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# Immunoreactivity of *Entamoeba histolytica* antigens with sera from amoebic patients

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**Abstract.** Immune sera from 15 patients with cured amoebic liver abscess were used to recognise the antigens of *Entamoeba histolytica* (HMI) by immunoblotting. The amoebic proteins most frequently recognised by sera from patients with cured amoebic liver abscess had molecular masses of 8, 13, 18, 22, 29, 38, 45, 67 and 94 kDa. Six plasma membrane-associated amoebic proteins of molecular mass 29, 38, 45–67 complex, 85 and 94 kDa were strongly recognised by such sera. Two plasma membrane-associated antigens of 108 and 129 kDa were not recognised by any sera. None of the crude or plasma membrane-associated antigens were recognised by sera from five patients of idiopathic ulcerative colitis, five patients of persistent giardiasis and five normal healthy subjects. Identifiction of such antigens, especially plasma membrane-associated antigens may pave a way to develop specific diagnostic and immunoprotective agents.

## Introduction

Amoebiasis is an important world wide disease caused by enteric protozoan, *Entamoeba histolytica*. The invading *E. histolytica* may produce minimal invasion in the gut or invade host tissue barriers to produce pathological consequences like amoebic colitis or amoebic liver abscess [18]. Interplay of virulence of *E. histolytica* and immune status of host have been ascribed for the clinical consequences of the disease processes [24]. Some degree of protective immunity has been shown to develop in human host following recovery from acute amoebic colitis [19]. Recurrence of an amoebic liver abscess following eradication of the infection is rather unusual in the human host [3]. An absence of any significant correlation in levels of anti-amoebic antibodies and clinical outcome of the disease does not necessarily indicate a failure in the development of the protective immune responses by human host [6, 28]. The development of an amoebic antigen-specific

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lymphocyte proliferative response, the generation of cytotoxic T cell activity directed against *E. histolytica* trophozoites and the production of interferon-γ-activating amoebicidal activity of macrophages in human host have been well documented [18–20]. We earlier have reported anti-plasma membrane (PM) amoebic antibodies as well as anti-PM antibody-dependent macrophage mediated cytotoxicity to *E. histolytica* modulating the amoebic infections in experimental hosts [21, 27, 29]. The immunization of the experimental host with amoebic PM proteins have also been shown to eliciate specific immune responses, resulting in protection to the experimental host upon intra-hepatic challenge with virulent *E. histolytica* [16, 26]. The *E. histolytica* trophozoites have been shown to be a complex mosaic of antigens [7, 12, 25]. Although monoclonal antibodies have been developed and used to identify amoebic antigens [13, 15, 22], the relevance of such antigenic molecules recognised by the monoclonal antibodies in relation to diagnosis or immunoregulation of the disease remained undefined. The precise antigenic determinents/polypeptides responsible either for elicitation of the immunoprotective response or for specific diagnosis have not been elucidated. Thus, the present study was carried out to investigate the recognition of the PM-associated antigens of *E. histolytica* by sera from patients with a cured amoebic liver abscess as a first step to identify immuno-relevant antigen(s).

## Materials and methods

### *Parasite and preparation of antigens*

Axenic *E. histolytica* (HM1) trophozoites were grown in trypticase panmede serum (TPS-1) medium [4]. A 48- to 72-h growth of *E. histolytica* trophozoites harvested in phosphate-buffered saline (PBS) pH 7.2 was sonicated at 23 kc/s with ten, 15-s bursts in MSE (Measuring and Scientific Equipments, UK) ultrasonic disintegrator [25]. The sonicated material was labelled as crude amoebic extract (CAE) antigen. The protein content of the CAE was determined [10].

### *Preparation of PM antigen*

PM was prepared from axenic *E. histolytica* (HM1) as described earlier [1]. Briefly, the *E. histolytica* trophozoites harvested and washed in PBS pH 7.2 were resuspended in a $2 \times 10^7$ cells/ml aliquot in PBS, pH 7.2 containing 10 mM $MgCl_2$ and 1 mg of concanavalin A (Con A). The cells were centrifuged to remove excess of Con A. The cell pellet was treated with phenylmethyl sulphonyl fluoride and $MgCl_2$. The homogenised cells were layered on a mannitol-sucrose gradient. The pellet was treated with 1 M α-methyl mannoside, homogenised and layered on 20% sucrose gradient. The vesiculated PM was collected from top of the gradient and concentrated by lyophilization. The purity of PM was checked by measuring the $Ca^{++}$-depenent ATPase activity [11]. The protein content of the PM was also estimated [9].

### *Clinical samples*

A total of 34 serum samples were collected from patients with confirmed and cured amoebic liver abscess, non-dysenteric amoebic colitis, other non-amoebic intestinal disorders like idiopathic ulcerative colitis, giardiasis and also from healthy subjects. The criteria for diagnosis of these subjects were as follows:

1. Confirmed amoebic liver abscess (15 patients). These patients had an enlarged tender liver and associated toxemia, demonstrable abscess on ultrasound, aspiration of anchovy sause pus which was either sterile on bacteriological examination or revealed *E. histolytica* trophozoites; had anti-amoebic antibody titres ($>1:64$) as determined by the indirect haemagglutination (IHA) technique and clinical recovery following specific anti-amoebic treatment with metronidazole and emetine.
2. Non dysenteric amoebic colitis (4 patients). These patients had gastrointestinal symptoms like vague abdominal pain, none had dysentery but their stool samples revealed the presence of *E. histolytica* cysts by formol ether method of concentration. All had anti-amoebic antibody titre ($>1:64$) in their sera.
3. Non-amoebic intestinal disorders (10 patients). This includes 5 patients of idiopathic ulcerative colitis confirmed by sigmoidoscopy and histological changes in biopsies, 5 patients of giardiasis having *Giardia lamblia* cysts or trophozoites in their stool samples. None had anti-amoebic antibodies in their sera as determined by IHA.
4. Apparently healthy individuals (Five subjects). These subjects were adults aged 20–30 years and resident of India since birth; had no ostensible symptoms and their physical examination revealed them to be normal. Repeated stool examination did not reveal trophozoites or cysts of *E. histolytica*; none had anti-amoebic antibodies in their serum samples.

### *Anti-amoebic hyperimmune serum (HIS)*

Anit-amoebic (HIS) was raised in BALB/c mice. Each mouse was given four subcutaneous injections of 0.2 mg of CAE protein per dose at weekly intervals. The first dose was emulsified in Freund's complete adjuvant, while the other three doses were emulsified in Freund's incomplete adjuvant. A week later the mice were bled and the pooled serum was labelled as anti-amoebic HIS.

### *Indirect haemagglutination test (IHA)*

Anti-amoebic antibody levels of amoebic patient and control sera were determined by IHA as described elsewhere [25] employing CAE as antigen.

### *Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)*

SDS-PAGE was carried out as described earlier [8]. Briefly, 50 μg of CAE protein or 25 μg of PM proteins/lane were subjected to electrophoresis on a 5% stacking and 10% separating gels under reducing and denaturing conditions in a electrophoretic cell (Bio Rad Laboratories, USA) at 25 mA current for 4 h.

### *Immunoblotting with patient or control sera*

Resoluted proteins on gels were either stained with 0.25% Coomassie blue or transferred to nitrocelloluse paper (NCP) of 0.2-μm pore diameter in a transblot cell (Bio Rad Laboratories, USA) at 200 mA current for 3 h [23]. The sheet was cut into strips and the transfer efficiency of amoebic proteins was checked by 0.1% amido black staining. The remaining protein-binding sites on the NCP were blocked with 3% bovine serum albumin (BSA) in Tris saline pH 7.4 for 3 h at 4°C. Strips were treated with 1:100 diluted anti-amoebic HIS or 1:50 diluted sera from either patient or control subject in Tris/saline containing 0.1% BSA for 3 h at 4°C. Strips were then washed thrice with Tris/saline containing 0.01% Nonidet-P-40. Strips treated with anti-amoebic HIS were incubated with 1:3,000 diluted anti-mouse immunoglobulin-horseradish peroxidase (HRP); IgG + A + M) conjugate (DakoPatts, USA), whereas strips treated with the amoebic

patient or control human sera were incubated with 1:3,000 diluted anti-human immunoglobulin – HRP (IgG + A + M) conjugate (The Binding Site Ltd., UK) for 1 h at 4°C. Strips were again washed with Tris/saline and reactions were developed with 4-chloro-1-naphtol (Sigma Chemical Co., USA) as substrate. The specificity of the antigens recognised was confirmed by failure of the sera from patients with confirmed *G. Lamblia* and from normal human subjects to detect *E. histolytica* antigens in immunoblots. The molecular weight standards used were carbonic anhydrase (29,000 Da) ovalbumin (45,000 Da) and BSA (66,000 Da). The molecular mass of amoebic antigens recognised were estimated by using logarithmic plot of migration of standards.

## Results

### *Membrane purity*

The specific enzyme activity of calcium-dependent ATPase of crude extract of *E. histolytica* trophozoites exhibited a mean value of 0.030 units (μmol inorganic phosphate released/min per mg protein) in four experiments (range 0.013–0.072 units) and the corresponding mean values for PM preparation was 0.132 units (range 0.100–0.240).

### *Antigen recognition*

A total of nine major amoebic polypeptides were recognised in crude amoebic extract by all the 15 sera from patients with cured amoebic liver abscess. These had a molecular mass of 8, 13, 18, 22, 29, 38, 45–67 and 94 kDa (Fig. 1, lanes B–D). The sera from non-dysenteric amoebic colitis also recognised the identical amoebic

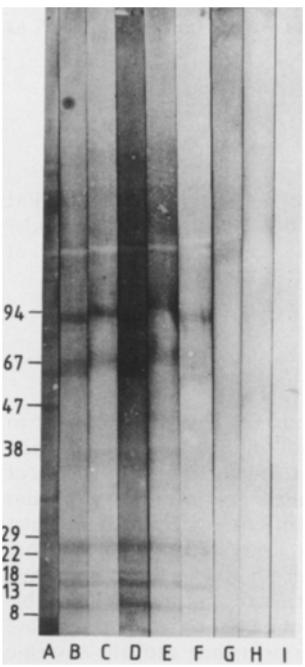


**Fig. 1.** Immunoblot of crude amoebic extract antigen (CAE) of *E. histolytica* (HM1). *Lane A:* Anti-amoebic hyperimmune serum. *Lanes B–D:* sera from patients with cured amoebic liver abscess. *Lanes E–F:* Sera from non-dysenteric amoebic colitis patients. *Lane G:* Serum from patient of giardiasis. *Lane H:* Serum from patient of idiopathic ulcerative colitis. *Lane I:* Pool serum from normal healthy subjects. Note that no antigen was recognised in *lanes G–I*

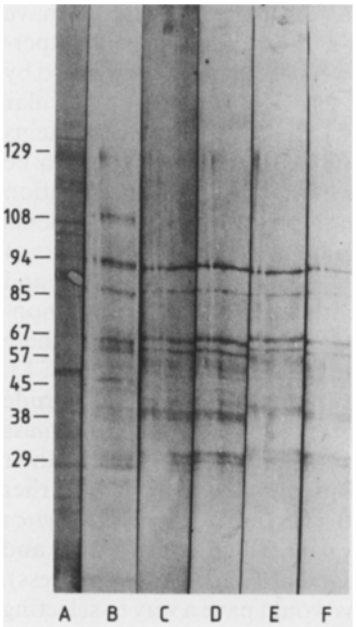


**Fig. 2.** Immunoblot of purified plasma membrane (*PM*)-associated antigens of *E. histolytica* (HM1). *Lane A:* Crude amoebic antigens recognised by anti-amoebic hyperimmune serum. *Lane B:* PM antigen recognised by anti-amoebic hyperimmune serum. *Lanes C–E:* Sera from patients with cured amoebic liver abscess. *Lane F:* Serum from non-dysenteric amoebic colitis patients

polypeptides (Fig. 1, lanes E, F). The sera from patients with idiopathic ulcerative colitis (Fig. 1, lane G), giardiasis (Fig. 1, lane H) and normal healthy subjects (Fig. 1, lane 1) did not recognise any of the amoebic proteins. The major PM-associated polypeptides recognised by all the 15 sera from patients with cured amoebic liver abscess had a molecular mass of 29, 38, 45–67 complex, 85 and 94 kDa (Fig. 2, lanes C–E). Sera from patients of non-dysenteric amoebic colitis also identified similar antigens (Fig. 2, lane F). The amoebic proteins of molecular mass 8, 13, 18 and 22 kDa recognised by sera from the patients with cured amoebic liver abscess in crude amoebic extract antigen were not recognised in the PM preparation (Fig. 2, lanes C–E) PM antigens of molecular mass 108 and 129 kDa were not recognised by any of the sera from clinical amoebic patients.

## Discussion

We have used 15 sera from patients with cured amoebic liver abscess as a probe to identify serologically reactive antigens of *E. histolytica*. Most of the previous studies have used whole amoebic antigens or soluble amoebic proteins in immunoblots with patient sera [2, 5]. However, precise surface or subcellular location of the important immunogenic antigens of *E. histolytica* remained unclear. It is likely that certain potentially potent antigens of PM may remain masked in soluble whole cell lysates. The PM of *E. histolytica* have been shown to contain 18 antigens ranging from 12 to 200 kDa [1]. Aust-Kettis et al. [2] identified at least six of seven antigenic polypeptides as components of the surface of *E. histolytica* by immunoaffinity chromatography with human sera. *E. histolytica* surface glycoproteins of molecular mass 20, 30, 81 and 150 kDa have been

implicated as major and important components of the parasite [14]. We have earlier shown immunoregulatory functions of PM-associated antigens in experimental amoebiasis [26, 29]. Nine amoebic antigens were found to be recognised by immune human sera of which four antigens of 23, 24, 37 and 90 kDa molecular mass were found to be surface-associated proteins [5]. Recently, amoebic proteins of molecular mass 24, 50, 70, 90, 112, 160 and 210 kDa have been shown to be surface-associated adhesins [17]. In our study we have employed a PM preparation along with crude amoebic extract antigens in immunoblots with sera from patients with cured amoebic liver abscess to identify most immunoreactive components of *E. histolytica*. Our data indicated recognition of 29, 38, 45–67 complex, 85 and 94 kDa proteins on the PM of *E. histolytica*. None of the serum samples from non-amoebic patients or apparently healthy subjects could recognise any of these antigens. We further noticed nine major amoebic antigens of molecular mass 8, 13, 18, 22, 29, 38, 45, 47 and 94 kDa to be recognised by immune human sera in crude amoebic antigen preparation. However, antigenic proteins with a molecular mass of 8, 13, 18 and 22 kDa appeared to have cytosolic localisation as indicated by their absence in immunoblot experiments with the PM preparation. We have earlier observed that the 29 kDa-protein is a major PM-associated antigen of *E. histolytica* [28] and have recently developed and used monoclonal antibodies to identify and characterise the 29-kDa surface-associated PM antigen of *E. histolytica* (in press). We feel that identification of PM-associated antigens would pave a way to selecting potentially immunogenic amoebic protein(s) which can be used as a candidate immunoprotective/diagnostic agent in future.

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