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The immunofluorescent detection of *Entamoeba histolytica* in pus using avidin–biotin system

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Summary

Based on biotin–avidin interaction, labelled proteins were used to develop an immunofluorescence technique to detect amoebae in samples of pus. Using this technique, *Entamoeba histolytica* has been demonstrated in 18 of 19 pus samples aspirated from amoebic liver abscess. None of the 17 control samples obtained from pyogenic abscess of non-amoebic origin showed the presence of *E. histolytica*. The test was specific, sensitive and easy to perform and is recommended for diagnosis of amoebic liver abscess.

Key words: Biotin–Avidin; Fluorescence; ALA

Introduction

Serological tests to detect amoebic antibody are frequently used in the diagnosis of amoebic liver abscess [1, 2]. Recently innovative sensitive techniques have been used to detect amoebic antigen in the sera [3, 4] and pus [5] which has further facilitated the diagnosis of invasive amoebic disease. Fluorescein-tagged antibodies have also been used to detect *Entamoeba histolytica* on tissues [6], pus [7] and cultures [8]. However, the latter techniques have not been extremely popular because of poor specificity [9]. Recently, the use of biotin–avidin system in the immunoenzymatic [10, 11] and immunofluorescence techniques [12] for various other antigen–antibody systems has enhanced the sensitivity and specificity of these tests. Very little information is available on the use of such a system in the diagnosis of parasitic diseases. Therefore, in the present study, avidin–biotin interaction was used to develop a sensitive and specific immunofluorescence technique for the detection of amoebae in the pus obtained from patients with amoebic liver abscess.

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Materials and Methods

Subjects

An indirect immunofluorescence technique for detection of *E. histolytica* using avidin–biotin interaction was evaluated in pus aspirates of 19 patients with amoebic liver abscess and 17 patients with other pyogenic abscesses. Amoebic liver abscess patients were admitted to the wards of the Department of Gastroenterology, All-India Institute of Medical Sciences, New Delhi. The diagnosis of amoebic liver abscess was confirmed according to WHO recommendations [13]. Besides clinical symptoms, all these patients showed space-occupying lesions in liver scan and/or ultrasound examinations. They were positive for amoebic antibody. The pus aspirated from the liver abscess was sterile for bacterial culture. The pus samples used as negative controls were from the pyogenic abscess of those patients who had inflammatory lesions of subcutaneous tissue and were being treated by the Department of Surgery of this Institute.

Technique

Entamoeba histolytica *antigen*

Amoebic antigen was prepared from cultures of *E. histolytica* strain NIH-200, grown axenically in Diamond's TPS-1 medium by the technique of Diamond [14]. The method of Lowry et al. [15] was used to determine the concentration of proteins in the solution. The antigen was stored frozen at −75 °C in small aliquots of 0.5 ml each.

Biotin-labelling of E. histolytica *antigen*

Biotinyl-*N*-hydroxysuccinimide (BNHS) (Sigma H-1759) was used for introducing biotin moieties into *E. histolytica* antigen using the technique of Guesdon et al. [10]. In short, 10 mg of amoebic antigen was dialysed against 0.1 M $NaHCO_3$ (pH 9.6) overnight at 4 °C and mixed with BNHS solution prepared by dissolving 17 mg of BNHS in 500 µl of *N,N*-dimethyl formamide (Sigma D-4254). The reaction mixture was incubated with an end-to-end mixer at room temperature for one h and then dialysed for 24 h at 4 °C against several changes for phosphate-buffered saline (PBS) (pH 7.2). After dialysis, an equal volume of double distilled glycerol was added and the conjugate was stored frozen at −75 °C in small aliquots. The optimum dilution of the conjugate was determined by using various concentrations.

Avidin–FITC

Fluorescence isothiocyanate labelled with avidin (Sigma A-2901) was used as a linkage molecule for biotin.

Purification of antiamoebic antibody by affinity chromatography

Antibodies to *E. histolytica* were purified by affinity chromatography. Ten mg of antigenic protein from axenically cultivated *E. histolytica* was coupled with CNBr-activated Sepharose-4B as per recommendations of Pharmacia Fine Chemicals, Sweden (Affinity Chromatography, Principals and Methods, p. 15) and stored at 4 °C. Antibodies to *E. histolytica* were isolated with saturated ammonium sulphate from

pooled human serum samples obtained from patients with amoebic liver abscess. These samples had high antibody titre as tested by the ELISA technique [1]. Briefly, the amoebic antigen-coupled Sepharose-4B was washed twice with PBS at 3000 rpm at 4 °C. The partially purified antibodies were incubated with *E. histolytica* antigen at 4 °C on an end-to-end rotary mixer overnight. Excess proteins were washed with PBS 3 times at 3000 rpm for 10 min each. The amoebic antibody was eluted twice with 4 ml of 0.05 M diethylamine on rotary mixer each for 90 min and pooled. The extract was dialysed against 0.1 M carbonate buffer pH 9.6.

Detection of amoebae in the pus

The procedure is outlined in Fig. 1. A thin smear of the aspirated pus was prepared on a glass slide and dried at room temperature. The slides were fixed with acetone (5 min) and washed with phosphate buffered saline (PBS) (pH 7.2). The slides were incubated with 1% bovine serum albumin (Sigma A-9647) in PBS for 10 min and washed with PBS 3 times, each washing of 5 min. As the first step in the assay, the slides were incubated with affinity-purified antibodies to *E. histolytica* (10 μg/ml) for 15 min. All the incubations were carried out at room temperature. The slides were washed thrice as above and incubated with 1:500 dilution of biotinylated *E. histolytica* antigen for 15 min. The slides were washed and finally incubated for 15 min with avidin-FITC. Complete clearance of free FITC was ensured with repeated washings (usually 5 times of 5 min each) and the slides were air dried at room temperature. The slides were observed by fluorescent microscopy (Zeiss photomicroscope III). The pus samples from other pyogenic abscesses served as a control group and were treated exactly in the same manner as for the pus from amoebic liver abscesses.

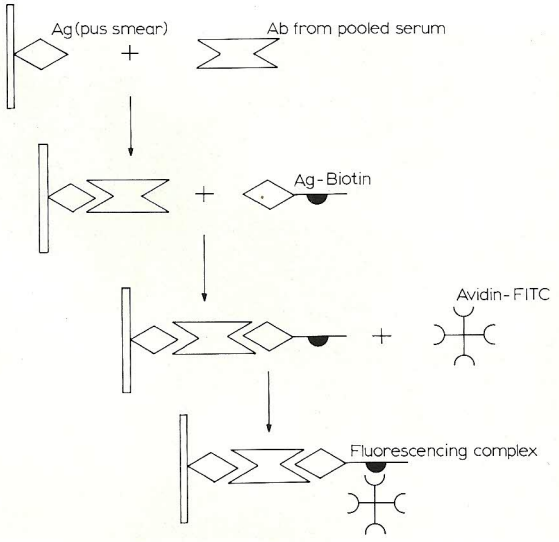

Fig. 1. Outlines of technique used in immunofluorescence detection of *E. histolytica* by avidin–biotin interaction.

Simultaneously, experiments were carried out by using pooled serum with an antibody titre of 1:200 from patients with amoebic liver abscess as an antibody source instead of affinity-purified amoebic antibody in all these patients.

Control tests were carried out in five patients each with amoebic liver abscesses and other pyogenic abscesses substituting phosphate-buffered saline and normal human serum (negative for amoebic antibody) instead of antibodies to *E. histolytica.* In yet another control study, pus from pyogenic abscess of two patients was mixed with amoebae from the sub-culture, a smear was prepared and stained as above.

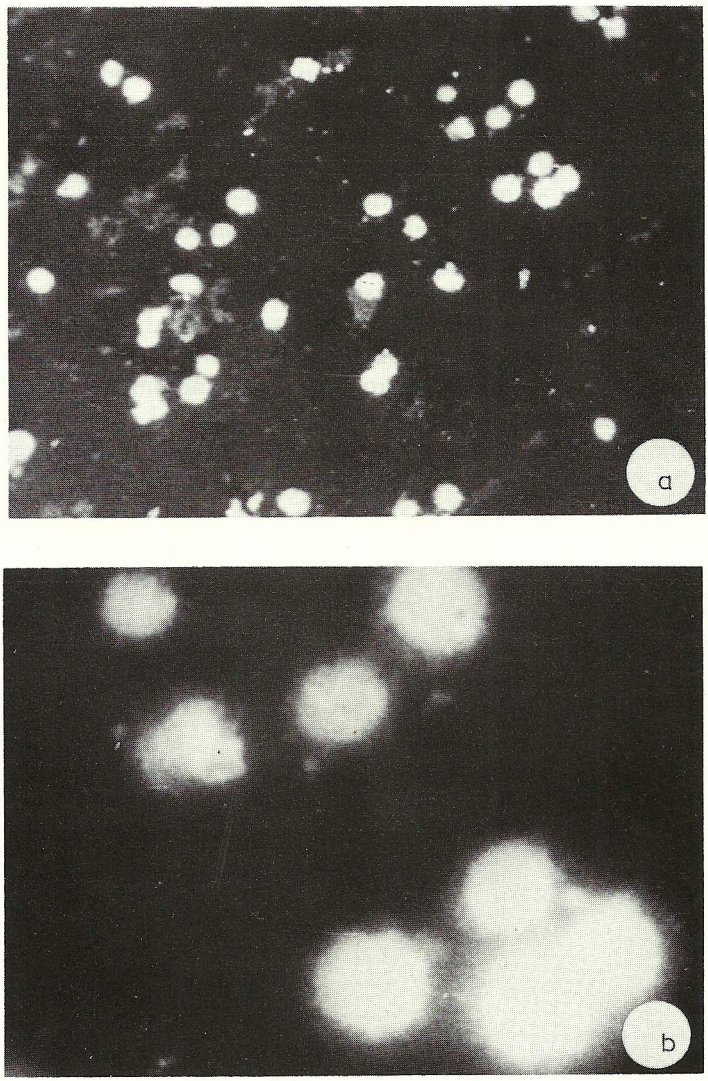

Fig. 2. Immunofluorescence of *E. histolytica* by avidin–biotin interaction in sub-culture is demonstrated under fluorescent microscope. (a) Amoebae (× 100). (b) Amoebae (× 400).

TABLE 1

IMMUNOFLUORESCENT DETECTION OF *E. HISTOLYTICA* IN PUS SAMPLES USING AVIDIN–BIOTIN INTERACTION

Groups	No. tested	No. Positive	% Positive
Amoebic liver abscess	19	18	95
Pyogenic abscess	17	0	0

Results

The slides prepared from sub-culture of *E. histolytica* were stained as control for comparison. The fluorescence in complete amoeba is shown in Fig. 2.

The results of the present study are shown in Table 1. Positive fluorescence showing complete amoebae was seen in 18 of the 19 test samples of pus from patients with amoebic liver abscess (Fig. 3). None of the 17 pus samples from pyogenic abscesses showed positive fluorescence. No difference in results was observed when either affinity purified amoebic antibody or pooled serum containing amoebic antibody was used.

In patients with amoebic liver abscess, when amoebic antibody was substituted with normal human serum or PBS, no fluorescence was seen on amoebae. However, background light fluorescence was observed, uniformly distributed, in patients with

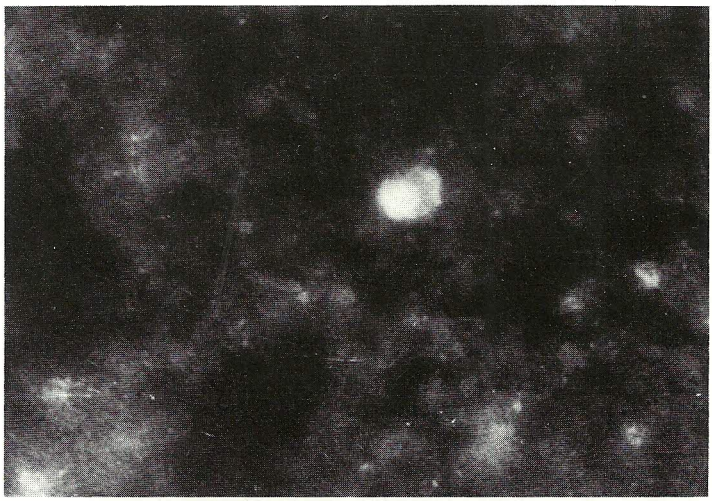

Fig. 3. Immunofluorescence of *E. histolytica* in pus samples from patients with amoebic liver abscess (× 400).

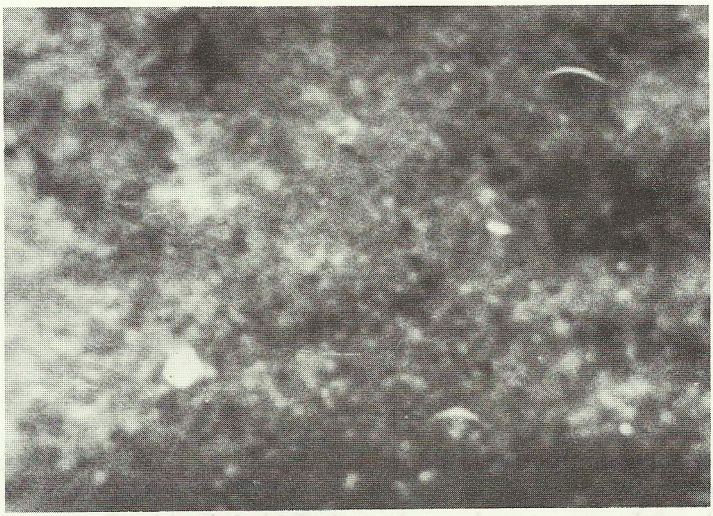

Fig. 4. Background light fluorescence uniformly distributed due to presence of antibodies to *E. histolytica* in pus samples from patients with amoebic liver abscess (× 400).

amoebic liver abscess (Fig. 4). In pus samples from pyogenic abscess, none of the samples showed any light fluorescence in background (Fig. 5). The control study with added amoebae in pus samples from pyogenic abscess showed no change in the character of the pattern and the background except that amoebae were seen giving fluorescence on its surface (Fig. 6).

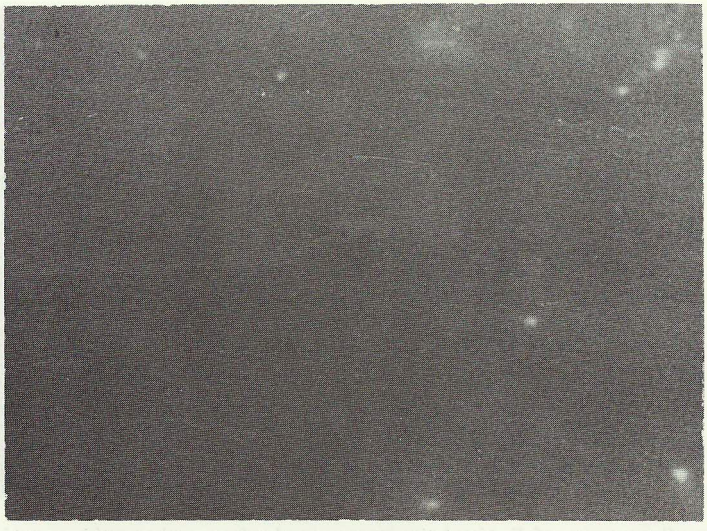

Fig. 5. No fluorescence was seen in pus samples from pyogenic abscess (× 400).

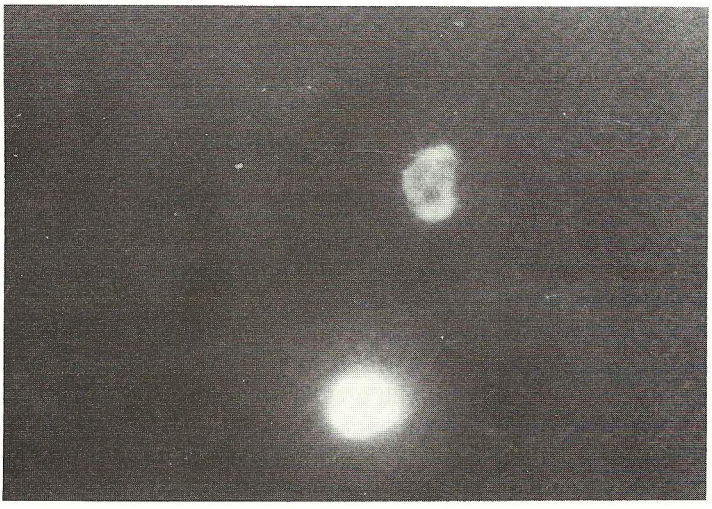

Fig. 6. No fluorescence was seen in pus samples. However, added amoeba from sub-culture could be demonstrated with avidin–biotin immunofluorescence technique (× 400).

Discussion

The present study demonstrates that the avidin–biotin system can be employed in immunofluorescent studies using the principle of double antigen sandwich technique. Using the present technique, the presence of *E. histolytica* could be demonstrated in 95% (18/19) patients with amoebic liver abscess. With 95% sensitivity, the test was definitely an improvement over our previous report of 86% [7], where a direct immunofluorescence technique was used to detect *E. histolytica* in the pus samples. The specificity of the avidin–biotin test was 100% as none of the negative controls, i.e., pus samples from pyogenic abscesses, showed any fluorescence. Pretreatment of the pus samples with normal human serum or phosphate-buffered saline did not allow binding of biotin-labelled antigen and subsequent avidin-FITC bridge resulting in negative fluorescence on the surface of amoebae. Specificity of the test was further confirmed by addition of amoebae to the pus samples from pyogenic abscesses. Figure 5 demonstrates no change in the character of the pattern and the background for fluorescence, with and without added amoebae. However, the amoebae could be picked up with avidin–biotin interaction (Fig. 6).

The failure of avidin–biotin system to pick up one of the 19 patients with amoebic liver abscess for positive fluorescence could be due to the absence of amoebae in the pus. Since invading amoebae in the abscess are usually localised to the peripheral regions, the pus from the central portion of the abscess may not have sufficient concentration of amoebae to be picked up in the smear. The possible amoebic degeneration by antibody-dependent cellular cytotoxicity [16] also cannot be ruled out in this patient as the pus is known to have high levels of complement [17].

The pretreatment of smear with bovine serum albumin helped in reducing the non-

specific background activity. The use of intermediary amoebic antibody and axenically prepared *E. histolytica* antigen as label with biotin made the test more specific and finally strong bridging interaction between the biotin and avidin helped the test to be more sensitive.

Amoebic antibody has been demonstrated to be present in pus from amoebic liver abscess [17, 18]. Since in the present system, the use of *E. histolytica* antigen–biotin label has been used, the presence of amoebic antibodies shows diffused fluorescence in the background (Fig. 4). However, this does not interfere with detection of amoebic antigen in the form of complete amoebae (Fig. 3). Pus from pyogenic abscess did not show any diffused fluorescence in the background.

Avidin–biotin technique is most useful in the demonstration of amoebae in the pus. Usually a positivity rate of up to 85% [19] is seldom reached [20] by an observation of pus under ordinary microscope. The present technique has helped to detect the amoebae in intact form in 95% of patients. The addition of either specific affinity-purified antibody or antibody from pooled human serum did not interfere with the test suggesting that it is not necessary to use purified antibody to run the test. It may be possible that in pus samples from patients with amoebic liver abscess, the amoebae were present in intact form or degraded form. The use of antiamoebic antibodies helped to pick up both. For the purpose of the test, only fluorescence on the intact amoebae was taken as positive. However, this problem was not encountered in pus samples from pyogenic abscesses. The test is quick to perform (less than 2 h) in comparison to 6–20 h by Enzyme Linked Immunosorbent Assay (ELISA) in our lab (unpublished data). In tropical countries like India where amoebic liver abscess is frequently confused with infected hydated cysts and pyogenic liver abscess, the use of this technique to demonstrate the etiological agent in the pus of liver abscess has diagnostic importance.

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