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A simple spot-test for circulating *Entamoeba histolytica* antigen-antibody complexes in patients with amoebic liver abscess

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An enzyme linked immunosorbent assay (ELISA) is described for the detection of *E. histolytica* immune complexes in serum. Antiamoebic antibody purified by affinity chromatography was used both to precoat strips of nitrocellulose membrane and as an enzyme carrier. These strips were incubated with samples of concentrated test serum and the enzyme conjugate. Following treatment with the peroxidase substrate 3-amino-9-ethylcarbazole the presence of *E. histolytica* antigens was visualized as red spots. Blocking of positive test sera with excess antibodies inhibited this reaction. Serum samples from 47 patients with amoebic liver abscess, 43 patients with other enteric diseases and 35 healthy controls were investigated. The spot test was positive in 75 per cent of patients with amoebic liver abscess, and in 12 per cent diseased controls whereas all the healthy controls were negative. The spot test is simple and sensitive and does not require prior separation of the immune complexes. The test is recommended as an aid to the diagnosis in patients suspected to have amoebic liver abscess.

Detection of circulating amoebic antigen provides a firm proof of ongoing invasive amoebiasis. Several recent studies have documented the presence of *Entamoeba histolytica* immune complexes in the sera of patients with amoebic liver abscess[1,2]. Both solid phase radioimmuno assay (SPRIA)[1] and enzyme linked immunosorbent assay (ELISA)[3,4] have been used for the detection of *E. histolytica* immune complexes. So far, these techniques require prior precipitation of the immune complexes requiring a minimum of 48 h to complete the test. On the other hand, enzyme immunoassays in which immune complexes are concentrated directly on filters following vacuum application have been used for detection of viral antigens[5-7]. Further, high protein binding membranes of nitrocellulose[8-11] or diazobenzyloxymethyl (DBM)[12] have recently been used for adsorption and concentration of proteins[13], thus obviating the requirements for prior concentration of immune complexes.

We have evaluated the diagnostic value of an ELISA for the detection of circulating *E. histolytica* immune complexes using nitrocellulose membranes as solid phase and the results are presented here.

Material & Methods

Patients and controls: Serum samples were collected from (*i*) 47 patients with amoebic liver abscess; (*ii*) 43 patients with enteric diseases other than amoebiasis, including viral hepatitis (16), intestinal tuberculosis (9) and giardiasis (18); and (*iii*) 35 healthy controls. The latter group included 19 employees and resident doctors of All India Institute of Medical Sciences (AIIMS), New Delhi, all negative for *E. histolytica* by stool screening and negative for antiamoebic antibodies[14]. The healthy control group also included 16 Norwegian students, all without history of amoebic disease. The patients with amoebic liver abscess were admitted to the wards of the AIIMS. According to current WHO recommendations[15], the diagnosis of amoebic liver abscess was based on clinical symptoms, positive scan and/or ultrasound examination, aspiration from abscess of sterile pus, positive serology and prompt response to treatment with metronidazole. The diagnosis of acute viral hepatitis was based on clinical evidence and strengthened by a five-fold or more increase in the transaminase levels. Intestinal tuberculosis was confirmed by histology and by the presence of circulating antibodies to *Mycobacterium tuberculosis* using an ELISA test[16]. All cases of giardiasis were positive for *Giardia lamblia* by stool examination.

Blood was obtained from fasting patients and the serum was separated by centrifugation at 3,000 rpm for 10 min, and stored at 4°C without preservative.

E. histolytica antigen for affinity chromatography: Antigen was prepared from cultures of *E. histolytica*, strain NIH-200, grown axenically in Diamond's TPS-1 medium[17]. Harvested trophozoites were washed with saline, once frozen and thawed, then sonicated for 5 min at 20 K cycles in an ultrasonic disruptor (MSC, UK) and finally centrifuged at 10,000 *g* for 30 min at 4°C. The protein content of the clear supernatant (the antigen) was determined according to Lowry *et al*[18] and stored frozen in small aliquots of 0.5 ml.

Purification of anti-amoebic antibody by affinity chromatography: Ten milligrams of the antigenic protein was coupled with CN Br-activated Sepharose 4 B as per recommendations of the manufacturers, Pharmacia Fine Chemicals, Sweden[19] and the sepharose-coupled protein was stored at 4°C in 0.1*M* phosphate buffer saline (PBS), *p*H 7.2 with sodium azide (1mg/ml). The ammonium sulphate precipitate of amoebic antibody from high titred pooled human serum was mixed with sepharose-coupled protein overnight. The amoebic antibody was eluted from the antigen-antibody complex with diethylamine and dialysed against 0.1*M* carbonate buffer, *p*H 9.6.

Antibody-enzyme conjugate: The affinity purified antibody to amoebic antigen was labelled with horseradish peroxidase (HRPO) (Sigma P-8375) according to Engvall[20] using the two-step glutaraldehyde technique. The conjugate was then stored frozen in equal amounts of distilled glycerol in small aliquots. Following checker board titrations the conjugate was used at the optimal dilution of 1:400 in 0.1*M* PBS.

Substrate: The peroxidase substrate 3-amino-9-ethylcarbazole obtained from Sigma

(A-5754) was prepared just before use. Four mg of the substrate was dissolved in one ml of N, N dimethylformamide (Sigma D-4254). While stirring, this solution was added to 14 ml of 0.1 *M* acetate buffer, *p*H 5.2 (21 ml of 0.1N acetic acid and 79 ml of 0.1 *M* sodium acetate) containing 0.15 ml of 3 per cent hydrogen peroxide. The mixture was filtered with Whatman filter paper number 1 before use.

Preparation of the strips: Nitrocellulose membrane sheets BA 83, 0.2 μm pore size (Schleicher and Schull, Dassel, West Germany) were cut into strips of convenient size, *e.g.*, 110 × 7 mm. The strips were soaked in PBS, activated for 1 h in 0.25 per cent glutaraldehyde and subsequently rinsed twice in PBS, for 15 min each time. Each strip was then incubated overnight in horizontally layered screw capped tubes containing the affinity purified antibodies, used at the optimal dilution of 20 μg/ml in PBS. After rinsing (as above) the strips were incubated overnight at 4°C in 1 per cent bovine serum albumin (Sigma A-9647) to block non-specific sites, rinsed again as above and finally stored dry in screw capped tubes for up to 4 wk at 4°C. The solutions containing antibodies to *E. histolytica* and bovine serum albumin were stored frozen at —20°C in stoppered tubes and could be reused 4-5 times over a few week without significant loss of activity. The strips were handled with tweezers throughout the procedure. All incubations were at room temperature.

Assay: The pre-coated strip was soaked in PBS for 5 min for equilibration and dried between two filter papers. The strip was then placed on the manifold, which was screwed up tightly and connected with a vacuum pump. Avoiding bubbles, each well of the manifold then received 50 μl of PBS followed by either 100 μl of the test serum, a positive or a negative control, as appropriate. Vacuum suction was allowed for 10 min. Before disconnecting suction each well was rinsed with distilled water (50 μl) and 2 × PBS (100 μl), and similar PBS washings were subsequently undertaken without suction. Each well then received 100 μl of antibody-enzyme conjugate. After 10 min of conjugate incubation the strips were removed from the manifold, rinsed for 5 min in 100 ml PBS, then for 2 × 5 min in 100 ml of acetate buffer *p*H 5.2 and were finally incubated for 15 min in the freshly prepared substrate solution. To stop the enzymatic reaction, the strips were transferred into water. A red spot, light or dark was considered as positive and absence of colour as a negative test (Fig. 1).

Blocking assay: Ten serum samples known to be positive for *E. histolytica* immune complexes were used to monitor the specificity of the test system by blocking with an excess of antibodies. For the blocking assay, the spot test was performed as above. However, each of the positive samples were first incubated for 2 h at 37°C with an equal volume of a spot test negative but antibody positive (titre 1:6400) serum from a patient with amoebic liver abscess.

Results

The spot test was found to be rapid and easy to perform. Once the strips were ready, the test could be completed in less than an hour including incubation periods of 30 min altogether. There was a sharp distinction between positive tests (red spots) and negative tests (white spots). Haemolysis of the test samples did not interfere with the intensity of the final colour product as

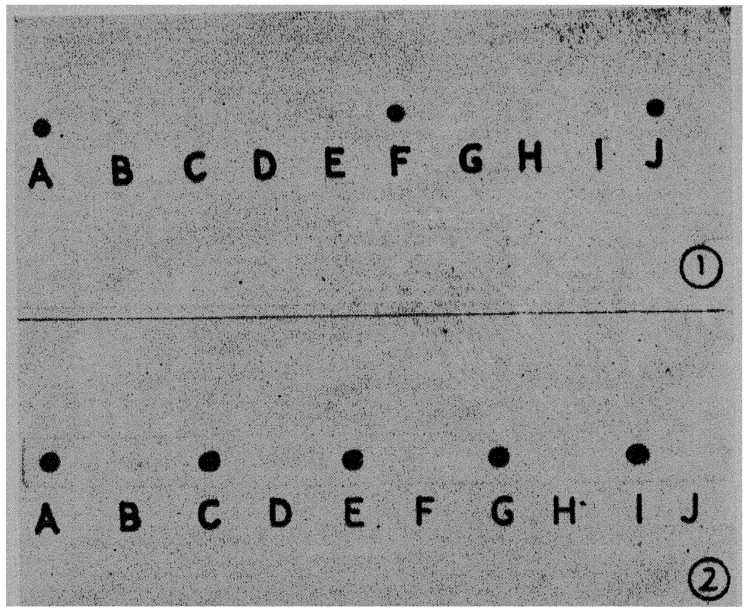

Fig. 1. *E. histolytica* antigens · Immunochemical detection of circulating *E. histolytica* antigens by spot test on nitrocellulose membrane. Dots shown correspond to amoebic liver abscess (A, B, C, E, F, H, I and J) and normal healthy controls (D and G). **Fig. 2.** Blocking-spot-test : The binding of antigen to the membrane was blocked by preincubating the test serum with high titred (1 : 6400) anti-amoebic antibody. The dark coloured spots (A, C, E, G and I) show presence of antigen in the test samples whereas the light coloured spots (B, D, F, H and J) reflect the blocking effect with specific antibodies in the corresponding test samples.

Table. Spot test positivity for circulating *E. histolytica* specific immune complexes in different groups of subjects

Group	No. tested	No. positive
Amoebic liver abscess	47	35 (74.5)
Viral hepatitis	16	2 (12.5)
Giardia lamblia infection	18	2 (11.1)
Intestinal tuberculosis	9	1 (11.1)
Normal healthy controls	35	0

Figures in parentheses are percentages

washings with distilled water completely removed any lysed erythrocytes. However, lipaemic samples blocked the sites physically resulting in incomplete binding and erratic results.

The results are summarized in the Table. Of the 47 serum samples from patients with amoebic liver abscess, 35 were positive in the spot test. In the control group of 43 patients with other intestinal diseases the mean positivity rate was 11.5 per cent and almost identical among patients with viral hepatitis, intestinal tuberculosis and giardiasis. None of the 35 healthy controls had a positive spot test. As shown in Fig. 2, the

addition of excess antibody to spot positive samples resulted in complete blocking and a negative test.

Discussion

Although the senstivity of the protein-A ELISA for antibodies[14] equalled that of the spot test for amoebic antigen, circulating antigens are the only proof of ongoing invasive disease. Traditional techniques for the detection of circulating *E. histolytica* immune complexes[1,2] are cumbersome and time consuming, and require precipitation of circulating immune complexes as an initial step. On the other hand, the technically simple dot immunobinding assays may not be sensitive enough to pick up the smallest quantities of antigen or antibody, as without the manifold only 1-2 μl of the serum sample can be utilized[10,21]. In the present assay, concentration of immune complexes on a nitrocellulose membrane significantly reduced the time required to run the test. Initial activation of the nitrocellulose membrane with glutaraldehyde increased the binding capacity of the amoebic antibody[10] and ensured complete coating as evidenced by a clear background. This facilitated the distinction between positive and negative samples and enabled us to detect even small amounts of antigen. In the present study, a large volume of the samples (100 μl) was concentrated on an area of 3 mm only, thereby increasing the efficiency of the assay.

The enzyme carrying antibody of the present spot test was purified from pooled human serum by affinity chromatography using antigens of axenically grown *E. histolytica* as solid phase. To test the specificity of the system a blocking test was carried out. Addition of excess antibodies to the serum samples blocked the antigenic sites and resulted in negative tests. This also proves that in spite of axenic growth and numerous laboratory passages, the involved immunogens of this strain still share common epitopes with invasive wild strains.

All 35 serum samples from the healthy controls were negative in the spot test, and 74.5 per cent samples from patients with amoebic liver abscess showed a positive test. It is not clear whether the negative results in the remaining patients were due to previous self treatment with amoebicides, a prozone phenomenon caused by antibody excess, or to other factors. As compared with the conventional ELISA technique[4], the spot test more often showed positive results with sera from the diseased controls. Two patients each from the viral hepatitis group and the giardiasis group, and 1 patient with intestinal tuberculosis showed positive spot tests. These patients were negative for anti-amoebic antibodies by ELISA[14] and parasite negative by single stool examination. The possibility of wide cross reactivity of the pre-coat antibodies seems unlikely due to the careful affinity purification procedure. Of the estimated 5-58 per cent asymptomatic *E. histolytica* cyst passers in India[22], about 15 per cent are expected to develop invasive disease[23]. Nutritional state, diet and alcoholism influence the prevalence of the disease in the carrier group[24-26]. Further, a high prevalence rate of amoebic infection has already been demonstrated in patients with non-A, non-B hepatitis[27]. Although detailed information on predisposing factors, other than hepatitis are not available it is assumed that these cases represent early subclinical amoebiasis. It is known that free antibodies may be absent in the early stage of amoebic infection[28].

The advantages of the spot test over the equally sensitive ELISA test[4] are: (*i*) The spot test may be performed at room temperature and does not require expensive equipment such as an ELISA reader; (*ii*) the use of a nitrocellulose membrane permits the reaction to be viewed simply against a white background. The complete lack of background staining makes it easy to discriminate between positive and negative reactions; (*iii*) as the procedure does not require the circulating immune complexes to be removed, the spot test is much quicker ($<$1 h) than ELISA (2-3 days); (*iv*) by using a manifold the antigen sample becomes concentrated to only 3 mm^2. This facilitates detection of very small amounts of the circulating immune complexes, thus increasing the sensitivity of the test; and (*v*) the spot test is much cheaper as the reagents can be used repeatedly.

A major drawback of the spot test is its requirement for centrifuged fasting serum as lipemic serum may give false results. Also the use of azide as serum preservative may interfere with the substrate 3-amino-9-ethylcarbazole.

We conclude that this simple antigen detecting spot test could be of considerable value for rapid diagnosis of amoebic liver abscess, and probably other invasive amoebiasis and for the differentiation between past and present infections. Although of equal sensitivity as the antigen detecting ELISA technique, the specificity of the spot test needs further evaluation.

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