The technique was further tested by mixing plaque samples from the three subjects and comparing the observed numbers of the five serotypes with the amount added. A comparison of the last two columns in Table 6 reveals that, in all instances, the recovery of each of the five serotypes equaled or exceeded the theoretical. With the exception of the anti-*A. naeslundii* serotype 3 data, the slight increase in cell counts can be attributed to (i) the greater numbers of each serotype in the pooled plaque which would increase the efficiency of staining through more uniform distribution of cells or (ii) the use of more dilute antiserum which would decrease nonspecific staining of background material and make stained cells easier to distinguish. However, neither explanation accounts for the 70% increase in *A. naeslundii* serotype 3 numbers; at this time we do not have an explanation for the increase. The 10 to 30% differences between the additive values of the serotype-specific counts and the counts obtained with the polyvalent antiserum are due to the unavailability of certain adsorbed antisera, most notably, *A. viscosus* serotype 1 antiserum and to a lesser extent a second *A. viscosus* serotype 2 antiserum. Unadsorbed sera prepared against the two latter strains were used to prepare the polyvalent serum. Despite these differences, this technique provides a relatively accurate and rapid method for differentiating the various species of *Actinomyces* present in the samples.

TABLE 5. *Efficacy of sample dispersion and recovery of A. viscosus serotype 2 from plaque samples*[a]

| DNA (μg) in total sample[b] | Plaque sample dilution | No. of *A. viscosus* serotype 2[c] | |
|---|---|---|---|
| | | Avg counts per field | Total cells ($\times 10^{-3}$) per μg of DNA |
| 0.923 ± 0.279 | 1:25 | 28.19 ± 1.01 | 36.24 ± 1.32 |
| | 1:125 | 5.66 ± 0.481 | 36.30 ± 1.30 |

[a] Plaque samples obtained from subject A. All values are the average of 10 determinations.

[b] Sample is DNase-treated sonic extract.

[c] A 10-μl sample reacted with absorbed anti-*A. viscosus* M100 serum.

## DISCUSSION

By using a technique similar to the one described by McKinney and Thacker (8), serotype-specific antisera have been prepared against representatives of three species of *Actinomyces* commonly found in the oral cavity of humans. The heterologous cross-reactions reported by others (2) were eliminated by column adsorption

TABLE 6. *Enumeration*[a] *of specific Actinomyces serotypes in single and combined plaque samples*

| Antiserum used | Cell count dilution[b] | No. of cells ($\times 10^{-3}$) in sample: A | B | C | Theoretical total in mixed sample[c] | Actual count in mixed plaque sample ($\times 10^{-3}$) |
|---|---|---|---|---|---|---|
| *A. naeslundii* serotype 1 (W826) | 0 | | 0.62 | | | |
| | 1:25 | 6.63 | | 1.78 | 9.03 | 10.2 |
| *A. naeslundii* serotype 3 (N16) | 1:5 | | 2.96 | 2.85 | | |
| | 1:25 | 4.66 | | | 10.48 | 17.94 |
| *A. israelii* serotype 1 (W855) | 0 | 0.165 | | | | |
| | 1:5 | | 4.69 | | | |
| | 1:25 | | | 7.20 | 12.03 | 13.36 |
| *A. israelii* serotype 2 (WVU307) | 1:5 | 0.06 | 3.54 | 0.57 | 4.14 | 4.31 |
| *A. viscosus* serotype 2 (M100) | 1:5 | | 1.23 | 0.615 | | |
| | 1:25 | 20.4 | | | 22.25 | 24.75 |
| Total identifiable species | | 31.91 | 13.04 | 13.02 | 57.93 | 70.56 |
| Polyvalent | 1:25 | 35.25 | 18.33 | 17.79 | 71.49 | 102.5 |

[a] Reported as cell numbers per microgram of DNA.
[b] Dilution of plaque sample necessary for fluorescent cell counts.
[c] Theoretical total equal to sum of patients A, B, and C.

procedures (8). In nearly every instance, the increase in specificity more than compensated for the decrease in titer resulting from the immune adsorption chromatography.

Relating the numbers of *Actinomyces* in plaque to the DNA content rather than to dry weight or protein content provides a consistent means for standardizing the procedure because these values can be roughly related to the total numbers of gram-positive bacteria remaining in the ultrasonically and DNase-treated material. The utility of this procedure is limited only by (i) the sensitivity of the DNA assay and (ii) the range and titer of the polyvalent antiserum. For example, some plaque samples collected from single sites (unpublished data) in the mouths of normal individuals did not contain sufficient bacteria to give reliable DNA values. Single-site evaluations of subjects with active cases of periodontal disease may be more easily performed because the pockets generally contain sufficient material for reproducible DNA determinations. The second limitation is probably less serious because orally derived strains of the three species of *Actinomyces*, *A. viscosus*, *A. naeslundii*, and *A. israelii*, appear to be taxonomically related and share one or more cell envelope antigens (2). It is obvious from the studies described here that any species of *Actinomyces* or groups of species can be identified and their numbers can be accurately measured with the appropriate antisera.

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