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Enzyme-Linked Fluorescence Assay: Ultrasensitive Solid-Phase Assay for Detection of Human Rotavirus

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Enzyme-linked immunosorbent assay (ELISA) has proven to be a useful assay system for the direct detection of infectious agents. However, when the usual color-producing substrates are employed, relatively large amounts of substrate must be hydrolyzed by the bound enzyme before detection can be achieved. We attempted to improve the sensitivity of ELISA by utilizing a substrate that yields a fluorescent product on enzyme action. The enzyme-linked fluorescence assay (ELFA) based on this principle was approximately 100 times more sensitive than the corresponding ELISA or radioimmunoassay for the detection of human rotavirus in a standard stool suspension. In addition, the ELFA for human rotavirus was capable of detecting antigen in six specimens that were negative by ELISA. Five of these specimens were obtained late in the course of confirmed rotavirus infections. ELFA provides a simple, reliable, ultrasensitive method for the rapid detection of viral antigen.

The diagnosis of infectious diseases has been greatly aided by the development of assay systems that can directly detect viral antigens in clinical specimens. Diagnostic techniques that have proven useful include immune electron microscopy (1), immunofluorescence microscopy (2, 6), radioimmunoassay (16), and enzyme-linked immunosorbent assay (ELISA) (4, 14). However, all of these assay systems possess some limitations. Immune electron microscopy requires an electron microscope and a skilled observer and is only applicable in cases where the antigen is morphologically identifiable (1, 10). Immunofluorescence microscopy requires subjective interpretation and often suffers from interference caused by background fluorescence present in specimens (6, 21; T. Williams, P. Bourke, and M. Gurwith, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 15th, Washington, D.C., Abstr. no. 234, 1975). Radioimmunoassay requires unstable and potentially hazardous radioactive isotopes and an expensive counting device (3).

Enzyme immunoassay, which is similar in design to radioimmunoassay but which utilizes an enzyme rather than a radioactive isotope as the immunoglobulin marker, does not have any of the above disadvantages (13, 14, 18). Its sensitivity derives from the fact that a single molecule of enzyme can react with a large number of substrate molecules, leading to the production of a visible color (4). This color can be measured either visually or with a colorimeter. However, the sensitivity of ELISA systems is limited by the ability to detect visible color (18). We postulated that the sensitivity of enzyme immunoassays could be improved by employing a substrate that could be detected in substantially lower concentrations than substrates that yield colored products. We thus developed an enzyme-linked fluorescence assay (ELFA) which utilizes a substrate that yields a fluorescent, rather than visible, product on interaction with the immunoglobulin-linked enzyme.

MATERIALS AND METHODS

Reagents. 4-Methyl umbelliferyl phosphate (MUP) and 3-*O*-methyl fluorescein phosphate were obtained from Research Organics, Cleveland, Ohio. Flavone 3-diphosphate triammonium salt was obtained from Chemical Dynamics Corp., South Plainfield, N.J. *p*-Nitrophenyl phosphate (NPP; type 104-5) was obtained from Sigma Chemical Co., St. Louis, Mo. Alkaline phosphate (calf intestine) was purchased from Sigma (type VII) and contained 1,025 units per mg of protein. Goat anti-guinea pig immunoglobulin G was purchased from Antibodies, Inc., Davis, Calif., and conjugated with the above alkaline phosphatase by the method of Engvall and Perlmann (4). Antisera to human rotavirus were prepared in goat and guinea pig as previously described (9). Although these antisera were prepared against serotype 2, they had cross-reactivity with other human and animal rotavirus serotypes (19, 23). (Antisera were provided by A. R. Kalica, National Institutes of Health.) All buffers were prepared in filtered, deionized water (Hydro Services, Rockville, Md.).

In preliminary studies, diethanolamine buffer

proved to be a satisfactory buffer and was used for all studies. An ionic strength of 0.03 M, $MgCl_2$ concentration of 10^{-5} M, and pH of 9.8 were found to be optimal and were used in subsequent studies.

Specimens. A total of 38 rectal swabs were obtained from 26 children living in the Baltimore, Md. area from January to March, 1979. Of these specimens, 25 were obtained from 15 children with diarrhea. The remaining 13 specimens were obtained from 11 healthy children to serve as normal controls. The swabs were placed in 1 ml of phosphate-buffered saline (pH 7.4) shortly after collection and stored at −20°C until testing. In addition, the sensitivity of the rotaviral detection systems was established by testing $\log_{10}$ dilutions in quadruplicate of a standard filtrate prepared from the stool of a gnotobiotic calf experimentally infected with human rotavirus (kindly supplied by A. Z. Kapikian, National Institutes of Health). Although the exact concentration of virus in this specimen is not known, electron microscopy has revealed characteristic rotaviral particles down to a dilution of 1:100 (9, 20).

Enzyme-substrate reactions. An equal volume of 10-fold dilutions of alkaline phosphatase and substrate were diluted to 100 μl in the above buffer and added to duplicate wells of a polyvinyl microtiter plate (Dynatech 220-24). After incubation at 37°C for 100 min, the contents of each well were transferred to a borosilicate glass tube (12 by 75 mm) and diluted with 900 μl of buffer. In the case of fluorogenic substrates, the resulting fluorescence was determined with an Aminco fluorocolorimeter (model J4-7439) utilizing 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). The amount of fluorescence was measured in arbitrary units. The fluorescence coefficient was calculated as previously described (5) by dividing the fluorescence in arbitrary units by the molar concentration of substrate. Utilizing this equipment, the fluorescence coefficient for quinine sulfate in 0.1 N sulfuric acid was 3.0×10^5.

In the case of colorigenic substrates, the absorbance at 405 nm was determined in a colorimeter (Spectronic 20). A dilution of enzyme was considered positive if it yielded a mean activity that was 2 standard deviations greater than the mean of five control wells to which buffer had been added in place of enzyme.

ELFA procedure. The ELISA procedure for the detection of human rotavirus (22, 23) was adapted to ELFA by the substitution of a fluorogenic substrate in place of the color-producing substrate usually employed. Optimal dilutions of reagents were determined by checkerboard titration (13). The assay for human rotavirus is outlined as follows. Goat anti-rotavirus serum, prepared as previously described, was optimally diluted in carbonate buffer, and 100 μl was added to each of the inner 60 wells of a polyvinyl microtiter plate (Dynatech 220-24). After an incubation overnight at 4°C, the plates were washed five times with phosphate-buffered saline (pH 7.4) containing 0.5 ml of Tween 20 per liter (PBS-Tween), as previously described, and a 100-μl sample of either a clinical specimen or a dilution of the rotavirus standard was added and incubated overnight at 37°C. After another washing procedure, a 100-μl volume of guinea pig anti-rotavirus serum diluted in PBS-Tween containing 1% fetal calf serum and 0.5% rotavirus-negative goat serum (PBS-T-S) was added (22) and incubated for 1 h at 37°C. The plates were washed, and a dilution of alkaline phosphatase-labeled goat anti-guinea pig immunoglobulin G diluted in PBS-T-S was added. After another 1-h incubation at 37°C and washing procedure, a 100-μl volume of MUP, diluted to 10^{-4} M in 0.03 M diethanolamine buffer (pH 9.8) containing 10^{-5} M $MgCl_2 \cdot 6H_2O$, was added. After a 100-min incubation at 37°C, the contents of each well were transferred to a borosilicate glass tube (12 by 75 mm) containing 900 μl of buffer, and the amount of fluorescence was determined as described above. A total of five wells containing PBS-Tween only was run with each test, and a specimen or dilution of standard was considered positive if it gave a fluorescence value greater than 2 standard deviations above the mean.

To eliminate the possibility of nonspecific reactivity (22), positive results were confirmed by the following assay. Wells in alternate rows of the microtiter plate were coated with goat anti-rotavirus serum as above, and the remaining wells were coated with an identical dilution of preimmunization serum from the same goat. Each specimen was run in four wells, two each coated as above. The positivity of a specimen was confirmed if the mean value in the wells coated with the anti-rotavirus serum was 2 standard deviations greater than the value in the wells coated with preimmunization serum.

ELISA. The ELISA for rotavirus was performed as above except that NPP (Sigma 104-5), diluted 1 mg/ml in the above buffer, was utilized as substrate. The amount of color produced was measured in a spectrophotometer as described above.

RESULTS

Enzyme-substrate system. The sensitivities of the enzyme-substrate systems are presented in Table 1. The use of MUP allowed for the detection of 5×10^{-19} mol of enzyme after a 100-min incubation at 37°C. This is approximately 100-fold less than can be detected by NPP, which is the color-producing substrate used in most ELISA systems that utilize alkaline phosphatase as the enzyme. The other fluorescent substrates showed less reactivity than MUP but more than NPP. In addition to its sensitivity, MUP was highly soluble and could be used at a concentration of 10^{-4} M without significant background fluorescence. This substrate was therefore used in subsequent experiments.

Detection of rotavirus. The comparison of ELISA and ELFA for the detection of logarithmic dilutions of a standard filtrate of human rotavirus is presented in Fig. 1. ELFA was capable of detecting a 10^{-5} dilution of the standard preparation, whereas the limit of detection by ELISA was a 10^{-3} dilution of the same filtrate.

Clinical specimens. Of 25 specimens from 15 children admitted to the hospital for acute

diarrhea between 1 January and 10 March 1979, 19 (76%) were ELFA positive for rotavirus and 13 (52%) were positive by ELISA. (Normal controls—13 specimens from 11 healthy children—were all negative by both systems.) Of the six specimens negative by ELISA but positive by ELFA, five were from three children with diarrhea who had stools obtained earlier in their hospitalization which were positive for rotavirus by both methods (Table 2). The specimens that were positive by ELFA but negative by ELISA were obtained towards the end of hospitalization, with the first such specimen noted on day 10 for patient A, day 6 for patient B, and day 8 for patient C.

All specimens positive by ELFA and/or ELISA were demonstrated to contain rotavirus by the confirmatory assay described above.

DISCUSSION

Enzyme immunoassays are solid-phase assay systems that are similar in design to radioimmunoassays but utilize an enzyme rather than a radioactive isotope as the immunoglobulin marker. This enzyme, when bound to the solid phase in a series of antigen-antibody reactions, interacts with added substrate to produce a measurable amount of product (4, 13, 14). The sensitivity of the assay is determined by the turnover rate of the enzyme and the detection limit of the substrate product (4). Most assays use an enzyme-substrate system that produces a reaction product with visible color (18). The sensitivity of these assays is therefore determined by the lowest concentration of this product that can be detected colorimetrically.

We postulated that the sensitivity of enzyme immunoassays could be markedly improved if substrates that were detectable in lower concentrations than the colorigenic ones could be used. Since fluorescent molecules are detectable in picomolar quantities by relatively inexpensive equipment (7), we elected to investigate substrates which yielded fluorescent products. In addition, since fluorescent substrates for alkaline phosphatase are available commercially at low cost and because alkaline phosphatase is widely used in ELISA systems, we chose to investigate this enzyme-substrate system.

Experiments with three commercially available substrates indicated that MUP provided the most sensitive system. The increased sensitivity of MUP, which has been noted by others, is probably due to its high fluorescent index and high turnover rate at the concentration employed (5, 7, 8). Utilizing this substrate, we were able to detect 5×10^{-19} mol of alkaline phospha-

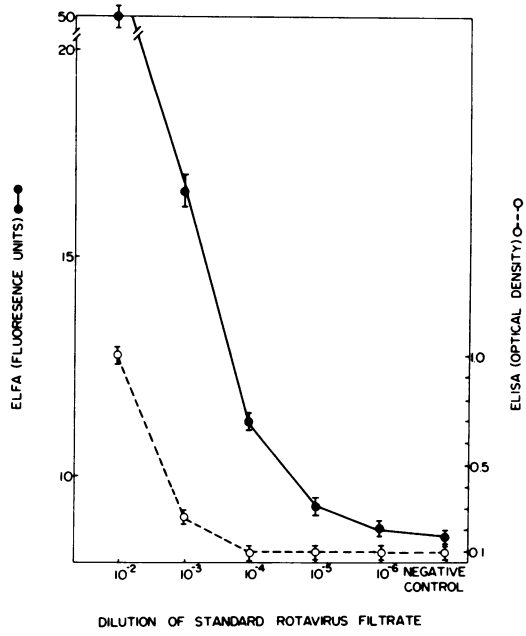

FIG. 1. *Titration curves for a standard rotavirus filtrate tested by ELFA and ELISA. Each dilution was tested in quadruplicate. In the ELFA assay, the 1:100,000 dilution was greater than 2 standard deviations above the mean of the negative control, whereas the equivalent endpoint dilution by ELISA was 1:1,000. This filtrate was positive for rotavirus at a dilution of 1:100 as tested by electron microscopy and 1:1,000 as tested by radioimmunoassay.*

TABLE 2. *Comparison of ELFA and ELISA for detection of rotavirus in serial specimens from three patients*

Patient	No. of stools	No. (%) positive by:	
		ELFA	ELISA
A	7	7 (100)	4 (57)
B	3	3 (100)	2 (67)
C	3	2 (67)	1 (33)
Total	13	12 (92)	7 (54)

TABLE 1. *Sensitivity of enzyme-substrate systems*

Substrate	End product	Concn (M)	Fluorescence coefficient[a]	Minimum detectable enzyme in 100 min (mols)
MUP	Fluorescent	10^{-4}	3.1×10^{6}	5×10^{-19}
MFP[b]	Fluorescent	10^{-4}	1.8×10^{5}	5×10^{-18}
FDP[b]	Fluorescent	10^{-3}	1.4×10^{4}	10^{-17}
NPP	Colored	10^{-2}	NA	5×10^{-17}

[a] Fluorescence of hydrolyzed substrate divided by the concentration (molar) of original substrate. Value for quinine sulfate in 0.1 M sulfuric acid was 3.0×10^{5} for the instrumentation described above. NA, Not applicable.

[b] MFP, 3-*O*-Methyl fluorescein phosphate; FDP, flavone 3-diphosphate triammonium salt.

tase after a 100-min incubation. This represents approximately 10^4 molecules of enzyme. Use of this substrate allowed for the detection of approximately 100-fold less enzyme than did the use of NPP, the colorigenic substrate usually utilized in ELISA reactions. In addition, MUP was found to be sufficiently stable and to have minimal fluorescence in the absence of enzyme.

Similar increases in sensitivity were noted when MUP was substituted for NPP in solid-phase immunoassays for the detection of human rotavirus, an important cause of infantile gastroenteritis (23). Utilizing such a system, a 10^{-5} dilution of a standard stool filtrate could be detected. This represents a 100-fold increase in sensitivity over the standard ELISA and a similar increase over electron microscopy. It has been reported that radioimmunoassay is capable of detecting this filtrate at a 10^{-3} dilution (9). Thus, ELFA is also 100 times more sensitive than this radioimmunoassay system.

The increased sensitivity of ELFA was also demonstrable for the detection of rotavirus in stools of children with diarrhea. Of such specimens, 19 (76%) were positive by ELFA, whereas only 13 (52%) were positive by ELISA. Five of the six ELFA-positive-ELISA-negative specimens were obtained from three children who had earlier specimens positive for rotavirus by both assay systems. In the sixth case, only a single specimen was obtained 6 days after the onset of diarrhea. Thus, the ELFA positivity in the face of ELISA negativity was probably due to the decreased concentration of virus particles in the specimens obtained late in the illness. The increased sensitivity of ELFA for the detection of human rotavirus would be useful for detecting antigen in the stools of newborns, where the amount of virus shed is low (12), and adults, where electron microscopic examination of the stools often fails to reveal viral particles in spite of serological evidence of rotaviral infection (11, 15).

Background fluorescence due to fluorescent material in biological specimens is a potential problem in microscopic immunofluorescence assays, especially in those performed on stool specimens (21; Williams et al., Program Abstr. 15th ICAAC, Abstr. no. 234, 1975). However, this was not encountered in the ELFA testing of 38 stool specimens, presumably because in solid-phase assays the original specimen is washed off the solid phase after the initial antigen-antibody reaction and therefore does not further participate in the reaction. In addition, we did not find fluorescence due to fluorescent material in wash solutions and glassware to be a problem. This problem, which has been troublesome in some fluorescence systems (17), was probably prevented by the use of filtered water and disposable equipment.

ELFA, utilizing MUP as the fluorogenic substrate, thus offers increased sensitivity in relation to the comparable ELISA system. In addition, any ELISA system that utilizes alkaline phosphatase can be adapted to ELFA, since only the substrate need be changed. ELISA systems that utilize other enzymes could also be adaptable to ELFA either by changing the enzyme to alkaline phosphatase or by utilizing a fluorogenic substrate for the enzyme used.

One disadvantage of ELFA is that a spectrofluorometer is required, and thus the results cannot be read visually, as is the case in some ELISA systems. Consequently, ELISA can be more convenient when visual reading is available and where maximum sensitivity is not required. However, in situations where sensitivity is important, ELFA offers a simple, safe, inexpensive method of detecting very small amounts of antigen.

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