Characterization of *Entamoeba histolytica* Antigens in Circulating Immune Complexes in Sera of Patients with Amoebiasis

K. Sengupta¹, P.K. Ghosh¹, S. Ganguly², P. Das², T.K. Maitra¹, and K.N. Jalan¹

¹Department of Immunology, Kothari Medical Centre and Research Institute, 8/3, Alipore Road, Calcutta 700 027, and ²National Institute of Cholera and Enteric Diseases, P-33, C.I.T. Road, Beliaghata, Calcutta 700 010, India

ABSTRACT

Isolated circulating immune complexes (CICs) from sera of patients with amoebiasis were characterized to determine *Entamoeba histolytica* antigens that participate in the disease process. In total, 116 serum samples were collected before starting anti-amoebic therapy, and their CICs were isolated by differential polyethylene glycol precipitation. The presence of amoeba-specific antigens in CICs was detected by antigen capture enzyme-linked immunosorbent assay (ELISA) and by immunoblot assay. Antigen capture ELISA showed significantly higher optical density (p<0.001) in all patients with amoebiasis than in the normal healthy controls and patients of non-amoebic hepatic disorder. Immunoblot assay detected amoeba-specific CICs in all 18 patients (100%) with confirmed amoebic liver abscess, 28 (80%) of 35 patients with clinically-suspected amoebic liver abscess, and 18 (78.26%) of 23 patients with amoebic colitis. No patients with non-amoebic hepatic disorders and healthy control subjects had any detectable level of amoebic antigens in CICs. Immunoblot assay revealed *E. histolytica* antigens of relative molecular masses of 35, 56, 70, and 90 kDa present in CICs of 64 of 76 patients with amoebiasis. The 35-kDa polypeptide was observed in 52 patients (81.25%). The results of the study suggest that the 35-kDa polypeptide antigen can be a diagnostic marker in active amoebiasis.

Key words: *Entamoeba histolytica*; Amoebiasis; Circulating immune complexes; Antigens; Antibodies

INTRODUCTION

Infection due to *Entamoeba histolytica* is one of the major health problems in developing countries. Demonstration of circulating antibodies to *E. histolytica* is widely used as a marker of immunodiagnosis for amoebiosis (1-3). However, the presence of anti-amoebic antibodies has no correlation with clinical intensity of the infection (3-5). In endemic areas, anti-amoebic antibodies persist for years even after complete recovery from the disease (1). The antibody response to *E. histolytica* Gal/GalNAC adherence lectin has been observed in patients with amoebiasis (6). The presence of anti-amoebic antibodies does not differentiate present from past infection (5-7). Therefore, the demonstration of amoeba-specific antigens in blood and pus would provide a better and more reliable means of diagnosis of invasive amoebiasis (8-10). Several test methods, such as counter-immunoelectrophoresis (9), enzyme-linked immunosorbent assay (ELISA) (11), and solid-phase radioimmunoassay (12), have been used for detecting *E. histolytica* antigens in circulating immune complexes (CICs) from sera of patients with amoebiasis. Subsequently, the presence of *E. histolytica*-specific
Gal/GalNAC lectin antigen in serum of amoebic liver abscess (ALA) and patients with amoebic colitis has also been reported (13-15).

Elevated levels of CICs have been reported in other parasitic diseases, such as bancroftian filariasis (16), Indian kala-azar (17), and onchocerciasis (18-19). Amoeba-specific antigens in CICs were demonstrated by solid-phase sandwich radioimmunoassay (12,20), anti-amoebic antibody-based micro-ELISA test (11,21), and a spot test on antibody-coated strips of nitrocellulose membrane (22). However, until now, no report is available on the characterization of amoeba-specific antigen(s) in CICs from sera of patients with amoebiasis. This lacuna of information promoted us to characterize amoeba antigens in CICs. In the present investigation, attempts were made to characterize E. histolytica-specific antigenic polypeptides present in CICs in sera of patients with amoebiasis.

MATERIALS AND METHODS

Clinical samples

Serum samples were collected from patients attending the Outpatients Department and/or Inpatients Department of Kothari Medical Centre, Calcutta, India. In total, 116 subjects who were not given any treatment prior to our collection of blood samples were included in the study. Consents were obtained from the patients and from the institutional review board for this study. Patients were categorized into the following five groups:

**Confirmed amoebic liver abscess** (18 patients): Amoebic liver abscess (ALA) was diagnosed based on the following criteria: (i) patients had enlarged tender liver, febrile-associated toxemia, and abscess demonstrated on ultrasound; (ii) patients had fever and pain in the epigastrium; (iii) bacteriologically sterile abscess aspirate; (iv) a positive serology for antibody to amoeba determined by an ELISA; E. histolytica antigen was also detected in aspirate by ELISA; and (v) improvement after treatment with an anti-amoebic drug.

**Suspected cases of amoebic liver abscess** (35 patients): All these patients had enlarged palpable liver, toxemia, fever, and pain in the epigastrium. Ultrasonographically, no abscess was demonstrated, and no aspiration was made in any of these cases. Patients showed high anti-amoebic antibody titre. Of the 35 patients given anti-amoebic treatment, 28 recovered.

**Amoebic colitis** (23 patients): These patients were confirmed by sigmoidoscopy, stool examination, and biopsy examination. Smear of stool was prepared in 0.85% saline and Lugol’s iodine, and was examined for presence of red blood cells within the trophozoite under microscope. The stool samples were inoculated in Robinson’s medium (23) and examined after 48-72 hours for growth of E. histolytica/E. dispar complex trophozoites. E. histolytica cysts were also concentrated by the formal ether method and inoculated in Robinson’s medium, and were observed under microscope for E. histolytica/E. dispar complex trophozoites after 48-72 hours of culture. In these patients, high anti-amoebic antibody titre in serum was observed. E. histolytica antigen in stool was detected by double-antibody sandwich ELISA (24).

**Patients with non-amoebic hepatic disorder** (20 patients): This group comprised four patients with hepatocellular carcinoma confirmed by fine needle aspirate cytology (FNAC) and seven patients with pyogenic liver abscess confirmed by bacteriological examination of pus. In this group, one patient had acute choledochitis, and eight patients had acute viral hepatitis. Although no E. histolytica antigen was detected in liver abscess aspirate, poor anti-amoebic antibody titre in sera was observed.

**Control group** (20 subjects): These patients were aged 22-35 years, and their sex and profession matched those of the patients group. They had no recent history of diarrhoea and dysentery, and stool samples were negative for E. histolytica/E. dispar complex trophozoites under microscopic, which was also confirmed by culture in Robinson’s medium. No E. histolytica/E. dispar-specific stool antigens were detected by double-antibody sandwich ELISA (24).

**Microscopy and culture**

Direct microscopic observation of collected liver abscess pus from patients with amoebic liver abscess revealed that 1 of 18 (5.87%) liver abscess specimens was positive for E. histolytica trophozoites, and their growth in culture medium was also observed (23). In the aspirate of patients with non-amoebic hepatic disorder, we did not observe any E. histolytica trophozoites either by direct microscopic examination or by their growth in culture medium (23).

Smears of stool samples prepared in 0.85% saline and Lugol’s iodine obtained from 18 ALA patients, 35 suspected ALA, and 20 patients of non-amoebic hepatic disorder did not show any E. histolytica/E. dispar complex
trophozoites or cyst under direct microscopic examination or by their growth in Robinson’s medium after 48-72 hours of culture. Direct microscopic examination of stool samples showed *E. histolytica/E. dispar* complex trophozoites and cyst in 19 of the 23 patients with amoebic colitis. Four patients showed only *E. histolytica/E. dispar* complex cyst. Stool culture was positive for *E. histolytica/E. dispar* complex trophozoites in 13 patients.

**Detection of stool antigen**

A monoclonal antibody-based sandwich ELISA was performed to detect stool antigen as described earlier (24). Briefly, a polystyrene 96-well ELISA plate (Nunc, Denmark) was coated with 3 µg/well rabbit anti-*E. histolytica* IgG (found optimal as determined in checker board titration) in carbonate-bicarbonate buffer pH 9.6 (sodium carbonate 1.59 g/L, sodium hydrogen carbonate 2.93 g/L in distilled water) and kept overnight at 4 °C. Excess antibody was removed by washing the plate twice with phosphate-buffered saline pH 7.2 containing 0.05% Tween-20 (PBST). Non-specific sites were blocked with 3% gelatin for two hours. The wells were then washed with PBST, and 100 µL of 1:50 dilution of faecal extract (1 g of faecal sample homogenized in 2 mL of PBS pH 7.2 centrifuged and collected supernatant) from which antigen was to be detected was added and incubated at 37 °C for one hour. The wells were washed thoroughly for four times with PBST and then incubated with 3 µg/100 µL/well (optimal concentration determined by checker board titration) of MAb NICE 11 for one hour at 37 °C. After thorough washings (5 times with PBST), goat anti-mouse IgG labelled with HRP was added and incubated for one hour. The plate was washed thoroughly, and substrate containing 10 µg of *o*-phenylenediamine dihydrochloride and 10 µL of 30% *H₂O₂* per 25 µL was added. This reaction was developed in the dark for 15 minutes, and then optical density was measured at 492 nm.

*E. histolytica/E. dispar* antigen was not present in the stool samples of 18 ALA patients, 35 suspected ALA patients, and 20 patients of non-amoebic hepatic disorder, and showed optical density equal to a normal healthy individual. However, patients with amoebic colitis showed the presence of *E. histolytica/E. dispar*-specific stool antigens 5-10 times higher optical density (p<0.001) than normal healthy individuals.

**Parasite antigen and anti-serum**

Amoeba antigen was prepared from axenic *E. histolytica* (strain HM1: IMSS) grown in TYI-S-33 medium (25). Briefly, *E. histolytica* trophozoites from 48-hour old culture were harvested and suspended in hypotonic buffer (sodium phosphate buffer, pH 7.4 containing 1 mM CaCl₂, 1 mM MgCl₂, 1 mM PMSF, 3 mM NEM, and 2 µM leupeptin). The cell suspension was homogenized using Potter-Elvejhem homogenizer, and the homogenate was then centrifuged at 10,000 g for 30 minutes. The clear supernatant was saved as the soluble antigen, and the yield of total protein was quantitated by the method of Bradford (26) using bovine serum albumin as the standard.

Antibodies to soluble antigen of *E. histolytica* were raised in an albino rabbit (weighing 1.5-2.0 kg) by giving four subcutaneous injections, at an interval of seven days, containing 2 mg of soluble antigen emulsified in an equal volume of Freund’s adjuvant and two intravenous doses with antigen alone. Seven days after the last dose, the rabbit was bled, and serum was separated. Immune sera were pooled, and the antibody titre was determined by ELISA. The immunoglobulin G fraction was purified from the pooled immune serum by affinity column chromatography using protein-A Sepharose CL-4B matrix (24).

**Enzyme-linked immunosorbbent assay**

ELISA was performed to determine anti-amoebic antibody titres in clinical samples (3). Briefly, 96-well microtitre ELISA plates (Nunc, Denmark) were coated with 2 µg/well *E. histolytica* soluble antigen in 50 mM carbonate-bicarbonate buffer pH 9.6 (sodium carbonate 1.59 g/L, sodium hydrogen carbonate 2.93 g/L in distilled water) and kept at 4 °C overnight. The plates were washed twice with PBS pH 7.2 containing 0.05% Tween-20 (PBST), and 200 µL of bovine serum albumin (BSA) was then added to 1% in PBST for one hour. After incubation, the plates were washed twice, and test sera were then added to the first well of each row at 1:50 dilution in PBST containing 1% BSA and applied serial dilutions. The plates were incubated at room temperature for one hour. The wells were then washed four times with PBST, and 100 µL of peroxidase-conjugated anti-human IgG (Jackson Immuno Research Laboratories, USA) was added and incubated for one hour. After thorough washings with PBST, citrate buffer containing 10 µg of *o*-phenylenediamine dihydrochloride (OPD) and 10 µL of 30% *H₂O₂* per 25 mL was then added. The
reaction was developed in the dark for 15 minutes, and optical density was measured in an ELISA reader at 492 nm.

**Polyethylene glycol precipitation of circulating immune complexes**

CICs of clinical samples were isolated by precipitation of sera with polyethylene glycol (PEG MW 8000, Sigma, USA) as described earlier (27). Briefly, 2 mL of serum was mixed with an equal volume of 5% PEG (final concentration 2.5%) in 0.1 M borate buffer, pH 8.5 and incubated at 4 °C overnight. The precipitates were separated by centrifugation at 1,500 g for 45 minutes at 4 °C. Thereafter, the precipitates were washed six times with 2.5% PEG in borate buffer and solubilized in 0.2 mL PBS, pH 7.2. The suspension containing immune complexes was dialyzed against PBS, pH 7.2 and stored at -80 °C in aliquots. The protein content was measured by the method of Bradford (26).

**Antigen capture ELISA**

The method used was a modification of double-antibody sandwich ELISA described earlier (24). Briefly, polystyrene 96-well ELISA plates (Nunc, Denmark) were coated with rabbit anti-*E. histolytica* IgG (2 µg/well affinity-purified IgG was found optimal as determined by checker board titration) in 50 mM carbonate-bicarbonate buffer pH 9.6. After incubation at 4 °C overnight, the wells were washed with PBST, and the non-specific sites were blocked with 2% gelatin (Sigma Chemical, USA) in PBST for one hour at room temperature. To each well, 100 µL of CICs containing 50 µg of immune complexes after thorough washings with PBST was added and incubated for two hours at 37 °C. The wells were washed four times with PBST, and 100 µL of peroxidase-conjugated anti-human IgG (Jackson Immuno Research Laboratories, USA) was added for one hour. After thorough washings with PBST, substrate buffer containing 10 mg of *o*-phenylenediamine dihydrochloride and 10 µL of 30% H₂O₂ per 25 mL was added. The colour reaction was developed in the dark and measured at 492 nm.

**Sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoblot assay**

The protein profiles of CICs were analyzed by 10% polyacrylamide gel under denaturing condition using Laemmli’s discontinuous buffer system (28). Briefly, 100 µg of protein of each CIC sample was boiled in sodium dodecyl sulphate sample buffer. Following electrophoresis, the protein bands were visualized by staining the gel with 0.2% Coomassie blue.

The electrophoretically-separated proteins of CICs were transferred onto nitrocellulose strips (29) and incubated with 3% gelatin to block the non-specific binding sites. The washed strips were incubated with 50 µg/mL of purified IgG of immune rabbit serum for two hours at room temperature. The antigen-antibody reaction was probed by anti-rabbit IgG-HRP (Jackson Immuno Research Laboratories, USA). The polypeptide bands were visualized by colour reaction developed in 0.05% 3-3’-diamino benzene dihydrochloride (Sigma, USA) in 20 mM Tris HCl buffer, pH 7.4 containing 0.03% H₂O₂.

**RESULTS**

**Determination of anti-amoebic antibody titre in sera**

The serum anti-amoebic antibody titres were detected in the patients’ group by ELISA and were plotted in Figure 1. Sera of all patients from confirmed ALA, suspected ALA, and amoebic colitis had an anti-amoebic antibody titre of ≥2000, whereas all the sera from control subjects and patients with non-amoebic hepatic disorders had a titre of ≤2000. Therefore, in our assay, a titre of 2000 was considered as the cut-off limit.
Determination of *E. histolytica* antigen in circulating immune complex

Antigen capture ELISA revealed that all 18 (100%) patients with confirmed amoebic liver abscess had amoebic antigens in CICs with a mean optical density of 2.2; 23 (65.71%) of the 35 suspected ALA patients had amoebic antigen-specific CICs with a mean optical density of 1.20; and 17 (73.91%) of the 23 patients with amoebic colitis showed amoebic antigen in CICs with a mean optical density of 1.55. Amoeba antigen-specific CICs were detected neither in 20 patients with non-amoebic hepatic disorders nor in 20 healthy individuals, the observed mean optical densities of whom were 0.55 and 0.25 respectively.

Immunoblot assay

Immunoblot assay revealed *E. histolytica* antigen-specific major immunoreactive polypeptides at 35, 56, 70, and 90 kDa in CICs obtained from the patients of confirmed ALA, suspected ALA, and amoebic colitis. The presence of amoeba antigen-specific polypeptides was observed in CICs isolated from all 18 patients (100%) of confirmed ALA cases, 28 (80%) of the 35 suspected ALA patients, and 18 (78.26%) of the 23 patients of amoebic colitis. None of the patients with non-amoebic hepatic disorders nor the healthy control subjects showed amoebic antigens in CICs (Table).

Immunoblot reactions showing amoeba-specific antigen polypeptides present in CICs in sera of patients of confirmed ALA, suspected ALA, amoebic colitis, non-amoebic hepatic disorder, and control group respectively are presented in Figure 2. The electrophoretically-separated CIC proteins of ALA subjects transferred onto nitrocellulose strips were also incubated with anti-human IgG HRP (Jackson Immuno Research Laboratories, USA) in a similar manner. However, no polypeptide band was seen (Fig. 3).

<table>
<thead>
<tr>
<th>Table. Recognition of amoebic antigen-specific circulating immune complexes in different groups of patient sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient group</td>
</tr>
<tr>
<td>Confirmed amoebic liver abscess</td>
</tr>
<tr>
<td>Suspected amoebic liver abscess</td>
</tr>
<tr>
<td>Amoebic colitis</td>
</tr>
<tr>
<td>Non-amoebic hepatic disorders</td>
</tr>
<tr>
<td>Healthy control</td>
</tr>
</tbody>
</table>

Fig. 2. Immunoblot assay represents *E. histolytica*-specific polypeptide antigens in CIC obtained from patients with confirmed amoebic liver abscess (Panel A); suspected amoebic liver abscess (B); amoebic colitis (C); non-amoebic hepatic disorder (D); and healthy control (E). Positions of protein molecular weight markers are shown in the left (M). The antigen–antibody reaction was probed by anti-rabbit IgG-HRP.
DISCUSSION

The elevated level of CICs in sera of patients and their disappearance after anti-amoebic therapy during infection due to *E. histolytica* were reported (21). These findings clearly suggest the importance of CICs in diagnosis of current amoebic infection and also relationship of CICs with pathogenesis of amoebiasis. In the present study, an attempt was made to characterize antigenic polypeptides of *E. histolytica* that participate in CICs in sera of patients with amoebiasis.

It was observed that all symptomatic amoebic patients contained an anti-amoebic antibody titre of ≥2000, but sera of non-amoebic patients never showed a titre of more than 2000. Thus, this value can be used as the cut-off limit from amoebic to non-amoebic cases. The presence of serum antibodies to amoebae means tissue invision by *E. histolytica* in the past or present. The prevalence of such antibodies in asymptomatic individuals of an endemic area can differentiate from symptomatic to asymptomatic amoebiasis.

Antigen capture ELISA showed significantly high (p<0.001) *E. histolytica*-specific circulating antigen in CICs of ALA patients, patients of suspected amoebic liver abscess, and patients with amoebic colitis compared to patients of non-amoebic hepatic disorders and normal healthy controls. Thus, antigen capture ELISA was useful in diagnosis of invasive amoebiasis. In consequence to our study, Mohimen *et al.* reported the presence of CICs in patients of asymptomatic *E. histolytica* carriers by sandwich radioimmunoassay and solid-phase antibody-specific RIA method (27). They showed a positivity of 61.53% and 76.92% by sandwich RIA and solid-phase antibody-specific RIA respectively. Vinayak *et al.* (21) reported 55% and 20% amoebic antigen-specific CICs in suspected ALA and symptomatic non-dysenteric intestinal amoebic patients respectively by micro-ELISA and suggested *E. histolytica* antigen-specific CICs in sera of patients with amoebiasis to be a reliable diagnostic approach in amoebiasis (10,11,30). We also observed that affinity-purified anti-*E. histolytica* antibodies did not cross-react with other parasitic antigens, such as *Giardia lamblia*, *Ascaris lumbricoides*, and *Taenia solium* as revealed by ELISA (data not shown). This suggests the specificity of the assay for amoebic antigens.

In total, 64 of the 76 patients of amoebiasis (confirmed ALA, suspected ALA, and amoebic colitis) revealed positive immune reactions at 35, 56, 70, and 90 kDa. However, the presence of 35-kDa polypeptide was observed in 52 patients (81.25%), which probably indicates its important role in systemic immune response during the disease. *E. histolytica*-specific polypeptides were detected in 28 of the 35 clinically-suspected ALA patients, whereas, by ELISA, amoeba-specific antigens were detected in 23 patients only. Thus, immunoblot analysis offered a greater sensitivity than the micro-ELISA test. Moreover, all these 28 patients, who showed amoebic polypeptides in CICs, responded well to anti-amoebic therapy with metronidazole. Whereas, seven patients, who did not show any *E. histolytica*-specific polypeptides in CICs, failed to respond to metronidazole. Five patients with amoebic colitis did not show any amoebic polypeptides in CICs by immunoblot assay. However, *E. histolytica*/*E. dispar* complex trophozoites/cyst were observed under direct microscopic observation and in culture medium; the reason may be the difference in tissue invasion.

Several major amoebic antigens, such as 29/30 kDa peripheral membrane protein (31), 52 kDa serine-rich surface protein (32), 125 kDa variable surface antigen (33), and 170 kDa Gal/GalNAc lectin, have been reported by various authors (34). These reported polypeptides were recognized by immune sera of patients with amoebic liver abscess (31-33). Ravdin *et al.* reported that immunoblotting of *E. histolytica* Gal/GalNAc adherence lectin, purified by monoclonal antibody immunoaffinity chromatography, confirms the immunogenic specificity of preparation where only 170 kDa heavy subunit of lectin was recognized by human anti-amoebic antibodies (6). Further, the presence of *E. histolytica*-specific Gal/GalNAc lectin antigen in the sera of patients with current amoebiasis which disappears after therapy has also been reported (13). In our study, we did not observe any of these reported major antigenic polypeptides present in CICs of patients with amoebiasis, and the reason for this is not known to us. A possible explanation could be that Calcutta is endemic for amoebiasis; so, infection and cure go side by side; anti-amoebic and antibiotic drugs are used indiscriminately, making it difficult to obtain true treatment history.

In summary, we have characterized amoebic polypeptides in CICs in sera of patients with amoebiasis by immunoblot assay. Interestingly, 35-kDa polypeptide antigen was observed in the majority (~81%) of patients with amoebiasis. The significance of recognition of these antigens needs further investigation.

**References**

1. Sengupta K *et al.* J Health Popul Nutr Sep 2002

2. Harpaz N *et al.* J Health Popul Nutr Sep 2002

3. Sengupta K *et al.* J Health Popul Nutr Sep 2002

4. Sengupta K *et al.* J Health Popul Nutr Sep 2002

5. Sengupta K *et al.* J Health Popul Nutr Sep 2002

REFERENCES


