Comparison of Fluorescent and Colorigenic Substrates for Enzyme Immunoassays

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A variety of substrates can be employed in enzyme immunoassays (EIAs) for the measurement of enzyme-labeled immunoreactants. We compared the sensitivities of a fluorescent and a colorigenic substrate in an EIA system for the measurement of *Haemophilus influenzae* purified polyribose phosphate. After a 10-min substrate incubation, the EIA in which the fluorescent substrate was used could detect 10 pg of polyribose phosphate per ml, whereas the EIA in which the colorigenic substrate was used required the addition of 640 pg of polyribose phosphate per ml to generate a positive reading. However, the use of longer substrate incubation periods led to an increase in sensitivity of the colorigenic EIA. After an incubation period of 240 min, the sensitivity was equal to that of the EIA in which the fluorescent substrate was used. These results suggest that the ultimate limit of sensitivity of EIA systems is determined by the nature of the antigen-antibody reactions. However, the use of high-energy substrates in EIA systems can allow for the attainment of maximal sensitivity after short enzyme-substrate incubation periods.

Enzyme immunoassays (EIAs) are attaining widespread usage for the measurement of antigens and antibodies in body fluids (10, 12, 14). EIA systems consist of a series of antigen-antibody reactions which result in the binding of enzyme-labeled immunoreactants to the solid phase (16). In most cases, the amount of bound enzyme is determined by the measurement of the concentration of visibly colored products generated by the enzyme-substrate reaction over a fixed period of time (13). The amount of enzyme-labeled immunoreactant which can be detected is thus limited by the detectability of colored substrate products. For this reason, a number of enzyme assays have been developed in which more sensitive substrate systems are used which can allow for the measurement of lower concentrations of substrate products and thus less enzyme-labeled immunoreactant (2, 6, 7). One such system makes use of a substrate which is converted to a highly fluorescent product on action with the enzyme (15). We compared the sensitivities of a fluorescent and a colorigenic substrate in the EIA for the measurement of *Haemophilus influenzae* polyribose phosphate (8).

MATERIALS AND METHODS

Purified polyribose phosphate (PRP) was obtained from Porter Anderson, University of Rochester School of Medicine, Rochester, N.Y. Burro anti-PRP was obtained from John Robbins, Bureau of Biologi-

cals, Bethesda, Md. Rabbit anti-PRP was purchased from the New York State Health Department. Goat anti-rabbit immunoglobulin G was obtained from Antibodies, Inc., Davis, Calif., and conjugated with alkaline phosphatase (type VII; Sigma Chemical Co., St. Louis, Mo.) by the method of Engvall and Perlmann (16). *p*-Nitrophenylphosphate, a colorigenic substrate, was purchased from Sigma Chemical Co. The fluorogenic substrate, 4-methylumbelliferyl phosphate, was purchased from Research Organics, Inc., Cleveland, Ohio.

The enzyme immunoassay for PRP was performed as previously described (8). Briefly, the wells of a polyvinyl microtitration plate (220-24; Dynatech Laboratories, Inc., Alexandria, Va.) was coated with burro anti-PRP diluted 1:10,000 in 0.06 M carbonate buffer, pH 9.6. After incubation overnight at 4°C, the plates were washed five times with phosphate-buffered saline (pH 7.4) containing 0.5% Tween 20 (PBST), and PRP diluted in fourfold steps, starting at 2.5 ng/ml, in PBST containing 0.5% gelatin and 0.5% normal horse serum was added to quadruplicate wells. After incubation for 1 h at 37°C, the plates were washed five times with PBST, and rabbit anti-PRP, diluted 1:1,000 in PBST, was added. After incubation for 1 h at 37°C, the plates were again washed, and alkaline phosphatase-labeled anti-rabbit immunoglobulin G, diluted 1:800 in PBST, was added. After another incubation for 1 h at 37°C, the plates were washed and substrate was added. In the case of the colorigenic substrate, *p*-nitrophenylphosphate was added at a concentration of 10^{-3} M in diethanolamine buffer, pH 9.6. In the case of the fluorogenic substrate, 4-methylumbelliferyl phosphate diluted to a concentration of 10^{-4} M in diethanolamine buffer was added. A higher concentration of fluores-

FIG. 1. Colorimetric and fluorescent substrates for EIA for the measurement of *H. influenzae* polyribose phosphate (PrP). The reactivity of *H. influenzae* antigen in EIA systems with varying substrate incubation times is shown. The colorimetric reactions are expressed in optical density units, and the fluorescent reactions are expressed in arbitrary fluorescent units (1 ml of 4-methylumbelliferone at a concentration of 10^{-9} mol/liter = 3 units). Vertical bars represent the standard error of the determinations. Points followed by an asterisk differ from this negative control value to a statistically significant extent ($P < 0.05$). Symbols: $\blacklozenge$, colorimetric substrate, 10-min incubation; ∇ , colorimetric substrate, 30-min incubation; $\bullet$, colorimetric substrate, 60-min incubation; $\square$, colorimetric substrate, 240-min incubation; $\blacktriangle$, fluorescent substrate, 10-min incubation; $\circ$, fluorescent substrate, 30-min incubation.

cent substrate could not be utilized owing to the solubility characteristics of the substrate. The colorimetric reactions were measured in a microplate colorimeter after a suitable incubation of the substrate at 37°C. In the case of the fluorogenic substrates, the measurement was performed by transferring 100 μ l of substrate to borosilicate tubes (10 by 95 mm) containing 0.9 ml of 0.1 M Tris, pH 8.8, and measuring the fluorescence in a fluorometer (Aminco Fluorocolorimeter) with a 50-W lamp and the following filters: incidence filter; Corning 7-60 (maximum band passage, 360 nm); secondary filters, Wratten 2A and Kodak 149-40-46 (470-nm sharp cutoff). At least eight negative control wells were run in each determination containing buffer only. A dilution of PRP was considered to be positive if it yielded a value which was 2

standard deviations greater than the mean of the negative controls.

RESULTS

The sensitivity of the EIA systems for the measurement of PRP are shown in Fig. 1. With a substrate incubation time of 10 min, the EIA in which the colorimetric substrate was used could detect PRP at a concentration of 640 pg/ml (64 pg per well). On the other hand, at this substrate incubation time, the EIA in which the fluorogenic substrate was used could detect PRP at a concentration of 10 pg/ml (1 pg per well). However, the sensitivity of the EIA with the colorimetric substrate improved with an increase in the time period during which the substrate was incubated in the microtiter wells. Thus, this EIA could detect PRP at a concentration of 160 pg/ml after a 30-min incubation, 40 pg/ml after a 60-min incubation, and 10 pg/ml after a 240-min incubation. Incubation for longer periods of time led to higher optical density values for both the antigen and control wells but did not increase the sensitivity beyond 10 pg/ml. In the case of the fluorogenic substrate, incubation of the substrate for periods of greater than 10 min led to increased fluorescence levels in the wells containing PRP at concentrations of greater than or equal to 10 pg/ml. However, this prolonged incubation time did not increase the endpoint sensitivity of the system beyond the limit of 10 pg/ml. Thus, the minimum amount of PRP which could be detected by both the colorimetric and fluorescent substrates was 10 pg/ml (1 pg per well).

DISCUSSION

The sensitivity of EIA systems is determined by a number of factors, including the kinetics of the antigen-antibody reaction and the detectability of the enzyme-labeled immunoreactants (1, 6, 13). The latter factor is determined to a great extent by the ability to detect the substrate product formed by the action of the enzyme on the substrate. Thus, assuming equivalent enzyme-substrate kinetics, the use of a substrate which releases the fluorescent molecule, 4-methylumbelliferone, should lead to the detection of approximately 100-fold less enzyme than the use of a substrate consisting of the yellow-colored compound, *p*-nitrophenylphosphate, since the fluorescent substrate can be detected by spectrofluorometry in concentrations 100-fold less than can the colored product by spectrophotometric methods (5, 8, 11). Similar results would be obtained by the use of other high-energy substrates, such as ones yielding chemiluminescent or radioactive molecules on enzyme action (9). However, the sensitivity of EIA systems is also limited by the ability of the antigen in the specimen to specifically bind with

the antibody on the solid phase and the other immunoreactants. This interaction is determined by the law of mass action which dictates that, for each antigen-antibody reaction, the concentration of antigen-antibody complex formed is proportional to the concentration of antigen, the concentration of antibody, and the affinity constant of the antibody (4, 13). If the concentration of antigen in the specimen is less than that which can be specifically bound to the solid-phase antibody, enzyme-labeled immunoreactants will not be bound and will not be specifically measured, regardless of the concentration or energy level of the substrate product formed by the enzyme substrate system.

These principles are demonstrated by the comparative sensitivities of the fluorescent and colorimetric EIA systems for the measurement of polyribose phosphate, the polysaccharide component of the cell wall of *H. influenzae* type b. The EIA system in which alkaline phosphatase-labeled immunoreactants and the fluorescent substrate 4-methylumbelliferyl phosphate were used could measure 10 pg of antigen per ml after a substrate incubation period of 10 min. On the other hand, the EIA in which the colorigenic substrate *p*-nitrophenylphosphate was used required the addition of 640 pg of antigen per ml for measurable amounts of product to be generated after a 10-min substrate incubation. However, the sensitivity of the EIA in which the colorigenic substrate was used could be improved by utilizing longer incubation periods and hence generating larger quantities of substrate product. An incubation of the substrate for 240 min led to the measurement of 10 pg of antigen per ml. Incubation for longer periods of time did not lead to an increase in sensitivity. Similarly, an incubation of the fluorescent substrate for more than 10 min did not lead to the measurement of amounts of PRP of less than 10 pg/ml. Thus, it is likely that 10 pg/ml (1 pg per well) was the minimum amount of PRP which could bind specifically to the solid-phase antibody under the conditions of the assay. This limit was probably determined by the affinity of the antibodies and the concentration of immunoglobulin which could be bound to the solid phase.

Fluorescent substrates are thus useful in EIA systems since they allow for the more rapid measurement of small concentrations of antigens within the detection limit of the system. However, the utility of fluorescent substrates is somewhat limited by the fact that the instrumentation required for fluorescent measurement is more difficult to use than that required for the measurement of visible color. The availability of simple instrumentation capable of measuring fluorescence in the small volume utilized in

solid-phase EIA systems or of UV light boxes allowing for the visual measurement of fluorescence reactions, as recently described by Forghani et al., would increase the practicality of the EIA systems which make use of fluorescent substrate (3). However, to make full use of the decreased reaction time offered by fluorescent substrates, attempts should be made to optimize the factors affecting the ultimate sensitivity of EIA systems. These include the concentration of antibody on the solid phase, the affinity constant of the antibodies, and the ratio of specific and nonspecific binding of enzyme-labeled immunoreactants to the solid phase. The optimization of these parameters, combined with the use of high-energy substrates, should lead to EIA systems capable of measuring small concentrations of antigens in clinical specimens in a rapid and reproducible fashion.

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LITERATURE CITED

1. Belanger, L. 1978. Alternative approaches to enzyme immunoassays. *Scand. J. Immunol.* 8(Suppl. 7):33-41.
2. Elkins, R. 1980. More sensitive immunoassays. *Nature (London)* 284:14-15.
3. Forghani, B., J. Dennis, and N. J. Schmidt. 1980. Visual reading of enzyme immunofluorescence assays for human cytomegalovirus antibodies. *J. Clin. Microbiol.* 12:704-708.
4. Frankel, M., and W. Gerhard. 1979. The rapid determinations of binding constants for antiviral antibodies by a radioimmunoassay. An analysis of the interaction between hybridoma proteins and influenza virus. *Mol. Immunol.* 16:101-113.
5. Fursman, R. W., R. C. McCarthy, H. Markowitz, and J. F. O'Brien. 1980. A rapid fluorescent enzyme assay of solid phase antibody bound prostatic alkaline phosphatase. *Clin. Chem.* 26:1028.
6. Harris, C. C., R. H. Yolken, H. Krokan, and I. C. Hsu. 1979. Ultrasensitive enzymatic radioimmunoassay: application to detection of cholera toxin and rotavirus. *Proc. Natl. Acad. Sci. U.S.A.* 76:5336-5339.
7. Konishi, E., S. Inasa, K. Kondo, and M. Hori. 1980. Chemiluminescence-linked immunoassay for detection of mumps virus antibodies. *J. Clin. Microbiol.* 12:140-147.
8. Pepple, J., E. R. Moxon, and R. H. Yolken. 1980. Indirect enzyme-linked immunosorbent assay for the quantitation of the type-specific antigen of *Haemophilus influenzae* type b: a preliminary report. *J. Pediatr.* 97:233-237.
9. Puget, K., A. M. Michelson, and S. Avrameas. 1977. Light emission techniques for the microestimation of femtomogram levels of peroxidase. *Anal. Biochem.* 79:447-456.
10. Segal, E., R. A. Berg, P. A. Pizzo, and J. E. Bennett. 1979. Detection of *Candida* antigen in sera of patients with candidiasis by an enzyme-linked immunosorbent assay-inhibition technique. *J. Clin. Microbiol.* 10:116-118.
11. Shalev, A., A. H. Greenberg, and P. McAlpine. 1980. Detection of attograms of antigen by a high-sensitivity enzyme-linked immunosorbent assay (HS-ELISA) using a fluorogenic substrate. *J. Immunol. Methods* 38:125-139.
12. Wisdom, E. B. 1976. Enzyme immunoassay. *Clin. Chem.* 22:1243-1258.

13. **Yolken, R. H.** 1980. Enzyme-linked immunosorbent assay (ELISA): a practical tool for rapid diagnosis of viruses and other infectious agents. *Yale J. Biol. Med.* **53**:85-92.
14. **Yolken, R. H., H. W. Kim, T. Clem, R. G. Wyatt, A. R. Kalica, R. M. Chanock, and A. Z. Kapikian.** 1977. Enzyme-linked immunosorbent assay (ELISA) for detection of human reovirus-like agent of infantile gastroenteritis. *Lancet* **ii**:263-267.
15. **Yolken, R. H., and P. J. Stopa.** 1979. Enzyme-linked fluorescence assay: ultrasensitive solid-phase assay for detection of human rotavirus. *J. Clin. Microbiol.* **10**:317-321.
16. **Yolken, R. H., P. J. Stopa, and C. C. Harris.** 1980. Enzyme immunoassay for the detection of rotavirus antigen and antibody, p. 692-699. *In* N. Rose and H. Friedman (ed.), *Manual of clinical immunology*, 2nd ed. American Society for Microbiology, Washington, D.C.