Comparison of Colorimetric, Fluorescent, and Enzymatic Amplification Substrate Systems in an Enzyme Immunoassay for Detection of DNA-RNA Hybrids

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Received 4 November 1988/Accepted 23 January 1989

The monoclonal antibody solution hybridization assay is a novel enzyme immunoassay for detection of RNA with a biotinylated DNA probe. To increase the sensitivity of this test, a fluorescent substrate and an enzymatic amplification cycling system were compared with a conventional colorimetric substrate for alkaline phosphatase. The fluorescent, cycling, and colorimetric substrates detected, respectively, 10, 10, and 100 amol of unbound alkaline phosphatase in 2 h. With a prolonged incubation period of 16.6 h, the conventional substrate measured 10 amol of the enzyme. In the immunoassay for RNA detection, the fluorescence and cycling assays were faster than that using the colorimetric substrate and reached an endpoint sensitivity of 3.2 pg/ml (0.16 pg per assay) of cRNA. However, longer incubation periods (16.6 h) for optimal generation of the colorimetric product led to a comparable level of sensitivity for the conventional substrate.

Solid-phase enzyme immunoassays (EIA) have been widely applied for rapid and sensitive detection of microbial antigens in body fluids (30, 31). We have described a homogeneous hybridization assay which uses the EIA format for detection of RNA viruses (27; F. Coutlee, R. Yolken, and R. Viscidi, submitted for publication; C. Newman, J. F. Modlin, R. H. Yolken, M. Bowman, and R. Viscidi. Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 490, 1987; R. Viscidi and R. H. Yolken, Abstr. VII Int. Congr. Virol., abstr. no. R.30.5, 1987). As for all EIA, the overall sensitivity of the test is determined by the kinetics of the antigen-antibody interaction, by the detectability of bound labeled immuno-complexes, and by the level of nonspecific reactivity of the antibody (background noise) (1, 21, 32).

In conventional EIA, the presence of a bound immunoreagent is magnified by conversion by a single molecule (here, alkaline phosphatase) of a large number of substrate molecules, leading to a detectable colored compound. The lowest detectable concentration of the end product determines the detection limit of an EIA (1, 36). Efficient enzymatic labeling strategies used, such as fluorescent (3, 4, 7–10, 12, 14, 25, 35), chemiluminescent (15), or radioactive (11) compounds, that are measurable at lower concentrations than visibly colored products could provide an alternative to increase the sensitivity of EIA. Enzymatically amplified cycling reaction represents another method of magnifying the signal produced by enzymatic reactions (16, 17, 19, 28). In the most extensively studied cycling assay (5, 6, 13, 22, 24, 26), each bound enzyme does not directly degrade a substrate to a colored compound but rather to a coenzyme which amplifies the signal by activation of coupled enzymatic reactions involving NAD-NADH recycling. This substrate system has been reported to be more sensitive and faster than conventional assays (26).

The purpose of this study was to compare the sensitivities of colorimetric, fluorescence, and cycling systems in the monoclonal antibody solution phase hybridization assay for detection of RNA. The most sensitive fluorescent substrate, methylumbellif erone (9), and a cycling assay based on NAD-NADH redox cycles were used here.

MATERIALS AND METHODS

Materials. Polyclonal goat anti-biotin antibody and p-nitrophenolphosphate (p-NPP) were purchased from Sigma Chemical Co., St. Louis, Mo. The fluorogenic substrate 4-methylumbelliferyl phosphate was purchased from Research Organics Inc., Cleveland, Ohio. The enzyme-linked immunosorbent enzyme amplification assay (catalog no. 9589S) was kindly donated by Bethesda Research Laboratories Inc., Gaithersburg, Md. The nick translation kit and bio-11-dUTP were purchased from Bethesda Research Laboratories. The F(ab') fragment of a mouse monoclonal antibody to DNA-RNA hybrids and labeled with alkaline phosphatase was kindly provided by Robert J. Carrico, Ames division, Miles Laboratories, Inc., Elkhart, Ind. Plasmid pSP65 and unconjugated alkaline phosphatase were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. The Riboprobe system for RNA transcription, RNasin RNase inhibitor, SP6 RNA polymerase, and DNase RQ came from Promega. 5-Diethylpyrocarbonate-treated water was produced by autoclaving deionized water with 0.1% 5-diethylpyrocarbonate.

Production of biotinylated probes by a nick translation reaction. Nick-translated probes were prepared with bio-11-dUTP by a standard protocol (23) supplied by the manufacturer. The reaction was performed at 15°C for 90 min. Unincorporated nucleotides were separated from the biotinylated probe by sodium acetate (final concentration, 0.3 M)-95% ethanol precipitation at -70°C for 2 h (19). After centrifugation at 10,000 × g for 15 min at 4°C, the pellet was washed with 70% ethanol. The sediment was air dried and suspended in 100 μl of Tris hydrochloride (50 mM; pH 7.2)-EDTA (2 mM).

Transcription reaction for production of target RNA. Single-stranded RNA targets were produced from plasmid pSP65 DNA. One microgram of pSP65 template was transcribed with SP6 polymerase in a 100-μl reaction volume by
a standard protocol (20). RNasin was added at 1 U/μl to protect single-stranded RNA. After 1 h of transcription, the DNA template was removed with addition of 1 U of RQ DNase for 15 min at 37°C. The RNA produced was extracted with an equal volume of phenol, followed by extraction in chloroform-isoamyl alcohol (24:1) (18). It was recovered by precipitation in sodium acetate-cold ethanol at −70°C as described above. The RNA pellet was suspended in 5-diethylpyrocatechate-treated water with diethyldithiocarbamate as an RNase inhibitor. The amount of RNA was estimated by measuring the absorbance at 260 nm. A ratio of >1.95 was accepted as an RNA preparation of high purity.

Hybridization solution assay. The enzyme immunoassay for detection of RNA with biotinylated DNA probes (monoclonal antibody solution hybridization assay) was performed as described previously (27). Briefly, 100 μl of half-log dilutions of pSP65 RNA transcripts in 5-diethylpyrocatechate-treated H₂O with 0.5% sodium dodecyl sulfate as an RNase inhibitor. The amount of RNA was determined by measuring the absorbance at 260 nm. A ratio of >1.95 was accepted as an RNA preparation of high purity.

RESULTS AND DISCUSSION

The enzymatic cycling amplification assay (Fig. 1) is based on the conversion by bound alkaline phosphatase of the phosphorylated form of NADP to free NAD. The NAD then serves as an enzyme cofactor for two enzymes which catalyze oxidation-reduction reactions. Alcohol dehydrogenase concomitantly transforms the NAD to NADH and also oxidizes ethanol into acetaldehyde. The cycling is completed with the oxidation of NADH back to NAD by diaphorase, which simultaneously reduces iodonitrotetrazolium violet to purple formazan. The dye is quantitated by measurement of its A₅₅₀. The selection of a redox cycle strictly specific for NAD-NADH in the presence of high concentrations of NADP is necessary for initial effective detection of alkaline phosphatase without subsequent removal of NADP. The enzymatic cycle can be completed at least 25,000 times per h (16).

The colorigenic, fluorogenic, and enzymatically amplified substrate systems were compared for the ability to detect half-log dilutions of unconjugated alkaline phosphatase. For evaluation of the enzyme concentration, a molecular weight of 140,000 for alkaline phosphatase was assumed. Twenty-five-microliter volumes of serial dilutions of alkaline phosphatase were added (in quadruplicate for each substrate), to wells of uncoated black and clear microtiter plates. Twenty microliters of each of the following substrates was then added per well: 1 mM p-NPP prepared in diethanolamine buffer; 0.1 mM 4-methylumbelliferyl phosphate in diethanolamine buffer: amplifier substrate (NADP) for 60 min, followed by the amplifier reagent (alcohol dehydrogenase and diaphorase) for an equal duration of time. With the fluorogenic and cycling substrates, 10⁻⁷ mol of the enzyme was

For the samples studied with the enzymatic amplification system, the final wash after the conjugate incubation was done six times with 50 mM Tris-hydrochloride (pH 7.4)-0.15 M NaCl-0.1% sodium azide. This washing buffer was prepared daily to minimize the risk of phosphatase contamination due to microbial growth. The standard washing solution (PBST) was not used, since the presence of phosphates can inhibit the enzymatic amplification reaction.

**Enzyme-linked immunosorbent enzyme cofactor amplification system.** All reagents were kept at 4°C and brought to room temperature before use. The substrate was reconstituted by addition of 12 ml of substrate diluent directly into a vial containing a fixed amount of lyophilized NADP. This solution was gently mixed until complete dissolution of the substrate. Fifty microliters of the reconstituted substrate was then added to each well and incubated for 20 min. The amplifier solution was reconstituted by dissolving the lyophilized enzymes (alcohol dehydrogenase and diaphorase) in 12 ml of amplifier diluent, and 50 μl was added to the substrate solution in each well without removing the substrate. As suggested in the instructions provided by the company, incubation times for substrate and amplifier steps were kept equal and the A₅₅₀ was measured in a spectrophotometer. All plates were blanked automatically by the reader against an antibody-coated well with no sample.

For all assays, a result was considered positive if the mean fluorescence or mean absorbance value exceeded the mean activity of the blank (DNA probe reactivity without RNA) plus 3 standard deviations. This threshold for positivity is illustrated on the graphs as a dark inverted triangle (detection cutoff). The values of fluorescence or absorbance plotted on the graphs are expressed as averages of four values from two independent experiments for each sample.

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detected after 120 min (Fig. 2). In agreement with previous publications (12, 13, 26, 30), the p-NPP substrate was less sensitive, detecting only $3.2 \times 10^{-17}$ mol of alkaline phosphatase in 120 min. However, extension of the incubation time up to 16.6 h (Fig. 3) allowed the colorimetric substrate to reach a detection level similar to that of the other substrate systems ($3.2 \times 10^{-18}$ mol of the enzyme). The fluorogenic substrate incubated overnight provided the most sensitive substrate, detecting $3.2 \times 10^{-16}$ mol of alkaline phosphatase.

The monoclonal antibody solution hybridization assay, described in more detail elsewhere (27; Coutlee et al., submitted; Newman et al., 27th ICAAC; Viscidi and Yolken, Abstr. VII Int. Congr. Virol.), combines the sensitivity and specificity of nucleic acid probes with the convenience of an EIA. In this assay, a biotinylated DNA probe is hybridized in solution with complementary RNA sequences. Biotin-labeled DNA-RNA hybrids are then treated as antigens and are first captured on a solid phase coated with an antibody to biotin. Following removal of unbound nucleic acids, an alkaline phosphatase-labeled monoclonal antibody to DNA-RNA (2, 29) is added to react with bound DNA-RNA hybrids. After a washing step, the substrate is added and the measurement of the product generated by enzymatic degradation of the substrate allows quantitation of biotin-labeled hybrids immobilized on the solid phase. In this study, we used a model system in which biotinylated plasmid pSP65 DNA was hybridized to complementary single-stranded pSP65 RNA transcripts. The influences of different substrate systems on the ability of the assay to detect biotinylated DNA-RNA hybrids as antigens were investigated.

The influence of incubation time on substrate and amplifier reactions was determined first for the enzymatic amplification procedure. Incubation times of 20, 40, and 120 min for the complete cycling reaction (10, 20, and 60 min for each reaction) were compared for the ability to detect half-log dilutions of RNA from 1,000 to 0.3 pg/ml (50 to 0.015 pg per assay). The results (Fig. 4) demonstrated that prolonged incubation led to higher optical densities for each RNA dilution. The 40- and 120-min incubations reached the same endpoint sensitivity of 3.2 pg/ml (0.16 pg per well). How-ever, detection cutoff (V), mean activity of the blank plus 3 standard deviations, +, Results superimposed.
ever, a total reaction time of 20 min was less sensitive, with an endpoint of 10 pg/ml (0.5 pg per assay). The greatest sensitivity was achieved when incubation times for the amplification and substrate steps were identical (data not shown).

Dilutions of RNA were tested in parallel in the monoclonal antibody solution hybridization assay using the different substrate systems. The titration curves and sensitivity endpoints for RNA detection with the colorigenic substrate and enzymatic amplification system are shown in Fig. 5, and the results with the fluorogenic substrate are presented in Fig. 6. With the conventional colorigenic substrate, 32 pg of single-stranded RNA per ml was detected after a substrate incubation time of 120 min. The sensitivity of the colorimetric substrate improved with overnight incubation (16.6 h), reaching the optimal level of detection of 3.2 pg/ml. However, after incubation for only 20 min, the fluorogenic substrate reached the optimum detection limit of 3.2 pg/ml of RNA. The sensitivity of the fluorescence assay was not improved by prolonged incubation times of up to 16.6 h (data not shown). Thus, with optimal incubation times both the colorigenic and fluorescence assays attained identical endpoints. Under optimal conditions, the enzymatic amplification assay reached the same RNA detection endpoint of 3.2 pg/ml in 40 min of substrate incubation.

Publications that advocate the use of more powerful substrate systems to improve EIA sensitivity report experiments in which hydrolysis of the colorigenic substrate was performed in short periods (5, 12, 13, 24, 26, 35). Our experiments support this conclusion when an incubation time of 120 min is used. The colorigenic substrate was then 10-fold less sensitive than the fluorogenic substrate. Under optimal conditions, colorigenic substrates reached sensitivity endpoints comparable to those of fluorogenic substrates (33, 34). As demonstrated in our study, the only advantage of fluorogenic substrates resides in the rapidity with which the assay can be performed. Cycling assays can increase by 250 times the absorbance values of enzyme-substrate systems (13). Practical applications of this technique resulted in a 20- to 70-fold increase in sensitivity over assays with colorimetric substrates (5, 13, 26). However, not unlike the experiments comparing fluorogenic and colorigenic substrates, the time allowed for hydrolysis of the colorigenic product was always limited to 30 to 60 min in these reports. In our experiments, allowing degradation of the substrate to proceed for longer periods increased the sensitivity of the conventional assay to levels achieved with the enzymatic amplification substrate. In contrast to previous publications, an enzymatic amplification system was not more sensitive than a conventional assay but only provided faster results.

**FIG. 4.** Comparison of incubation times with the enzymatic amplification assay system for detection of RNA in the monoclonal antibody solution hybridization assay. The reactivities of biotinylated DNA-RNA hybrids with the monoclonal antibody to DNA-RNA with various amplification incubation times are shown. After solution hybridization, biotinylated DNA-RNA hybrids were captured on a plate and reacted with anti-DNA-RNA antibody labeled with alkaline phosphatase. Equal incubation times for the substrate and amplifier steps of 10 (+), 20 (+), and 60 (+) min were used to quantify the bound label. Absorbance values were measured at 492 nm. The data are the means of four values from two experiments. Detection cutoff (▼), mean reactivity of DNA probe without RNA plus 3 standard deviations; OD, optical density.

**FIG. 5.** Colorimetric substrates for detection of DNA-RNA hybrids in the monoclonal antibody solution hybridization assay. Half-log dilutions of RNA were hybridized in solution with a biotinylated DNA probe. Hybrids were sampled on a microtiter plate coated with anti-biotin antibody and detected with anti-DNA-RNA antibody. The bound conjugate was quantitated by addition of various substrates. p-NPP (1 mM) in diethanolamine buffer was incubated for 2 h (□) or 16 h (+), and the cycling assay was used with an overall incubation time of 40 min (+). The data are the means of four values from two experiments. Detection cut-off (▼), mean reactivity of DNA probe plus 3 standard deviations; OD, optical density.

**FIG. 6.** Fluorogenic substrate for the monoclonal antibody solution hybridization assay. The experiment was as described in the legend to Fig. 5, except for the substrate used: 0.1 mM methylumbelliferyl phosphate in diethanolamine buffer incubated for 20 min.
Other factors besides the detectability of the end product affect the sensitivity of the monoclonal antibody solution hybridization assay. An RNA concentration of 1.2 pg/ml probably represents the minimal concentration of nucleic acid which can bind specifically to the microtiter plate. For example, amplification of the signal from nonspecific reactions can raise the background noise without increasing the endpoint sensitivity. In our experiments, nonspecific reactivity was reduced to a minimum by use of the F(ab')2 fragment of the antibody, which eliminated nonspecific interactions with the Fc portion of the immunoglobulin.

The enzymatic amplification assay studied here was revealed to be sensitive, simple, reliable, and rapid. The principal advantage of fluorescence and cycling assays is to reduce the time required to perform the assay to reach maximal sensitivity. The availability and widespread use of a microtiter plate colorimeter for measurement of colored end products of cycling assays is an advantage over fluorescent substrates. The cycling systems products can be measured on a standard spectrophotometer, while fluorescent substrates require the use of a fluorometer not readily available to all laboratories. The monoclonal antibody solution hybridization assay completed by cycling reactions represents a simple and adaptable technique for nonisotopic detection of RNA.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AI 00625-01 from the National Institute of Allergy and Infectious Diseases and a Medical Research Council fellowship to F.C.

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