Double-Label Fluorescence Immunoassay of Bacteria

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Fluorescence of sensitized bacterial suspensions, reactive with either fluorescein-labeled or rhodamine-labeled antiglobulins, could be quantitatively distinguished in dual-labeled preparations by fluorescence immunoassay.

Antiglobulins differentially labeled with fluorescein or rhodamine have been widely used to identify antigenically distinct bacterial or mammalian cells by microscopic techniques in a single experimental sample (3, 5). The potential applicability of this methodology to the simultaneous determination of different organisms or serotypes of organisms from single dental plaque samples is especially apparent (1, 6, 8). In this note, we show that the fluorescence of specifically sensitized bacterial suspensions reactive with either fluorescein-labeled or rhodamine-labeled antiglobulins can be quantitatively distinguished in dual-labeled preparations by fluorescence immunoassay.

Fluorescein isothiocyanate- and tetramethylrhodamine isothiocyanate-conjugated goat antisera to rabbit immunoglobulin G (Cappel Laboratories, Inc., Cochranville, Pa.) were used to measure binding of antibody to *Actinomyces viscosus* suspensions by fluorescence immunoassay (4). Two 1-ml portions of a stock bacterial suspension (absorbance at 660 nm = 0.32, about 10^6 colony-forming units per ml) were pelleted by centrifugation ($1,265 \times g$ for 5 min) in test tubes (13 by 100 mm). The pellets were resuspended in 600 μ l of rabbit antibody to *A. viscosus* (1:100) prepared as previously described (4). After reaction for 30 min at room temperature with frequent mixing, the cell preparations were washed three times (2 ml per wash) in phosphate-buffered saline by centrifugation ($1,265 \times g$ for 5 min). One of the washed cell pellets was resuspended in 600 μ l of fluorescein-conjugated antiglobulin (1:80) and the other in 600 μ l of rhodamine-conjugated antiglobulin (1:20). Preliminary experiments had demonstrated that the respective amounts of fluorochrome-conjugated reagents used were sufficient to saturate the number of sensitized *A. viscosus* used in these suspensions. The labeled bacterial suspensions were then washed three times with phosphate-buffered saline (2 ml per wash) by centrifugation ($1,265 \times g$ for 5 min) and diluted to a final volume of 2 ml in phosphate-buffered

saline. Unlabeled bacterial cells were similarly washed and diluted in phosphate-buffered saline to a final concentration equivalent to that of the labeled cells. Portions of these cell preparations were then mixed to give varying proportions of fluorochrome-labeled cells to unlabeled cells or fluorescein-labeled cells to rhodamine-labeled cells. Each final mixture of cells contained the same concentration of cells as the starting suspensions, in a volume of 400 μ l. Fluorescence was measured as previously described (4), with a Farrand MK I spectrofluorometer (Farrand Optical, Co., Inc., Valhalla, N.Y.) modified to accept a model 1140 quantum photometer (Princeton Applied Research Corp., Princeton, N.J.), except that a 300- μ l semimicro-cuvette assembly (Farrand Optical Co.) was used for all readings. Band widths for both excitation and emission were fixed at 10 nm by appropriate slits.

Data were fitted by linear least-squares analysis. Variances associated with the calculated slopes and intercepts (2) were compared by the *F* test. When variances were not significantly different, *t* tests were used to determine statistical significance. The data from which these comparisons were made are presented in Table 1 and in Fig. 1 and 2.

As shown in Fig. 1, when fluorescein-labeled organisms were mixed in varying proportions with unlabeled organisms and measured under spectroscopic conditions specific for fluorescein, a linear dose response was seen (correlation coefficient = 1.00, Table 1). If the unlabeled organisms were replaced with an equal quantity of rhodamine-labeled organisms, the dose response was unaffected (correlation coefficient = 0.99). Conversely, when rhodamine-labeled organisms were mixed in varying proportions with either unlabeled organisms or fluorescein-labeled organisms (Fig. 2) and measured under conditions specific for rhodamine, a linear fluorescence dose response resulted (correlation coefficients = 1.00 and 1.00, respectively). As shown in Table 1, the slopes of the dose-response curves for detection of either fluorescein- or rhodamine-labeled or-

TABLE 1. Parameters of fluorescein- and rhodamine-sensitized bacterial emission data

Assay ^a	Calculated parameter			
	Correlation coefficient ^b	Slope (count/s per % labeled cells $\pm$ 1 SD ^c)	Coefficient of variation of slope (%)	Y-intercept (count/s $\pm$ 1 SD ^c)
Fluorescein emission (λ_E = 485 nm; λ_A = 525 nm)				
F + unlabeled	1.00	2,620 $\pm$ 105	4.01	-4,790 $\pm$ 6,430
F + R	0.99	2,620 $\pm$ 140	5.36	-9,230 $\pm$ 8,930
Rhodamine emission (λ_E = 535 nm; λ_A = 580 nm)				
R + unlabeled	1.00	220 $\pm$ 7.02	3.20	-419 $\pm$ 430
R + F	1.00	213 $\pm$ 5.02	2.35	-311 $\pm$ 307

^a λ_E , Excitation wavelength; λ_A , wavelength at which emission was analyzed. F, Fluorescein labeled; R, rhodamine labeled.

^b Fluorescence and percentage of labeled cells.

^c SD, Standard deviation.

FIG. 1. Fluorescence dose response of fluorescein-labeled organisms in the presence of rhodamine-labeled organisms (●) or of unlabeled organisms (○). Fluorescence was excited at 485 nm, and emission was analyzed at 525 nm with crossed polarizers. Background measured under these conditions with unlabeled organisms ($4.5 \text{ counts/s} \times 10^3$ [Kcps]) was subtracted from each experimental observation.

ganisms were not significantly different when the fluorescent organisms were mixed with either unlabeled organisms or organisms labeled with the other fluorophore ($P > 0.10$ for all relevant pair comparisons; t tests). Similarly, all Y-intercepts calculated from the least-squares analysis did not vary significantly from 0 ($P > 0.30$ for all relevant comparisons; t test). Therefore, under the spectroscopic conditions used for the dual-labeled suspensions, resonance energy

FIG. 2. Fluorescence dose response of rhodamine-labeled organisms in the presence of fluorescein-labeled organisms (●) or of unlabeled organisms (○). Fluorescence was excited at 535 nm, and emission was analyzed at 580 nm with crossed polarizers. Fluorescence background measured under these conditions with unlabeled organisms ($1.65 \text{ counts/s} \times 10^3$ [Kcps]) was subtracted from each experimental observation.

transfer from fluorescein to rhodamine (9) was not significant, and quantitative fluorescence immunoassay of fluorescein- and rhodamine-labeled suspensions was feasible.

When the ratios of the fluorescence dose-response slopes for fluorescein- and rhodamine-labeled organisms with unlabeled organisms were compared, the rhodamine assay was 11.9 ± 0.61 (1 standard deviation) times less sensitive than the fluorescein assay. We have not yet determined whether this apparent inefficiency was the result of the particular rhodamine-con-

jugated antiglobulin reagent used, the relatively inefficient response of our photomultiplier tube at the wavelengths used, or other factors. It should be noted, however, that the lack of sensitivity of the rhodamine assay did not appear to affect its precision (7), as assessed by the coefficient of variation of the slope of the fluorescence dose-response curves. In fact, the rhodamine assay was more precise than the fluorescein test (coefficient of variation of the slope was 2 to 3% for rhodamine, as compared to 4 to 5% for fluorescein, Table 1). More experimentation would be required to statistically verify this conclusion.

A final important consideration in the use of fluorescence immunoassay methods in bacterial systems is the magnitude of fluorescence-specific signal with respect to background. The data shown in Table 1 and Fig. 1 and 2 were all corrected by subtraction of the background contribution of cells in buffer without any fluorescence detection reagent; the remaining net counts therefore reflect only the fluorescence-specific signal. If one arbitrarily defined an uncorrected signal that is two times more than background as being significantly different and calculated the corresponding percentage of labeled cells from the regression equations for mixtures with unlabeled cells derived in Table 1, then the rhodamine system could be used to measure samples with about 9.4% labeled cells, whereas the fluorescein system would detect as little as 3.6% labeled cells. These detection limits would be correspondingly lowered if more favorable assumptions were made on the nature of a "significant" difference, i.e., if precision of the readings were high.

In conclusion, this simple model system demonstrated that simultaneous quantitative fluorescence immunoassay with fluorescein- and rhodamine-conjugated reagents is feasible. Un-

der the spectroscopic conditions described, interference of one fluorophore with the other appeared negligible. Sensitivity limits were determined by background considerations and the precision of the fluorescence readings.

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