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Rapid Identification and Quantitation of Small Numbers of Microorganisms by a Chemiluminescent Immunoreaction

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A method (patent pending) for rapidly identifying and quantitating small numbers of microorganisms was developed based on the specific immunoreaction of microorganisms with homologous antibodies linked by conjugation to peroxidase. The high sensitivity of the method is due to the use of a chemiluminescent reaction for the determination of the enzyme. The reaction was performed on Alcar supports with low nonspecific adsorption. The very low noise achieved permitted the detection of as few as 30 to 300 bacterial cells.

The need for a method of rapid identification of small amounts of microorganisms poses a problem in both the medical and the environmental fields. The immunological methods are the most sensitive and specific in actual use, and the immunoenzymatic methods are gaining increased importance. The most commonly used enzymes in those assays are β-galactosidase, alkaline phosphatase, and peroxidase, and in all cases final detection is based on direct color comparison or spectrophotometric determination after enzymatic action on suitable substrates (4, 5, 7).

A more sensitive method for the quantitation of peroxidase is based on the determination of photons emitted during the oxidation of pyrogallol with hydrogen peroxide (1).

This paper describes an immunoenzymatic assay for bacterial determination using a chemiluminescent method for quantitation of peroxidase-labeled antibody bound to *Serratia marcescens* test organisms.

MATERIALS AND METHODS

Chemicals used. Chemicals used were: pyrogallol, perhydrol, NaCl, and phosphates (Merck); peroxidase RZ-2.6, tris(hydroxymethyl)aminomethane, and Tween 20 (Sigma); Aclar 33C, a chlorotrifluoroethylene polymer (Allied Chemicals International); diethylaminoethyl-cellulose (Bio-Rad); protein A (Pharmacia); anti-rabbit immunoglobulin (goat) and anti-rabbit immunoglobulin-peroxidase conjugate (Miles Yeda); nutrient broth, nutrient agar, and tryptone (Difco); RH_{363} and ST_{334} (gift from H. Frenckel, Agricultural Research Institute, Beit-Dagon, Israel).

Preadsorption solution. The preadsorption solution contained 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.6, in saline–normal goat serum–*Salmonella* supernatant (see below) in the proportion 10:2:4.

***Salmonella* supernatant.** *Salmonella* sp. (Dublin) was grown overnight in nutrient broth at 37°C in a rotatory shaker (New Brunswick, 150 rpm). The bacteria were separated by centrifugation, suspended in 1.5 growth volumes of saline, and centrifuged. The supernatant obtained was used in the preparation of the preadsorption solution.

S. marcescens. *S. marcescens* was grown overnight at 28°C on slants prepared with 2% tryptone, 0.5% NaCl, and 1.5% nutrient agar. Bacteria were suspended in saline and brought to an optical density of 0.600 at 500 nm. This suspension contained 3×10^8 colony-forming units per ml (determined on nutrient agar) and 4.5×10^8 cells per ml (determined in a Petroff-Hausser counting chamber). Subsequent dilutions were performed in saline containing 20 μg of the wetting agent RH_{363} per ml.

Anti-*Serratia* immunoglobulin. Rabbits were inoculated by four weekly intravenous injections of 1 ml of increasing concentrations (5×10^6 to 10^8 cells) of *S. marcescens*. Two weeks after the last injection, the animals were bled, the serum was separated, and the immunoglobulin was purified by chromatography on diethylaminoethyl-cellulose equilibrated with 0.03 M phosphate, pH 8.6 (10).

Conjugation of immunoglobulin with peroxidase. Conjugation of 10 mg of peroxidase to 5 mg of immunoglobulin was performed according to Avrameas and Ternynck (2), but the remaining free peroxidase was not separated from the conjugate. The final volume obtained was 1.5 ml. Before use, the preparation was kept for at least 1 week at 4°C.

Protein A-peroxidase conjugate. Protein A-peroxidase conjugate was prepared according to Avrameas and Ternynck (2), and peroxidase-antiperoxidase soluble complex was prepared according to Sternberger et al. (8).

Fixation of bacteria on Aclar strips. A series of 1-μl portions of saline containing 20 μg of RH_{363} per ml and increasing concentrations of bacteria (3×10^6, 10^7, 3×10^7, 10^8, and 3×10^8 cells per ml) were dropped at 7-mm intervals on an Aclar 33C strip (4 by 45 mm). The suspensions were quickly dried by a temperature gradient; the Aclar strip in a petri dish was put over a beaker containing hot water, and the cover was cooled with solidified CO_2. When the strip was dry, a 5-μl drop of methanol was added to each spot and left to evaporate.

Immunoreaction. The Aclar strip with the fixed bacteria was immersed into a 1-ml test tube containing the preadsorption solution for 5 min and then transferred into another 1-ml test tube containing 20 μl of conjugate diluted with 1 ml of preadsorption solution (immunoreaction solution). After 20 min at room temperature, the strip was removed and washed three times with 1 ml of 0.005 M tris(hydroxymethyl)aminomethane, pH 7.6, in saline.

Determination of peroxidase. The strip was cut in portions corresponding to each bacterial spot, each piece was introduced into a reaction cuvette, and 50 μl of 0.2% pyrogallol in 0.18 M phosphate buffer, pH 6.5, was added. Each cuvette was introduced into a 760 Luminescence Biometer (DuPont Instruments), and the reaction was started by injection of 50 μl of 0.37% H_2O_2 in 0.18 M phosphate buffer, pH 6.5. The light emitted was determined by repeated measurements at 6-s intervals up to maximal value. The sensitivity was set at 8.0 and the minimal exponent value was set at 3. Also, the time course of light emission was recorded (Varian F80).

RESULTS

The conjugation of peroxidase to immunoglobulin did not affect the ability of the enzyme to induce light emission upon reaction with pyrogallol and H_2O_2. Light emission increased with the square of the enzyme concentration; i.e., double concentration of enzyme produced four times the amount of light, etc. (Fig. 1). The time course of light emission depended on the concentration of enzyme present (Fig. 2), higher enzyme concentrations reaching maximal light emission values in shorter times.

The best technical conditions that we found for the bacterial determination are those described in Materials and Methods. Omission of the preadsorption step resulted in a high blank value. The *Salmonella* supernatant component

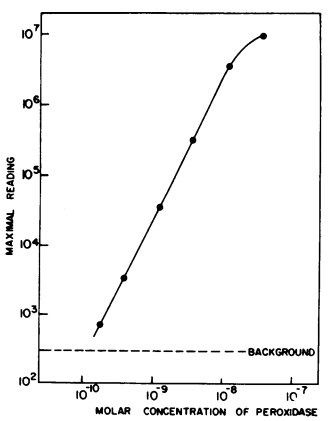

FIG. 1. *Titration of peroxidase by chemiluminescence, performed as described in the text using 5 μl of peroxidase solution instead of the strip with bacteria.*

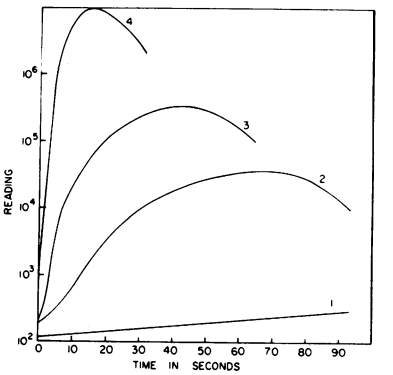

FIG. 2. *Time course of light emission. Peroxidase used: (1) none, (2) 5 × 10^{-9} g, (3) 1.5 × 10^{-8} g, (4) 1.5 × 10^{-7} g.*

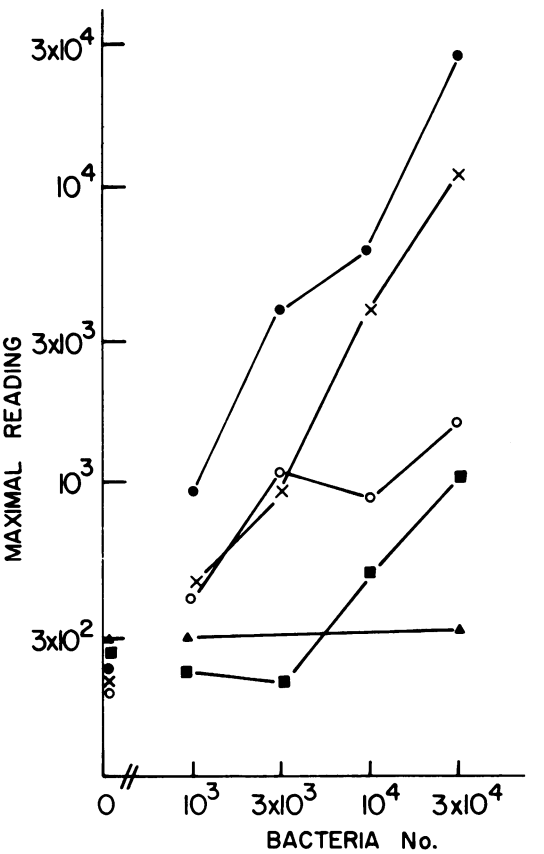

FIG. 3. *Titration of S. marcescens with increasing amounts of conjugate. Conjugate added to 1 ml of immunoreaction solution: (▲) 3 μl, (■) 5 μl, (○) 10 μl, (×) 15 μl, (●) 30 μl.*

of the preadsorption solution was found when testing *Salmonella* as the "wrong bacteria" for anti-*Serratia*-peroxidase conjugate. The values obtained with *Salmonella* were consistently

lower than those without any bacteria. Dilution of the *Salmonella* and, later, the use of supernatant prepared as described in Materials and Methods brought us to the desired low background. Characterization of the active component, and whether it acts on the conjugate or on the Aclar sheet, is under study.

Purification of the conjugate by ammonium sulfate precipitation, gel filtration, or ultracentrifugation did not increase the sensitivity of the test, probably due to the loss of conjugate by adsorption on the glassware, gels, tubes, etc., used in these processes. For this reason, analysis by ultracentrifugation on a sucrose gradient was not conclusive. It showed that only 10 to 20% of the immunoglobulin was conjugated to peroxidase, but this figure was probably too low.

At high concentrations of conjugate (1:3), 10 to 30 bacteria consistently showed signals above the control, without bacteria, but quantitation in this range is difficult. Normally, the conjugate prepared as described was used in dilutions of 1:75 to 1:30. At lower concentrations, the conjugate was completely adsorbed to the glass test tube (Fig. 3). A conjugate solution diluted 1:50 (20 μl/ml) could be repeatedly used without loss of activity (Fig. 4).

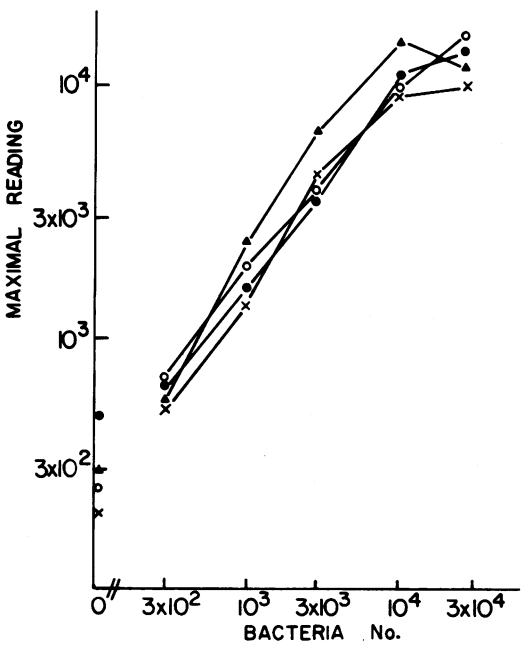

FIG. 4. *Repeated use of same conjugate solution. Eight strips, as described in the text, were incubated for 20 min, one after the other, in the same immunoreaction solution containing 20 μl of conjugate. Other details were as in the text. Symbols:* (○) *first strip,* (●) *third strip,* (▲) *fifth strip,* (×) *eighth strip.*

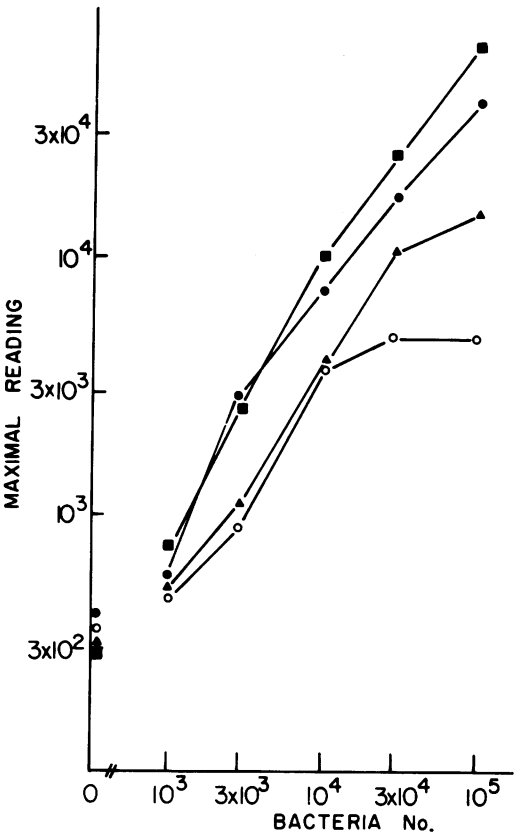

FIG. 5. *Influence of wetting agents in bacterial suspensions. Bacteria were suspended in saline* (○) *or saline containing the following substances:* (▲) *Tween 20 (100 μg/ml),* (●) RH_{363} (100 *μg/ml),* (■) ST_{334} (100 *μg/ml).*

The reaction between the conjugate and the bacteria was mostly over in 10 min at room temperature (20°C), but shorter periods of reaction may be used if the number of bacteria expected is over 10^3 to 10^4. Incubation for longer than 10 min did not improve the results. The optimal temperature for the immunoreaction was around 20°C, although there was no great difference between 17 and 37°C.

When a drop of bacterial suspension is dried by evaporation on a solid surface, most cells are found in a thin ring on the periphery of the drop, as seen with a microscope. By the use of wetting agents, we were able to obtain a better distribution of the bacteria on the Aclar strip and improve the results (Fig. 5). We chose to use RH_{363} for our routine work. Best results were obtained with 20 μg of this compound per ml. Double-layer techniques, with antibacteria serum and peroxidase attached to either anti-rabbit immunoglobulin (goat) or staphylococcal

protein A, and the unlabeled antibody-enzyme method described by Sternberger et al. (8) can be used with the chemiluminescent reaction. The sensitivity depends on the reagents used. We were able to detect 100 to 300 bacteria (Fig. 6).

Test with body fluids. *Serratia* suspensions were serially diluted in saline and urine from 10^6 to 10^8 cells per ml, and 1-μl portions were tested. Direct tests of bacteria in serum could not be performed because the dried serum proteins formed a crust that fell from the Aclar surface. Therefore, bacteria were diluted in serum (10^6 to 10^8 cells per ml), centrifuged, suspended in the same volume of saline, and tested. The results (Fig. 7) show that urine and serum did not interfere with the assay.

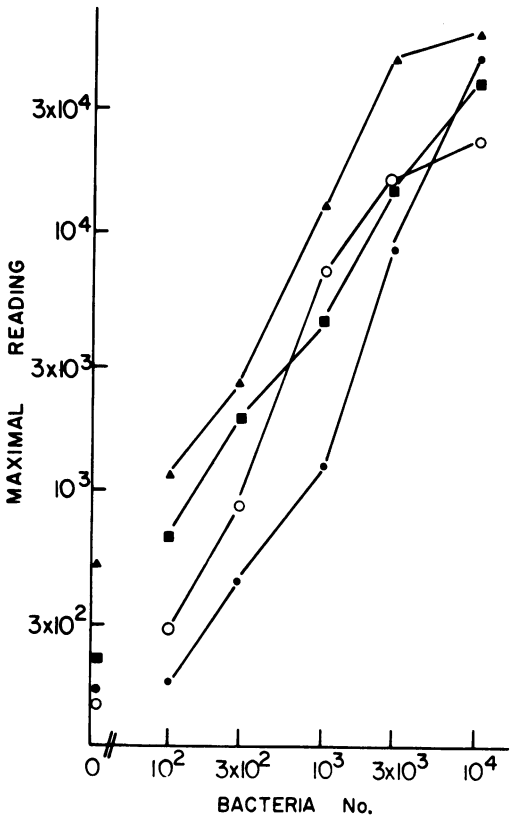

FIG. 6. *Determination of S. marcescens with double and triple immunoreactions. Bacteria were placed on an Alcar strip as in the text. All immunoreactions were performed at room temperature for 20 min. Preadsorption solution and washing were as in the text. Anti-S. marcescens serum, goat anti-rabbit, goat anti-rabbit-peroxidase conjugate, and protein A-peroxidase conjugate were used as 20 μl/ml of solution. Peroxidase-antiperoxidase soluble complex was used as 100 μg/ml. Symbols:* (▲) *single-layer, anti-S. marcescens-peroxidase conjugate;* (■) *double-layer, anti-S. marcescens rabbit serum followed by goat anti-rabbit-peroxidase conjugate;* (●) *double-layer, anti-S. marcescens rabbit serum followed by protein A-peroxidase conjugate;* (○) *triple layer, anti-S. marcescens rabbit serum followed sequentially by goat anti-rabbit immunoglobulin serum and by peroxidase-antiperoxidase soluble complex.*

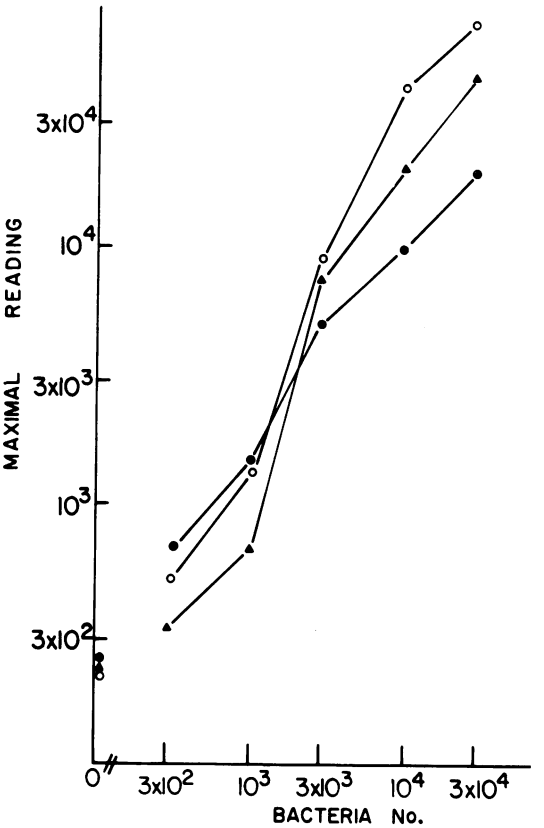

FIG. 7. *Test of S. marcescens diluted in urine and serum. Symbols:* (●) *bacteria diluted in saline,* (○) *bacteria diluted in urine,* (▲) *bacteria diluted in serum, centrifuged, and suspended in saline.*

DISCUSSION

The immunoreaction between antibacteria immunoglobulin-peroxidase conjugate and bacteria results in a very high concentration of the enzyme around the microorganisms. As seen, the amount of light emitted by the reaction of peroxidase with pyrogallol and hydrogen peroxide is proportional to the square of the enzyme concentration. This provides for a very high light emission by the bacteria antibacteria immunoglobulin-peroxidase complex in comparison with the amount of peroxidase present and, therefore, for a high sensitivity, which enables the detection of as few as 30 to 300 bacterial cells. The high concentration of antibacteria im-

munoglobulin that can be bound to the cell does not allow space for a further marked amplification in the number of immunoglobulin molecules by the use of double- and triple-layer methods (8). Since immunoreactions obey the mass law (6), a higher concentration of conjugate is needed to detect 30 cells than that needed to detect 3,000.

The leveling-off of light emission at high concentrations of peroxidase (see Fig. 1) seems to be due to self-absorption by the colored products formed by the reaction. Colored product formation is directly proportional to enzyme, and the production of colored products by the enzyme linked to up to 30,000 to 100,000 bacterial cells is negligible.

The applications of the method described are not confined to use in bacterial or viral determinations. A variety of immunoassays parallel those in actual use were tried with very good results, as, for instance, competitive assays similar to radioimmunoassays (in preparation).

The method described offers obvious advantages over methods based on immunoradiometry (3, 9). It is more sensitive and faster, and the shelf lives of the reagents are much longer. Preparations with ^{125}I, which is the most widely used isotope in immunoradiometry, have to be substituted every 4 to 6 weeks, whereas conjugates with peroxidase or peroxidase-antiperoxidase soluble complex are known for their long lifetimes (2, 8), and in our case preparations over 1 year old did not show appreciable loss of activity.

The assays are adaptable to automation and can be performed in any laboratory without extra precautions, and, in contrast to work with radioactive isotopes, the reagents used are innocuous and no health or environmental hazards are involved.

In conclusion, the method described brings the advantageous enzyme-linked immunoassays into a much higher sensitivity range.

ACKNOWLEDGMENTS

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