Enzyme linked protein-A: an ELISA for detection of amoebic antibody

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Abstract

Enzyme linked protein-A was used to develop an enzyme linked immunosorbent assay (ELISA) system for the detection of circulating antibodies to amoebic antigen. The specificity of protein-A to bind IgG only through Fc receptors, makes the test more specific for the detection of IgG antibodies to amoebic antigen. The ELISA system was used to detect amoebic antibody in control subjects (56), patients with amoebic liver abscess (79) and *Entamoeba histolytica* cyst-passers (10) and the results compared with those of indirect haemagglutination assay (IHA). The ELISA was more sensitive and detected 74·7% of cases with amoebic antibody in amoebic liver abscess compared with 66·7% detected by IHA. The test was more specific, sensitive and easy to perform and is recommended as a test of choice for the serological diagnosis of amoebic liver abscess.

Introduction

Circulating antibodies to amoebic antigen have been used as diagnostic markers for amoebic liver abscess. A number of serological tests (PRAKASH *et al.*, 1969; KRUPP, 1966; JEANES, 1966) is available for the diagnosis and epidemiology of amoebiasis. In recent years more emphasis has been laid on diagnosis of amoebiasis by enzyme linked immunosorbent assay (ELISA) (BOS *et al.*, 1975; YANG & KENNEDY, 1979; AGARWAL *et al.*, 1981). This test is comparable to that of the radioimmunoassay (VOLLER *et al.*, 1977). The proteins used as carriers for enzymes have been either specific antibodies or anti-immunoglobulins, which should be monospecific or monoclonals; it is difficult to obtain monospecific antibodies in certain diseases. Recently protein-A has been used as carrier for different tracers such as FITC (LANGONE, 1980), radio-nucleotides (LANGONE *et al.*, 1977) and enzymes (NYGREN & HANSSON, 1981; OUDENAREN *et al.*, 1981). Protein-A obtained from *Staphylococcus aureus* has an extraordinary affinity for Fc regions of IgG subclasses 1, 2 and 4 of most of the species tested (GODING, 1978), including man, without inhibiting antigen-antibody reaction. It is a single polypeptide chain of molecular weight 42 000 and can be easily labelled with horse-radish peroxidase (HRPO) (NYGREN & HANSSON, 1981).

In this report we describe an ELISA system for the detection of antibodies to amoebic antigen by using enzyme-linked protein-A. The test has been compared with the conventional indirect haemagglutination assay (IHA).

Materials and Methods

The following groups of subjects were selected for the serological studies for amoebic antibody by the techniques of ELISA and IHA:

S.No.	Groups	Number studied
1.	Healthy controls	56
2.	Amoebic liver abscess	79
3.	*Entamoeba histolytica* cyst passers	10

Criteria for selection of subjects:

1. Normal healthy controls: This group included males and females of different socioeconomic status. There was no recent or past history of amoebic diseases in these subjects and stool examinations did not show cysts or vegetative forms of *E. histolytica*.
2. Amoebic liver abscess: The diagnosis of amoebic liver abscess and asymptomatic cyst passers was made as per WHO recommendations (WHO Expert Committee, 1969).

Entamoeba histolytica *antigen*

Amoebic antigen was prepared from cultures of *E. histolytica*, strain NIH-200, grown axenically in Diamond's TP S-1 medium by the technique of DIAMOND (1968). The method of LOWRY *et al.* (1951) was used to determine the concentration of proteins in the solution. The antigen was stored frozen in small aliquots.

Conjugate

Protein-A from *Staphylococcus aureus* (Sigma, P6650) was labelled with horse-radish peroxidase (HRPO) (Sigma P8375) by the method of ENGVALL (1980) using the two-step technique with glutaraldehyde (Sigma G5882). The conjugate was then stored in an equal amount of glycerol in small aliquots.

Assay

All sera were stored at −20°C until tested in dilutions of 1:100 and further in two-fold dilutions in positive cases. The ELISA test was performed as detailed below. The wells of the Nunc polyvinyl microtitre plates were precoated with 100 µl of amoebic antigen in a dilution of 5 µg/ml in carbonate buffer 0·1 M, pH 9·2. The plates were kept at room temperature overnight, usually 18 to 20 hours. After washing three times for 5 min with phosphate buffered saline (PBS) containing 0·05% Tween 20 (PBS-T), 200 µl of 0·5% gelatin in PBS-T was added into each well. The plate was incubated at 37°C for one hour. After three additional washings, each for 5 min, with PBS-T, 100 µl of 1:100 dilution in PBS-T of the sample to be tested for amoebic antibody and the positive and negative control were added to the plate. The plate was then incubated for one hour at 37°C and 30 min at 4°C. After another three 5 min washings, 100 µl of 1:2000 dilution of the protein-A-HRPO conjugate in PBS-T containing 20% foetal calf serum (FCS) was added and the plate incubated at 37°C for one hour and 30 min at 4°C. The wells were washed five times for five min each time with PBS-T, and 100 µl of the freshly prepared substrate (orthophenylene diamine (OPD), 5 mg/10 ml of citrate buffer pH 5·0 made by mixing 98·6 parts of 0·1 M citric acid and 101·4 parts of 0·2 M disodium orthophosphate and containing a final concentration of 0·006% hydrogen peroxide) and the plates incubated in the dark at room temperature. After 5 to 30 min (depending upon the amount of

colour developed), the reaction was stopped by adding 100 µl of 4 M sulphuric acid to each well. The optical density (OD) at 493 nm was measured in a double beam spectrophotometer (Beckman Model 25 spectrophotometer) equipped with microcuvettes, or in a Dyanatec micro-ELISA mini reader.

The test was considered positive with OD of 0·54 or above. This cut off number was obtained by taking 2 S.D. (2 × 0·13) above the mean value (0·28) in healthy subjects.

Indirect haemagglutination assay (IHA)

Indirect haemagglutination assay was carried out as described by PRAKASH (1974).

Results

The results of enzyme linked-protein A ELISA tests are presented in Fig. 1. 53 of the 56 normal healthy controls were negative at 1:100 dilution. Three subjects were positive at 1:100 and two of these were positive at 1:200 dilution. Among the asymptomatic *E. histolytica* cyst passers (10 subjects), three were negative and seven were positive in 1:100 dilution. None of them was positive in dilutions above this.

13 of the 79 serum samples from patients with amoebic liver abscess were negative in the dilution of 1:100. Seven were positive only in dilution of 1:100 and the remainder were positive in dilutions of 200 and above.

The positivity rate of IHA in 1:162 dilution was 1·8% in normal healthy controls, 0% in asymptomatic *E. histolytica* cyst passers and 66·7% in patients with amoebic liver abscess.

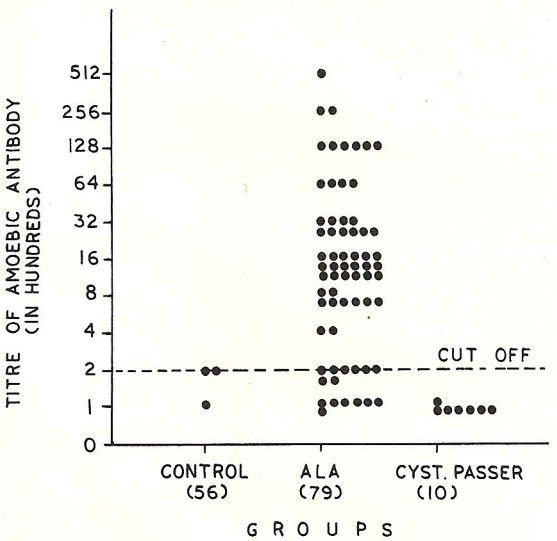

Fig. 1. Titres of amoebic antibody in different groups

Discussion

Enzyme linked-protein A ELISA test was positive in 1:100 titres in 5·4% of healthy controls, 70·0% of asymptomatic *E. histolytica* cyst passers and 83·5% in patients with amoebic liver abscess. The corresponding positivity rates in 1:200 dilution for the three groups were 3·6%, 0% and 74·7%, respectively. Above this dilution, none of the subjects from the healthy control group and asymptomatic cyst passers was positive although 64·6% of amoebic liver abscess patients were. If we consider 1:100 dilution as the cut-off point for positive tests, the sensitivity of ELISA would be 94·6% and the specificity 83·5% for diagnosis of amoebic liver abscess. However, if we take the cut-off point as 1:200 dilution, the sensitivity of the test is 96·4% with a specificity of 74·7%. With further dilution, though the sensitivity is 100%, the specificity is reduced to 64·6%. It is recommended that a dilution of 1:200 and above should be taken as a positive test for further evaluation by other investigators.

At this recommended cut-off point, the ELISA test is more sensitive than the standard IHA test. It detected eight more subjects with amoebic liver abscess than had the IHA and only one subject positive by the IHA was missed by the ELISA at 1:200 dilution. The positivity in a small number of healthy controls could be due to invasive amoebiasis, i.e., amoebic dysentery or a liver abscess some time in the past which may have stimulated antibody production.

With non-significant differences, ELISA and IHA seem to have similar sensitivity and specificity for the diagnosis of amoebic liver abscess. ELISA has the advantage of being a relatively simple, quick, stable and inexpensive test compared to the IHA test. It is therefore recommended to clinical service laboratories for the diagnosis of amoebic liver abscess.

In the present ELISA system, protein-A has been used to conjugate with horse-radish peroxidase to make the system more specific. The advantages of the use of protein-A for conjugation over immunoglobulins is that this molecule is more stable to heat and denaturing agents due to its smaller size. It can be coupled easily to the enzyme (OUDENASEN, 1981) and the binding of IgG is complete and quick within seconds (GODING, 1978). The use of protein-A has conveniently replaced the use of the second antibody and has made the new ELISA system more stable, specific and quick to perform.

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