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Microvolume, Kinetic-Dependent Enzyme-Linked Immunosorbent Assay for Amoeba Antibodies

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We describe a microvolume enzyme-linked immunosorbent assay based on enzyme rate kinetics. Antigens from *Entamoeba histolytica* were adsorbed in wells of disposable polystyrene strips containing 12 flat-bottom wells. After exposure to the serum of a patient and peroxidase-labeled anti-human immunoglobulin G, the rate of color change in specific substrate was determined by eight sequential readings of individual wells over a 2-min period with a microcomputer-controlled model MR-600 automated plate reader. The changes in absorbance readings were converted to slope values for each well by the microcomputer. Thus, 12 samples were read, and results were printed in ca. 3.5 min. Assay conditions are described and data are presented to show that this assay is quantitative for antibody and antigen concentration with a single-tube (well) dilution.

Enzyme-linked immunosorbent assay (ELISA) has become a widely used serological assay since its introduction in 1972 (3). For quantitative measurements, most applications require testing of multiple dilutions of serum yielding an endpoint expressed as a dilution of the test serum. ELISA reactions based on enzyme rate kinetics (K-ELISA), in contrast, yield quantitative, linear data from testing a single dilution of serum. The K-ELISA was introduced by Tsang et al. (9) and has been used to quantitate antibodies (8) as well as antigens (6, 7). Barlough et al. (1) utilized a computer-assisted, semiautomated K-ELISA assay to quantitate antibodies to viruses in feline sera. Slope values can be converted to activity units (10) of the measured ligand, which permits direct comparison of different reagents. The K-ELISA is capable of detecting nanogram quantities of purified protein (10) and can retain this level of sensitivity in the presence of nonspecific competitors.

This report describes a micromodification of the K-ELISA which is based on reagent volumes and hardware commonly used in traditional ELISA methods. We feel this method can have practical diagnostic as well as research applications.

MATERIALS AND METHODS

All chemicals (reagent grade or better) unless specified otherwise were obtained from Mallinckrodt, Inc., St. Louis, Mo. Urea and Tris were from Schwartz-Mann, Division of Mediscience, Spring Valley, N.Y., and polyoxyethylene sorbitan monolaurate (Tween 20) was from Sigma Chemical Co., St. Louis, Mo. ABTS (2,2'-azino-di-[3-ethyl benthiazolin-sulfonate]) (9) was obtained from Boehringer Mannheim, Indianapolis, Ind.

Peroxidase-conjugated immunoglobulin G (IgG) (goat anti-human IgG) was prepared and kindly provided by V. Tsang, Centers for Disease Control, Atlanta, Ga.

Two lots of *Entamoeba histolytica* antigen were employed as sensitizing agents. These antigens were crude, freeze-dried preparations derived from sonically disrupted, axenically cultured HK-9 amoebae (4). The contents of each vial, prepared from ca. 2.5×10^6 amoebas, were dissolved in a solution of 1.0 ml of 8.0 M urea, 0.05 M Tris-hydrochloride, 0.3 M KCl, and 2 mM EDTA, (pH 8.00). Antigen thus prepared was held at +4°C for up to 4 weeks without loss of activity.

Protein concentrations were determined by the method of Bradford (2) with reagents from Bio-Rad Laboratories, Richmond, Calif., with human albumin and IgG as standards (Sigma).

Sera obtained from confirmed cases of amebiasis and from persons with no history of amebiasis were used to evaluate *E. histolytica* antigens. Reactions were carried out in Immulon 2 Removawell strips (Dynatech Laboratories, Inc., Alexandria, Va.).

In the microvolume K-ELISA, all reagents were used in a volume of 200 μl. Antigen diluted in Tris-potassium chloride buffer (0.05 M Tris, 0.3 M KCl, 2 mM EDTA adjusted to pH 8.00 with HCl) was added to each well of a strip, and strips were incubated in a 37°C water bath for 3 h. The strips were wrapped in plastic film and held at 4°C until the next day. Excess antigen was removed from wells by aspiration, and the wells were filled with a wash solution containing 0.01 M Na_2HPO_4/NaH_2PO_4, 0.15 M NaCl, and 0.3% Tween 20 (pH 7.2) (PBS-Tw). The wells were washed with three changes of PBS-Tw. Serum diluted in PBS-Tw was added to each well and allowed to incubate for 1 h in a 37°C water bath. After this incubation, the unreacted serum was removed by aspiration, and the wells were washed three times in PBS-Tw. Peroxidase-conjugated goat anti-human IgG, diluted 1:300 in PBS-Tw, was added to each well. After a 1-h incubation at 37°C, the excess conjugate was removed, and the wells were washed three times with PBS-Tw. After the last wash, PBS-Tw was left in the wells, and the strips were covered before being read.

A 40-mM stock solution of ABTS in deionized water could be kept at 4°C up to 3 months before use. Immediately before use, the ABTS was diluted to 0.2 mM in 50 mM citric acid adjusted to pH 4.0 with NaOH. Hydrogen peroxide was added to a final concentration of 1 mM in the ABTS-cirate buffer (5). The diluted substrate was kept at room temperature in a covered, amber bottle.

One strip of 12 wells was placed in a Removawell strip holder, and the PBS-Tw was removed by aspiration. The strip holder was placed in the carrier of a model MR-600 Microplate Reader (Dynatech), and 200 μl of substrate solution was added to each of the 12 wells simultaneously with a 12-channel pipettor (Titertek; Flow Laboratories,

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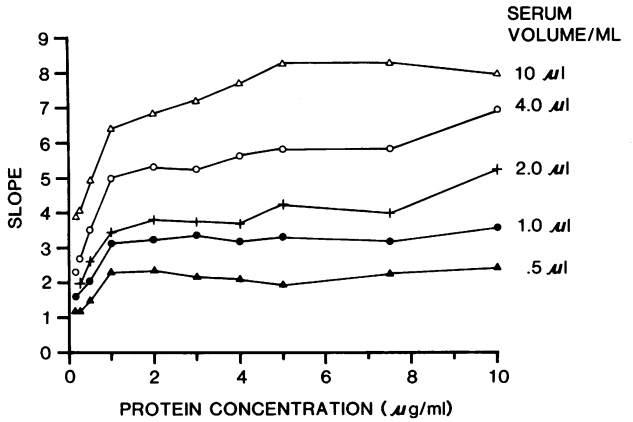

FIG. 1. Determination of antigen saturation dose of *E. histolytica* antigen. Antigen (protein) concentrations range from 0.0625 to 10 μg/ml. Minimum saturation level is at 1 μg/ml. Serum concentrations are as noted.

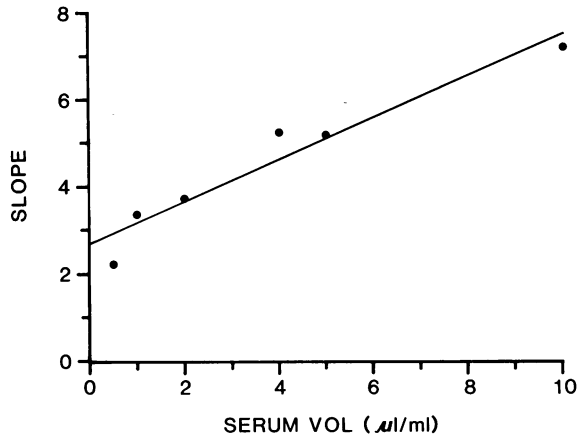

FIG. 2. Assay for antibody concentration. *E. histolytica* antigen is in excess at 3 μg/ml. Serum is the rate-limiting ligand. ($r^2 = 0.932$).

McLean, Va.). Reading of the strips was initiated by computer command in a program in which the strip was indexed continuously through eight complete cycles, and the absorbance at 410 nm of each well at each reading was recorded in memory. The time interval between the sequential readings of each well was 15 s.

When readings were completed, absorbance values for each well were printed, and slope and correlation coefficient (r) values were calculated and printed. The slope value was calculated from the regression formula:

$$\text{slope} = \frac{\Sigma(X \cdot Y) - (X \cdot Y)/n}{\Sigma X^2 - (\Sigma X)^2/n}$$

where X = sequential reading number, Y = absorbance at 410 nm, and n = number of absorbance readings taken. The resulting value was multiplied by 10^3 for reporting purposes. The time required for reading and printing results for 12 specimens was ca. 3.5 min.

Optimal antigen or antibody concentrations or both were determined by a series of block titrations. For an antigen titration, wells were sensitized with a series of antigen concentrations based on protein content. Up to 12 antigen concentrations could thus be accommodated on each strip. Typically, a single dilution of antibody would be added to each well of a strip. Tests were run with four replicates for each antibody dilution tested.

A similar procedure was used for antibody optimization in which the strips were sensitized with an excess of antigen and reacted with a series of antibody concentrations.

RESULTS

The precise quantitation of antibody or antigen afforded by the K-ELISA is based on holding all reactants in excess except the ligand to be measured, which must be rate limiting.

In a typical titration of *E. histolytica* antigen with different antiserum concentrations, antigen saturation occurs at a protein concentration of ca. 1 μg/ml (Fig. 1). At antigen concentrations at or above the saturation level, the reaction rate (slope) is serum-concentration dependent (Fig. 2). Direct correlation is indicated by the regression line. The highest dilution of antibody tested (1:2,000) is below the optimal serum concentration, based on an r^2 value of 0.932 over the dilution range 1:2,000 to 1:100 and an r^2 of 0.970 over the range of 1:1,000 to 1:100.

When antigen is held as the rate-limiting ligand and antibody is in excess, there is a linear relationship between reaction rate and antigen (protein) concentration. Figure 3 shows this relationship for *E. histolytica* antigen. For antigen (protein) concentrations between 0.125 and 1.0 μg/ml with serum in excess at 10 μl/ml (1:100 dilution), the slope is directly proportional to protein concentration ($r^2 = 0.996$) used for sensitization.

We compared antigen saturation curves from two lots of *E. histolytica* antigen tested with a single serum sample (Fig. 4). The protein content of lot 34 antigen was 0.986 mg/ml and that of lot 45 was 0.897 mg/ml. Each antigen was diluted to the range of 0.125 to 10 μg/ml for sensitizing the wells. Antigen saturation occurred between 1 and 2 μg/ml with both antigens, and the curves are almost identical.

Reproducibility of the assay was determined by calculating the coefficient of variation (CV) for replicate samples (usually four) within a particular run. The CV rarely exceeded 10%, and the average of the CV values for a run never exceeded 10%. The usual CV was <6%. To determine the

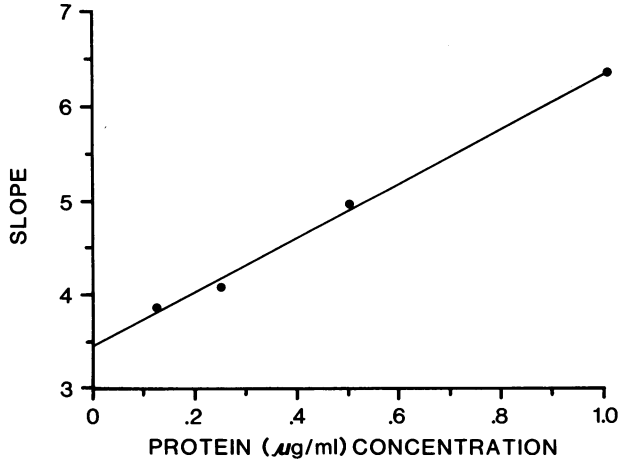

FIG. 3. Assay for antigen (protein) concentration. Antigen is *E. histolytica* lot 45. Serum concentration is in excess at 10 μl/ml (1:100 dilution). $r^2 = 0.996$.

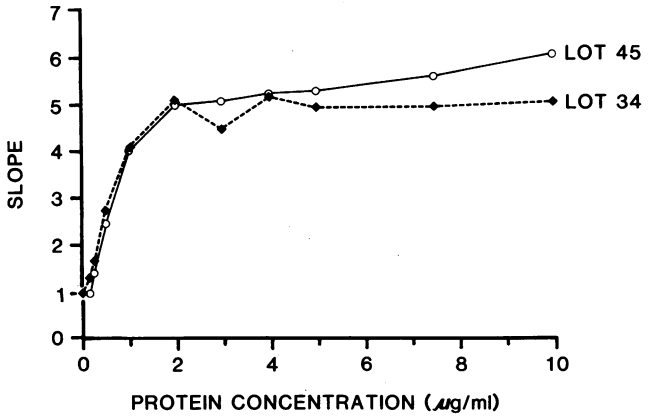

FIG. 4. Comparison of two lots of crude *E. histolytica* antigen. Serum concentration of the patient = 4 μl/ml.

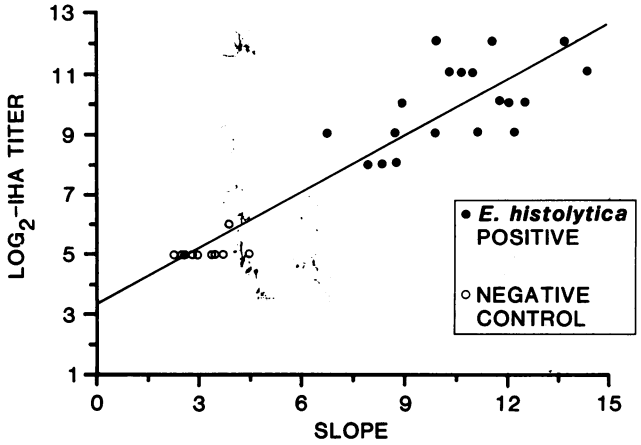

FIG. 5. Comparison of K-ELISA and IHA results. Solid line is the regression line.

reproducibility at a representative slope value in the antibody-limiting configuration, we tested strips sensitized with 3 μg of *E. histolytica* antigen per ml with the serum of a patient diluted to a concentration of 4 μl/ml. These tests were repeated six times over a 6-day period. The mean slope value for 160 observations was 7.12 (± CV = 8.3%).

K-ELISA slope values and indirect hemagglutination titers of sera from 22 amebiasis patients (20 with documented tissue invasion, 2 with asymptomatic cyst passers) and 9 negative controls were compared (Fig. 5). Only 1 of 22 confirmed cases had a K-ELISA slope value less than 6. This patient, an asymptomatic cyst passer, had an indirect hemagglutination titer of 1:32. The other cyst passer had an IHA titer of 1:512 and a K-ELISA slope of 8.8. The highest slope value found among negative, control sera was 4.5.

DISCUSSION

Reported here is a simple, rapid, and sensitive K-ELISA system, patterned after the K-ELISA devised by Tsang et al. (9, 10). This assay has a sensitivity in the nanogram-per-milliliter range and can quantitate antibodies in the serum of patients by a single-tube assay. By using a computer-controlled microplate reader, 12 samples can be read, and results can be printed in less than 4 min.

ABTS was used as the substrate chromogen in our assay because it is stable, does not present a possible carcinogen hazard (5), and retains its linearity for well beyond the initial 2-min recording period (data not shown).

By using conditions described herein, the saturation dose of the sensitizing agent was found to be in the area of a 1-μg/ml range of total protein. This is the same order of magnitude reported by Engvall and Perlmann (3) and by Tsang et al. (9). We have used an antigen-sensitizing dose of 3 μg/ml (protein concentration) when quantitating antibody levels to ensure an excess of antigen. Under these conditions, serum diluted 1:250 (4 μl/ml) permits single-dilution quantitation of antibody concentration.

Two lots of *E. histolytica* crude antigen were used to demonstrate the sensitivity of this modified K-ELISA procedure. In studies not reported here, we have evaluated partially purified antigen from *E. histolytica* and *Schistosoma mansoni*, and they were found to function in this assay in a similar manner. Additional studies are planned to demonstrate the value of this assay with antigens from other parasitic protozoa.

The reproducibility of this assay has been monitored by calculating mean, standard deviation, and CV for four replicates of most samples. Occasionally, one of four replicates was obviously out of range, giving a CV value up to 20%. We suggest that all tests be conducted in duplicate, and specimens showing differences greater than 10% be retested to ensure highest quality results.

The K-ELISA has been shown to be a sensitive assay for antibodies as well as antigens (10). By holding all assay components in excess relative to the ligand (antigen or antibody) to be measured, the rate of color development in the substrate chromogen is directly proportional to the concentration of the rate-limiting ligand.

We have shown that the K-ELISA can be utilized on a microvolume scale and read accurately with a commercially available reader designed for microplate assays utilizing traditional methodology. We feel that this assay can be useful in the serodiagnostic setting in which large numbers of specimens must be processed. It can also be useful in the research laboratory for assaying antigen or antibody levels accurately and with a sensitivity in the nanogram range.

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