Quantitative Fluorescent Immunoassay of Antibodies to, and Surface Antigens of, *Actinomyces viscosus*

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Optimal conditions for a fluorescence immunoassay of antibodies to, and surface antigens of, *Actinomyces viscosus* ATCC 19246 are described. In the standard fluorescence immunoassay, 10^8 colony-forming units of *A. viscosus* reacted with an antibody preparation, were washed, and then were treated with an excess of fluorescein-conjugated goat anti-rabbit immunoglobulin G. After another set of washes, fluorescence was determined in a spectrofluorometer; in most cases excitation was at 485 nm, with emission measured at 525 nm. These conditions minimized interference from light scatter and stray light. Under appropriate conditions, antibodies to *A. viscosus* could be readily determined, with the fluorescence of the specific antibody-treated cells more than five times the fluorescence of controls treated with normal rabbit serum. Organisms coated with specific antibody could be detected at levels approaching 10^5 colony-forming units per ml. The standard fluorescence immunoassay procedure was readily adapted to the measurement of either particulate or soluble surface antigens of *A. viscosus* by competition of the antigen with a fixed amount of antibody in the standard assay system; the competition resulted in an antigen dose-dependent inhibition of fluorescence. The fluorescent immunoassay system thus appears to be a general one that could be applied to other microbial systems as well.

The immunofluorescent technique originally described by Coons et al. (9, 10) has many applications in the rapid qualitative detection of microorganisms in clinical materials, including detection of *Actinomyces* species in human dental-plaque samples (8, 19). Quantitation of fluorescent microorganisms from such specimens is difficult. Recent work with solid-phase fluorescent immunoassays (FIA), however, has demonstrated that antigens or antibodies can be quantitated by the measurement of immunospecific fluorescence associated with individual, derivatized carrier beads (2, 4, 17) or suspensions of such beads (3). We report here that suspensions of the microorganism *A. viscosus*, a suspected periodontal pathogen of humans and animals (1, 14, 15), can be used to directly assay antibodies to, and surface antigens of, that organism by simple FIA procedures. This FIA methodology thus appears to be a general one potentially applicable in the clinical setting to quantitative determinations of microorganisms or of antibodies directed to their surface components.

MATERIALS AND METHODS

Organisms. *A. viscosus* ATCC 19246 was obtained directly from the American Type Culture Collection and maintained aerobically by passage on blood agar

plates. The organism was characterized periodically by colonial morphology, Gram stain, and biochemical reactions. Bacterial suspensions were prepared from 60-h cultures of the organism grown aerobically at 37°C in *Actinomyces* broth (Baltimore Biological Laboratory, Cockeysville, Md.) by centrifugation ($1,256 \times g$ for 10 min) and three washes with 100 mM phosphate buffer (PB), pH 7.0. For plate counts, serial 10-fold dilutions of the stock bacterial suspension were diluted in PB, and portions were plated in triplicate on brain heart infusion agar (Baltimore Biological Laboratory) plates supplemented with 2% yeast extract (Difco Laboratories, Detroit, Mich.). Absorbance at 660 nm was determined on serial twofold dilutions of the stock bacterial suspension with a double-beam spectrophotometer (GCA/McPherson Instrument, Acton, Mass.). Under these conditions, an absorbance of 0.32 corresponds to 10^8 colony-forming units (CFU) per ml.

Lactobacillus acidophilus ATCC 4356 was obtained directly from the American Type Culture Collection and maintained aerobically by passage on Rogosa SL agar (Difco Laboratories) plates. Bacterial suspensions were prepared from 60-h cultures of the organism grown aerobically at 37°C in Rogosa SL broth (Difco Laboratories) by centrifugation ($1,256 \times g$ for 10 min) and three washes with PB.

Ra-a-19246 production. A suspension of washed *A. viscosus* ATCC 19246 was adjusted to 10^{10} CFU/ml. A 1-ml amount of this suspension was injected intravenously into the peripheral ear vein of New Zealand white rabbits three times per week for 1 month. After

a 1-week rest period, the rabbits were exsanguinated, and the sera (Ra-a-19246) were collected and stored at -20°C .

Soluble antigen preparation. A pellet of *A. viscosus* (300 mg wet weight) was resuspended in deionized water (15 ml) and disrupted with an ultratip sonic oscillator (New Brunswick Scientific Co., New Brunswick, N.J.) at full power for 15 min in an ice bath. The disrupted suspension was then clarified by two centrifugations ($800 \times g$ for 10 min), and the clarified supernatant fluid was centrifuged ($100,000 \times g$ for 90 min). The supernatant fluid from the last centrifugation step was used as the "soluble" antigen preparation. Antigen preparations were stored at -20°C .

FIA. A fluorescein-conjugated goat anti-rabbit immunoglobulin G serum (Fl-Go-a-RaG) (Miles Laboratories, Inc., Elkhart, Ind.) was used for FIA of antibodies bound to *A. viscosus*. In brief, a standardized amount of *A. viscosus* (10^8 CFU) was pelleted by centrifugation ($1,256 \times g$ for 5 min) in test tubes (6 by 50 mm). The pellet was resuspended in 300 μl of either an Ra-a-19246 dilution in PB or 300 μl of the corresponding dilution of pooled normal rabbit serum (Pel-Freez, Rogers, Ark.) as control. After reaction for 30 min at room temperature with intermittent mixing, the cells were washed three times (500 μl /wash) in PB by centrifugation ($1,256 \times g$ for 5 min). The washed pellets were resuspended in an Fl-Go-a-RaG dilution (300 μl) and incubated for 15 min at room temperature with intermittent mixing. The suspensions were then centrifuged ($1,256 \times g$ for 5 min) and washed (500 μl /wash) three times with PB. The washed cells were finally resuspended in 2.5 ml of PB, and the fluorescence of the mixture was determined as detailed below. It must be noted that centrifugation steps in this FIA are critical; incomplete resuspension of the pellets or loss of materials during washes leads to erratic results. For this reason, it is essential to use "swinging-bucket" rotor types for the centrifugation steps.

The FIA described above for the detection of antibodies to *A. viscosus* can be modified to detect *A. viscosus* surface antigens by the incorporation of a preincubation step of antigen with a standardized amount of Ra-a-19246 for 30 min at room temperature. After centrifugation ($1,256 \times g$ for 5 min) to remove whole cells if they were used as inhibitors, the mixture was added to a standardized pellet of *A. viscosus*, and the FIA was completed as described above. A fluorescence control was included with buffer instead of antigen in the preincubation step. Inhibition was calculated as follows: percent inhibition = $(1 - \text{experimental fluorescence/control fluorescence}) \times 100$.

Fluorescence measurements. Most fluorescence measurements were made on an unmodified MKI spectrofluorometer (Farrand Optical Co., Inc., Valhalla, N.Y.) and recorded as measurements of photocurrent in nanoamperes. Some readings were also made by replacing the photomultiplier of the MKI with a model 1140 quantum photometer (Princeton Applied Research Corp., Princeton, N.J.) and R212 photomultiplier tube (Hamamatsu Corp., Middlesex, N.J.) and measuring fluorescence as photon-counting rate in the digital mode (at root mean square percentage of deviation of 1.3%) or as nanoamperes in the

electrometer mode. Crossed film polarizers (Farrand Optical Co.) were used in most cases in an attempt to minimize interference from light scatter (5, 11). However, comparison studies not detailed here showed little or no effect of polarizers on experimental-control fluorescence ratios under the optimum conditions described below. Similarly, comparison studies of the effect of slit width on experimental-control fluorescence ratios (21) showed little advantage in the use of narrow slits. Accordingly, 10-nm band pass slits were used for most of the experiments described below. Spectra were obtained by scanning the analyzer monochromator at 20 nm/min and recording fluorescence on a SR-255B strip chart recorder (Heath Co., Benton Harbor, Mich.).

RESULTS

Stability of Fl-Go-a-RaG-labeled bacterial suspensions. To determine optimum conditions for the FIA, it was necessary to determine excitation and emission spectra with labeled microorganisms. A suspension of *A. viscosus* was reacted with Ra-a-19246 (1:100 dilution) and Fl-Go-a-RaG (1:80 dilution) in the standard FIA procedure. Fluorescence emission was excited at 485 nm and analyzed at 525 nm. As shown in Fig. 1, fluorescence of the cell suspension was virtually constant over 10 min of continuous observation. This suggests that neither quenching of fluorescence of the fluorophore nor settling of the organisms was significant over a time period longer than that necessary to obtain the spectra shown below. Stability of the fluorescence was seen with or without crossed polarizers.

Choice of optimum excitation wavelength of Fl-Go-a-RaG-labeled *A. viscosus*. Emission spectra were obtained for a suspension of labeled organisms prepared as described in the "stability" studies, with excitation at two

FIG. 1. Stability of Fl-Go-a-RaG-labeled *A. viscosus*. Excitation was at 485 nm, and emission was analyzed at 525 nm with crossed polarizers.

different wavelengths. As Fig. 2A shows, excitation at 495 nm gave slightly more fluorescence signal than at 520 nm (the emission maximum of fluorescein [8]), but the peak was poorly resolved from the large scatter signal centered at 495 nm. In contrast, excitation at 485 nm (Fig. 2B) permitted resolution of the fluorescence signal from scatter, albeit with some apparent loss of fluorescence intensity. We felt that this small loss of intensity was minimal compared with the relative absence of scatter interference and for this reason chose 485 nm as our standard excitation wavelength for the studies described below. The use of crossed polarizers gave no better resolution of the fluorescence peak when excitation was at 495 nm.

Choice of optimum emission wavelength of Fl-Go-a-RaG-labeled *A. viscosus*. An emission spectrum was obtained for a suspension of labeled organisms prepared as described in the

stability studies with excitation at 485 nm. As Fig. 3A shows, a strong fluorescence signal with a maximum at about 520 nm was obtained. A control was treated in exactly the same way except that pooled normal rabbit serum rather than Ra-a-19246 was used in the initial sensitization step. Figure 3B shows that this control has low fluorescence in the 520-nm region. Controls in which no Fl-Go-a-RaG was present or in which PB was substituted for Ra-a-19246 and Fl-Go-a-RaG showed virtually no fluorescence at 520 nm (Fig. 3C). At 525 nm, some loss of fluorescence intensity occurred with the fluorescent-labeled organisms. However, we chose to use this wavelength to analyze emitted fluorescence so as to further minimize possible light scattering problems with little concomitant loss of sensitivity.

Determination of optimum amount of Fl-Go-a-RaG. Pellets of 10^8 CFU of *A. viscosus* were reacted with Ra-a-19246 (1:100 dilution) and various dilutions of Fl-Go-a-RaG in the standard FIA procedure. As Fig. 4 shows, the Ra-a-19246 bound to the *A. viscosus* was effectively saturated by Fl-Go-a-RaG at dilutions less than 1:80. At the 1:5 dilution, nonspecific uptake of Fl-Go-a-RaG appeared to occur on control as well as sensitized cells; this resulted in some decrease in the experimental-control fluorescence ratio. At dilutions of the Fl-Go-a-RaG greater than 1:80, the experimental-control fluorescence ratio also fell as the amount of Fl-Go-a-RaG became limiting.

Titration of rabbit antibodies to *A. viscosus* surface antigens by FIA. Pellets of 10^8 CFU of *A. viscosus* were reacted with dilutions of Ra-a-19246 or dilutions of pooled normal rab-

FIG. 2. Effect of excitation wavelength on emission spectrum. (A) Excitation at 495 nm. (B) Excitation at 485 nm. Both spectra were obtained without polarizers at a scan rate of 20 nm/min.

FIG. 3. Emission spectra: (A) *A. viscosus* reacted with Ra-a-19246 and Fl-Go-a-RaG, (B) *A. viscosus* reacted with normal rabbit serum and Fl-Go-a-RaG, (C) *A. viscosus* reacted with buffer or Ra-a-19246. All spectra obtained with crossed polarizers at a scan rate of 20 nm/min. Excitation was at 485 nm for all spectra.

bit serum and FI-Go-a-RaG (1:80). Figure 5 shows that the bacterial suspension appeared to be saturated with antibody at dilutions of 1:80 or less of the Ra-a-19246. At higher dilutions, the amount of specific fluorescence dropped rapidly; this resulted in a decrease in experimental-control fluorescence ratios.

Sensitivity of fluorescence immunoassay for the detection of *A. viscosus*. Pellets of 4×10^8 CFU of *A. viscosus* were reacted with Ra-a-19246 (1:40 dilution) or normal rabbit serum (1:40 dilution) and FI-Go-a-RaG (1:20 dilution) in the standard FIA procedure. The final pellet was resuspended to 4 ml in PB to give a cell concentration of 10^8 CFU per ml. Serial twofold dilutions of the mixture were made in PB, and fluorescence was determined. Figure 6 shows

FIG. 4. Dose response of fluorescence of antibody-coated and control *A. viscosus* cells to varying amounts of FI-Go-a-RaG. Excitation was at 485 nm; emission was analyzed at 525 nm with crossed polarizers. The numbers in parentheses indicate the ratio of experimental to control fluorescence for each point.

FIG. 5. Dose response of fluorescence to varying amounts of Ra-a-19246 or normal rabbit serum in the standard FIA (excess FI-Go-a-RaG). The numbers in parentheses indicate the ratio of experimental to control fluorescence for each experimental point.

FIG. 6. Dose response of fluorescence of dilutions of *A. viscosus* cells treated with either Ra-a-19246-FI-Go-a-RaG or normal rabbit serum-FI-Go-a-RaG. The numbers in parentheses indicate the ratio of experimental to control fluorescence for each experimental point.

that the fluorescence intensity of the normal rabbit serum-treated cells declined rapidly to values approximating those seen with only buffer in the cuvette. Cells treated with Ra-a-19246 showed specific fluorescence as determined by experimental-control fluorescence ratios of more than one at all concentrations examined ($\geq 10^5$ CFU/ml). The experimental-control fluorescence ratio dropped at higher dilutions as the fluorescence-specific signal began to approach the limiting value of the buffer alone. This fact, therefore, limited the ultimate sensitivity of the assay to values significantly above those of appropriate control tubes.

Inhibition of the FIA by particulate antigens. Inhibition of specific immunofluorescence of *A. viscosus* was tested with either intact *A. viscosus* ATCC 19246 or the unrelated gram-positive rod *L. acidophilus* ATCC 4356. Varying amounts of inhibitor bacterial suspension were incubated with Ra-a-19246 (1:100 dilution, 300 μ l) for 30 min at room temperature with intermittent mixing. Removal of the inhibitor sus-

pension by centrifugation ($1,256 \times g$ for 5 min) permitted testing of the reaction mixture supernatant fluids for residual antibody in the standard FIA procedure. Ra-a-19246 at the same dilution without inhibitor was tested to give a control fluorescence value (no antibody bound by inhibitor). Figure 7 shows that fluorescence inhibition by intact *A. viscosus* diminished as the number of organisms in the inhibition mixture diminished. About 12.5×10^6 CFU were required for minimal detectable inhibition. *L. acidophilus* was entirely unreactive as an inhibitor.

Inhibition of the FIA by soluble antigens. The soluble antigen preparation (800 $\mu\text{g}/\text{ml}$) derived from *A. viscosus* ATCC 19246 was serially diluted in PB in 150- μl amounts and incubated with Ra-a-19246 (1:100 dilution, 150 μl) for 30 min at room temperature. The mixture was then added to a pellet of 10^8 CFU of *A. viscosus*, and residual antibody was determined in the standard FIA procedure. Figure 8 demonstrates that inhibition by soluble antigen could be readily demonstrated, although the amount of antigen tested was not sufficient for complete inhibition.

DISCUSSION

The use of fluorescent labeling techniques to detect antibodies to, and surface antigens of, microorganisms is a widely accepted practice in microbiology (6). However, these methods are generally used in qualitative determinations and suffer from a number of problems emphasized by the Automation in the Medical Laboratory Sciences Review Committee of the National Institute of General Medical Sciences (16). These problems include: (i) a lack of uniformity in

FIG. 8. Inhibition of fluorescence of the standard FIA by a soluble antigen preparation derived from *A. viscosus*.

sample preparations; (ii) instability of reagents during use; (iii) elimination of stray or scattered light in both excitation and emitted light; (iv) a need for better control of antibody specificity and avidity; and (v) a lack of standard reference materials for the calibration of fluorescence-measuring systems.

Recent developments with solid state FIA (3) suggested to us that these problems might be avoided to some degree by the use of spectrofluorometry rather than the fluorescence microscope as a detection system. Our FIA uses fluorescein-labeled antiglobulin as a reagent to detect antibodies bound to a suspension of *A. viscosus*. We have found this procedure to be a rapid one and to be readily reproducible if proper precautions are taken during the centrifugation steps to eliminate losses during washing. The use of cell suspensions for fluorescence measurements also has the advantage that reaction conditions are standardized insofar as is experimentally possible for every cell in the mixture. Observational difficulties derived from variations in smear thickness in conventional slide methods, therefore, are eliminated.

Our studies have also shown that quenching of fluorescence over reasonable observational periods is not a problem under the conditions of monochromatic excitation employed here; this contrasts with the observations of others with conventional fluorescence microscope techniques. These differences might be attributed to photodecomposition reactions (20) that might become more significant under the conditions of greater photon flux used in the usual conditions of excitation in fluorescence microscopy. The

FIG. 7. Inhibition of fluorescence of the standard FIA by intact *A. viscosus* or *L. acidophilus* organisms.

use of monochromated light appears to minimize such problems by the elimination of most light at extraneous wavelengths. There appears to be no appreciable settling of organisms over the observational periods used in this study.

Because stray and scattered light can present major problems in the fluorescence measurement of particulate systems (5, 11, 21), we attempted to minimize these interferences by careful selection of excitation and analyzer wavelengths. The final values chosen were 485 nm for excitation and 525 nm for emission. Whereas these values were offset from "optimal" values with some resulting loss in sensitivity, we found that these conditions appeared to effectively minimize scatter problems. The need for crossed polarizers or narrow slits in fluorescence determinations of these bacterial suspensions was thus eliminated. Because of these facts, conventional filter fluorimeters may be adequate for the FIA described here; however, we have made no such instrument comparisons. Such fluorimeters have already been demonstrated to work in other FIA systems (3).

We have found that a commercial preparation of Fl-Go-a-RaG functions well as detection reagent in our FIA; however, at low dilutions ($\leq 1:5$), this reagent shows immunologically non-specific uptake on both antibody-treated or control organisms. This uptake may reflect the presence of natural antibodies in the Fl-Go-a-RaG specific to *A. viscosus* or "nonspecific" events of an undefined nature. We have made no attempts to distinguish these possibilities because dilutions of the detection reagent can be used to saturate the bound immunoglobulin where non-specific binding is not significant.

The FIA procedure we describe here for the detection of antibody to *A. viscosus* appears to be as sensitive as direct fluorescent microscopic examination of antibody-coated organisms in terms of titers of reagents necessary to give positive results (T.P. Gillis, M.S. thesis, Louisiana State University School of Graduate Studies, New Orleans, 1976). However, the FIA we describe here has the significant advantage that the presence or absence of fluorescence and its relative amount are determined instrumentally without any need to resort to complicated verification procedures in order to standardize observations between different individuals (7, 13).

We have further shown that our FIA procedure can be inhibited by either soluble or particulate antigen preparations. Accordingly, this FIA procedure can be standardized on the basis of standard curves relating the amount of immunoglobulin bound to fluorescence with a standard lot of fluoresceinated antiglobulin or in

terms of the amount of (stable) antigen necessary to inhibit a previously standardized antibody system to a given degree (12). Alternatively, a standardized fluorescein preparation could be used to establish a concentration standard curve and specific fluorescence in experimental assays related to this standard. Variable quenching effects (due to self-absorption) and light-scattering effects would make the fluorescence values seen in experimental assays apparent rather than absolute, but, in any case, problems with variations between instruments and variations in instrument sensitivity due to variations in lamp output with time or electronic drifts would be minimized (21). The use of standard curves in other FIAs (3) is routine.

Under the conditions of the standard FIA described here, suspensions of *A. viscosus* on the order of 10^5 CFU/ml can be detected directly (Fig. 6). An alternative to the direct determination of organism by the FIA method would be the inhibition of fluorescence of a standardized bacterial system by an unknown sample. The sensitivity of this procedure, however, is determined by the concentration of the standardized bacterial suspension used. As shown in Fig. 7, when 10^8 CFU of *A. viscosus* are used in the standard FIA system, the minimum detectable level of inhibitory *A. viscosus* is on the order of 1.25×10^7 CFU/ml, about 100 times less sensitive than the direct assay. This apparent difference in sensitivity is largely artificial, however, because if 10^7 or 10^6 CFU had been used in the standardized suspension, one would expect the detection limits to be correspondingly lowered.

In conclusion, the FIA system we have described here provides a rapid, sensitive method to quantitate antibodies to, and surface antigens of, *A. viscosus*. The method appears to be a general one and eliminates some of the problems associated with conventional fluorescence microscopic techniques. This FIA system may readily be applied to immunological inhibition studies.

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