Indian J Med Res 81, June 1985, pp 575-579

Detection of *Entamoeba histolytica* in the pus of amoebic liver abscess by immunofluorescent technique

M. Irshad, B.M. Gandhi, G. Chaudhuri & B.N. Tandon

Department of Gastroenterology & Human Nutrition
All India Institute of Medical Sciences, New Delhi

Revised article received February 22, 1985

An immunofluorescence technique has been developed to detect amoebae in samples of pus. Using this technique, *E. histolytica* has been demonstrated in 12 of 14 pus samples aspirated from amoebic liver abscess, but in none of the 8 control samples from pyogenic abscess of non-amoebic origin. None of the liver biopsy samples obtained from the patients of amoebic liver abscess showed the presence of *E. histolytica*. This technique was found to be simple, quick and reliable to establish the etiological diagnosis of liver abscess.

The definitive diagnosis of amoebic liver abscess is mainly based on a positive serological test for amoebic antibodies. Amoebic antigen as well as amoebic antibodies have also been demonstrated by counterimmunoelectrophoresis (CIEP) technique in pus samples obtained from liver lesions[1,2]. *Entamoeba histolytica* is known to be present in the abscess[3], yet it has rarely been shown in the pus aspirated from liver abscess. Goldman[4] used fluorescein tagged antibody to identify *E. histolytica* and *E. coli* in the cultures. Parelkar *et al*[5] have also used immunofluorescence technique for the staining of *E. histolytica* in the tissues. The present study was undertaken to develop an immunofluorescence technique for the detection of amoebae in the 'pus' obtained from the patients of amoebic liver abscess.

Material & Methods

The test samples of 'pus' were aspirates from patients of amoebic liver abscess whose confirmative diagnosis had been established on the accepted clinical, serological and liver scan/ultrasonographic criteria. The pus samples used as negative controls were from the pyogenic abscess of those patients who had inflammatory lesions of subcutaneous tissues and glands. Liver biopsies were obtained from an area away from the amoebic liver abscess in eight patients.

Isolation and purification of amoebic antibody : Anti-amoebic antibody was isolated from the serum of a patient of amoebic liver abscess who had a high antibody titre by indirect haemagglutination test

(1 : 354294). The isolation was done by the salting out method using ammonium sulphate as the precipitating salt. To 20 ml of the serum, was added solid ammonium sulphate to make a final concentration of 1·03 *M*. The precipitate formed in 6 h at *p*H 7·0 was removed by centrifugation. After adjusting the *p*H of the supernatant to *p*H 7·0, its salt concentration was raised to 1·65 *M*. The protein precipitate which was formed in 5 h was collected and dialysed against 0·05 *M* sodium phosphate buffer, *p*H 7·2. The protein was further purified by chromatography on a Sephadex G.200 column[6].

Conjugation of antibody with FITC : The antibody isolated and purified as described above, was tested for its specificity by CIEP using pure amoebic antigen. Then the antibody was conjugated to fluoroisothiocyanate (FITC) by a slightly modified method of Clark and Shepard[7] Briefly, the antibody preparation was thoroughly dialysed against 0·5 *M* sodium bicarbonate buffer, *p*H 9·5. The *p*H of protein solution was adjusted to 9·5 with 0·01 *M* NaOH solution. The dry FITC powder was dissolved in few drops of the same buffer and then added to protein solution in the ratio of 0·05 mg FITC per mg of protein. Incubation was carried out at 4°C for 24 h followed by thorough dialysis against PBS, *p*H 7·2 to remove unbound FITC. Finally, this conjugate was purified by gel filtration technique on Sephadex G-25 column as mentioned earlier. The conjugate so prepared was preserved at 4°C. Before use, it was diluted with PBS, *p*H 7·2 containing 1 per cent BSA. The ratio of fluorescein : protein in the present conjugate was calculated and found to be 3 : 1.

Detection of amoebae in the pus : A small drop of last aliquot of aspirated pus was put on the glass slide and a thin smear was made. The smear was dried at room temperature and then fixed with acetone (5 min). The smear was then flooded with PBS, *p*H 7·2 containing 1 per cent BSA for 10 min followed by washing with water. Later, it was incubated with diluted conjugate at room temperature for nearly 30 min. After incubation was complete, the slide was washed first with water and then with PBS, *p*H 7·2, at least thrice for 5 min each. Finally the smear was dried at room temperature and observed under fluorescence microscope. The positive test meant the presence of fluorescence in the patients' sample similar to the reference control of axenic amoebic culture. Besides the negative and positive controls, various other controls were also used in this study. In each control, a thin smear of axenic amoebic culture was made and treated differently before final staining. In the first case, the smear was pretreated with normal serum for 30 min before applying the above conjugate. The second smear was first treated with unlabelled specific antibody for 30 min and then stained with the conjugate. In the third case, the amoebic smear was stained with FITC-labelled normal globulin instead of the above conjugate. Controls were used with specifically quenched conjugates.

Detection of amoebic antigen in liver tissue : For the detection of amoebic antigen or antigen-antibody complex in liver tissue samples, a thin section (4μ) of liver biopsy material was fixed on a glass slide and treated with conjugate in an identical manner as described for pus. After washing, the tissue section was mounted

in PBS containing 20 per cent glycerol and observed under UV microscope as indicated.

Protein estimation : The protein content, at different steps of the above method was determined by the method of Lowry *et al*[8] using BSA as standard protein.

Results

The results have been shown in the Table. Positive fluorescence was noted in 12 of the 14 test samples of 'pus'. Two test samples were false negative as both had high amoebic antibody titres diagnostic for amoebic liver abscess. The fluorescence was found to be negative in each of the eight pyogenic negative control pus samples and eight liver biopsy specimens from the patients of amoebic liver abscess. The results of different controls used indicated that there was no change in the fluorescence after treating the amoebae with normal serum. On the other hand, pretreatment of amoebae with unlabelled specific antibody remarkably reduced the fluorescence. Similarly, no fluorescence was seen by treating the amoebae with FITC-labelled normal globulin.

Table. Immunofluorescent detection of *E. histolytica* in the pus and amoebic antigen in liver biopsy samples from patients with amoebic liver abscess

Name	Amoebic antibody (CIEP)	Amoebic antibody (IHA)	Immunofluorescence of the pus for *E. histolytica*	Immunofluorescence of liver biopsy sample for amoebic antigen
MLD	+	1 : 54	+	—
JB	+	1 : 1458	+	—
PS	—	1 : 1458	—	—
JS	+	ND	+	—
SP	+	1 : 1453	+	—
RC	+	1 : 13122	+	—
ND	+	1 : 354294	+	ND
SJ	+	1 : 1458	+	ND
RN	+	1 : 4374	—	ND
RS	—	1 : 162	+	—
GS	+	1 : 486	+	ND
MS	+	1 : 4374	+	ND
GSB	+	ND	+	—
IH	+	1 : 4374	+	ND

The amoebic antibody in serum was tested by counterimmunoelectrophoresis (CIEP) and indirect haemagglutination assay (IHA). The titres as shown in the Table were determined by IHA technique. The identification of amoebae in the pus was done in reference to the positive control of axenic amoebic culture treated with antibody-FITC conjugate identically. ND, test not done

Discussion

The demonstration of the etiological agent in the pus aspirated from the liver lesion of amoebic liver abscess patients has been a major problem. Microscopic detection of live amoebae in fresh 'pus' samples is only rarely possible. The present fluorescence technique demonstrated *E. histolytica* in 85·7 per cent (12/14) of patients with amoebic liver abscess. The absence of fluorescence in pus from all the pyogenic abscess samples indicated the high specificity of this test. The specificity of this test was further confirmed by the results of various controls used. Pretreatment of axenic amoebic culture with normal serum before applying labelled specific antibody did not inhibit the fluorescence. However, pretreatment of amoebic antigen with unlabelled specific antibody abolished the fluorescence when it was subsequently stained with labelled antibody. Similarly, no fluorescence was observed after the treatment of antigen with FITC-labelled normal globulin. These results stress on the high specificity of this test. In fact, the high specificity observed in the present method may be attributed to the high purity of amoebic antibody used for conjugation and a ratio of 3 : 1 for FITC: protein in the conjugate. While the former helps in specific binding of conjugate to amoebae, the latter produces a distinct fluorescence distinguishing the amoebae from other like cellular structure giving a dim fluorescence due to the absorption of conjugate non-specifically.

The negative response in two samples from patients of amoebic liver abscess may be ascribed to several factors. These cases may be either true negative or false negative. In the latter case the patient may have received the treatment that degenerated the amoebae and thus it was too late to do the test to demonstrate degenerated amoebae. Invading amoebae in the abscess are mainly localised in the peripheral region and if the pus for smear was from the most central portion of the abscess, the detection of amoebae may not have been possible. An alternative possibility is that the surface receptors on amoebae were blocked by specific antibodies present in the pus[1,9,10] and prevented binding of the conjugate to amoebae to give the fluorescence. Even the possibility of amoebic degeneration by antibody dependent cellular cytotoxicity[11] cannot be ruled out as the 'pus' is known to have high levels of complement[9]. However, the exact reason of true negative fluorescence at present is not known.

The inflammatory changes in the liver tissue of amoebic liver abscess patients have been suggested to be due to the presence of immune complexes. Such immune complexes between amoebic antigen and antibody have been reported in serum[12-14] and considered to be involved in antigen elimination. It had been assumed that these complexes get deposited in liver tissue also and bring about inflammatory changes on sites other than focal abscess. However, these complexes when tested by the fluorescence method, could not be demonstrated in any of the liver biopsy samples in the present series of patients. The negative fluorescence does not indicate an absolute absence of the complex or free antigen. It is also possible that the complex is present but it does not have exposed antigenic sites to bind the conjugate.

Our results suggest that in referral centres where the facility for fluorescence microscopy exists, this technique would be of much use for the demonstration of etiological agent in the pus of amoebic liver abscess patients. The technique is simple, quick and reliable and can be used for clinical service and research purposes.

Acknowledgment

The authors thank the Norwegian Agency for International Development (NORAD), Norway, for providing financial assistance for this study. Prof. Claus Ola Solberg, Dean, University of Bergen, Norway, assisted this project as a co-investigator for the NORAD Collaborative Study on Amoebiasis at the All India Institute of Medical Sciences, New Delhi.

References

1. Mahajan, R.C., Ganguly, N.K., Chitkara, N.L., Dutta, D.V. and Chhuttani, P.N. Decttion of amoebic antigen in liver pus by counterimmunoelectrophoresis - a preliminary report. *Indian J Med Res* **62** (1974) 1462.
2. Mahajan, R.C., Ganguly, N.K., Chitkara, N.L., Dutta, D.V. and Chhuttani, P.N. Precipitating antibodies against *Entamoeba histolytica* in liver pus - preliminary communication. *Indian J Med Res* **63** (1975) 229.
3. Brandt, H. and Perez Tamayo, R. Pathology of human amoebiasis. *Hum Pathol* **1** (1970) 351.
4. Goldman, M. Use of fluorescein - Tagged antibody to identify cultures of *Entamoeba histolytica* and *Entamoeba coli*. *Am J Hyg* **59** (1954) 318.
5. Parelkar, S.N., Stamm, W.P. and Hill, K.P. Indirect immunofluorescence staining of *Entamoeba histolytica* in tissues. *Lancet* **i** (1971) 212.
6. Irshad, M., Yahiya Khan, M. and Salahuddin, A. Salting-out behaviour of buffalo immunoglobulin-G. *Indian J Biochem Biophys* **18** (1981) 246.
7. Clark, H.F. and Shepard, C.C. A dialysis technique for preparing flourescent antibody. *Virology* **20** (1963) 642.
8. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. Protein measurement with the Folin phenol reagent. *J Biol Chem* **193** (1951) 265.
9. Ravi, V.V., Mithal, S., Malaviya, A.N. and Tandon, B.N. Immunological studies in amoebic liver abscess. *Indian J Med Res* **63** (1975) 1732.
10. Stevens, D.L., Taylor, R.G., Everett, E.D., Owensy, L. and Mc Nitt, T.R. Amoebic liver abscess, Report of a case presenting with non-reactive serologic tests for *Entamoeba histolytica*. *Am J Gastroenterol* **72** (1979) 234.
11. de la Torre, M., Oritiz-Oritiz., L. de Hoz, R. and Sepalveda, B. Action de suero humano immune Y de la gammaglobulina antiamigiana sobre cultives de *E. histolytica*. *Arch Invest Med* (Mex.) **4** Suppl 1 (1973) 567.
12. Onyemelukwe, G.C. and Onyewotu, I.L. Amoebic liver abscess-serum anticomplementary screening for immune complexes. *Trans R Soc Trop Med Hyg* **75** (1981) 613.
13. Hartman, D.P. and Meerowitch, E. Evidence of autologous IgG reacting with antiamoebic antibodies in human sera. *Clin Immunol Immunopathol* **14** (1979) 467.
14. Ganguly, N.K., Mahajan, R.C., Prothasis, A.F., Anand, B.S., Dilawri, J.B., Sharma, S. and Chandonani, R.F. Antigenaemia in cases of amoebic liver abscess. *Indian J Med Res* **71** (1980) 213.

Reprint requests : Dr B.N. Tandon, Professor of Medicine, Department of Gastroenterology and Human Nutrition, All India Institute of Medical Sciences
Ansari Nagar, New Delhi 110029