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FEMS Immunology and Medical Microbiology 27 (2000) 23–30

FEMS IMMUNOLOGY AND MEDICAL MICROBIOLOGY

www.fems-microbiology.org

Partial characterization of a 36-kDa antigen of *Entamoeba histolytica* and its recognition by sera from patients with amoebiasis

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Received 19 February 1999; received in revised form 19 July 1999; accepted 30 July 1999

Abstract

A 36-kDa antigen of axenically grown pathogenic *Entamoeba histolytica* (HM1-IMSS) was eluted from the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-resolved crude amoebic extract antigens. The immunoreactivity of this partially purified 36-kDa antigen with monoclonal antibody (MoAb) $3D_{10}$ altered significantly ($P < 0.01$) after heat and trypsin treatment but remained unaltered after treatment with sodium metaperiodate ($P > 0.5$), thereby indicating the protein nature of the epitope recognized by MoAb $3D_{10}$. The epitope was found to be localized on the surface as well as in the cytoplasm of the *E. histolytica* trophozoites with the majority of it in the cytoplasm. In addition, this epitope was also found to be present on the cyst form of the parasite. The 36-kDa molecule was recognized by the sera from 29 (85%) of the 34 patients with amoebic liver abscess and five (83%) of the six patients with amoebic colitis. No serum samples from asymptomatic cyst passers, from patients with non-amoebic hepatic or intestinal disorders and apparently healthy subjects had antibodies that reacted with this 36-kDa molecule. The immune responses in man to this 36-kDa amoebic molecule indicate a potential specific role for this molecule in invasive amoebiasis.

Keywords: Amoebiasis; Amoebic coproantigen; Amoebic liver abscess; *Entamoeba histolytica*

1. Introduction

Amoebiasis is an important world wide disease caused by an enteroinvasive protozoan parasite, *Entamoeba histolytica*. *E. histolytica* infection results in approximately 50 million cases of invasive amoebiasis and up to 100 000 deaths every year [1]. Cytolysis of the target cells by *E. histolytica* is initiated by the adhesion of *E. histolytica* trophozoites through surface lectins via specific receptors [2], followed by the cytolysis of the target cells through the action of various virulence factors like proteolytic enzymes [3–6], cytotoxins [7,8] and pore forming peptides [9]. Thus, surface-associated molecules and various cytotoxic molecules are important in mediating the host parasite interactions. However, studies of the individual antigens and their biochemical and immunological properties are lacking, with the exception of a few, like the 220-kDa protein that is inhibited by *N*-acetyl-D-glucosamine [10], *N*-acetyl galactosamine inhibitable adherence lectin [11], a 125-kDa surface antigen [12], 96-kDa protein [13] and a 29-kDa surface-associated protein [14].

Complete biochemical and immunological characterization of various amoebic antigens is of paramount importance in understanding the pathogenesis and the immunoregulation of the disease. Furthermore, these studies would be helpful in selecting the potential vaccine candidates and the antigens important for the diagnosis/prognosis of the disease. Therefore, the precise antigenic determinants/polypeptides responsible for either elicitation of the immunoprotective response or for the specific diagnosis need to be elucidated. Recently, we identified a 36-kDa amoebic coproantigen by monoclonal antibodies (MoAbs) and showed its potential to diagnose the current amoebic infection in an enzyme-linked immunosorbent assay (ELISA) system [15]. The present study was carried out to partially characterize this 36-kDa amoebic coproantigen and to investigate its recognition by serum samples from patients with amoebiasis.

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0928-8244/00/$20.00
PII: S0928-8244(99)00156-X

2. Materials and methods

2.1. Parasite and the preparation of antigen

Axenic *E. histolytica* (HM1-IMSS) was grown in TYIS-33 medium [16]. A 48–72-h growth of *E. histolytica* trophozoites harvested in phosphate-buffered saline (PBS) pH 7.2 was sonicated at 23 Kc/s with six 30-s bursts in MSE (Measuring and Scientific Equipments, UK) ultrasonic disintegrator [17]. To the sonicated material, 0.5% of Triton X-100 was added and the material was kept at 4°C for 2 h. This material was labelled as 'crude amoebic extract' (CAE) and to it, phenylmethylsulfonylfluoride (1 mM), *N*-(*N*-(L-3-transcarboxirane-2-carbonyl)-L-leucyl)-agmatine (E-64, 20 μM) and ethylenediamine tetraacetic acid disodium salt (EDTA, 2 mM) were added. The protein content of the material was determined [18].

2.2. MoAbs

A panel of MoAbs developed by us was used to identify the epitope on 36-kDa amoebic antigen. A MoAb $3D_{10}$ belonging to the IgG_1 isotype recognized a 36-kDa amoebic coproantigen in stool supernatant from confirmed cases of amoebiasis and was used in this work. Further, it also recognized three polypeptides with approximate molecular masses of 36, 25 and 17 kDa in the Western immunoblots of the CAE [15]. MoAb producing cells were propagated as ascites in the peritoneal cavities of the pristane-primed Balb/c mice. The ascitic fluid was drained and centrifuged at $7500 \times g$ for 20 min to settle the hybrid cells and the peritoneal exudate cells. The MoAb from the ascitic fluid was purified by affinity chromatography by using protein G-Sepharose-4B fast flow column and finally suspended in PBS, pH 7.2 [19].

2.3. Partial purification of 36-kDa antigen

The 36-kDa amoebic antigen was partially purified by excision from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-resolved CAE proteins on the polyacrylamide gels by using a molecular mass marker which co-migrated with the protein of interest. Briefly, CAE proteins were resolved by SDS-PAGE on a preparative gel. A small slice of the gel corresponding to 36-kDa antigen migration was cut from the gel. The antigen from the gel was eluted out by using the elution buffer (50 mM Tris, 1% SDS, 100 mM NH_4HCO_3, 0.1 mM EDTA and 150 mM sodium chloride, pH 8.8) [20]. The antigen thus eluted was desalted and concentrated by ultrafiltration using filter with a cut off molecular mass of 10 kDa.

2.4. SDS-PAGE and Western immunoblotting

The integrity, purity and the immunoreactivity of this partially purified antigen were checked by rerunning this antigen on SDS-PAGE [21] and by Western immunoblotting [22]. Briefly, 100 μg of CAE protein/lane and 10 μg of partially purified 36-kDa antigen/lane were subjected to SDS-PAGE on 4% stacking and 10% separating gels under reducing and denaturing conditions in an electrophoretic cell (Bio-Rad Labs, USA) at 25 mA for 4 h. Resolved proteins from the gels were either stained with Coomassie blue or transferred on to nitrocellulose papers (0.22-μm pore size) at 200 mA for 3 h. The strips were blocked with 3% bovine serum albumin (BSA). Strips containing the CAE antigens were incubated with mouse anti-CAE antibodies and the strips containing the partially purified 36-kDa antigen were incubated with either mouse anti-CAE antibodies or with MoAb $3D_{10}$, followed by incubation with anti-mouse immunoglobulin-HRP conjugate (Bio-Rad Labs, USA). The color reactions were developed with diaminobenzidine (Sigma Chemicals, USA) as the substrate (5 mg 3,3′-diaminobenzidine in 10 ml PBS, pH 7.2, and 5 μl H_2O_2).

2.5. Physico-immunochemical characterization of 36-kDa antigen

Characterization of 36-kDa antigen was achieved by measuring its immunoreactivity to MoAb $3D_{10}$ following exposure to (a) heat, (b) trypsin and (c) sodium metaperiodate in a micro-ELISA system [14]. The sensitivity to heat was determined by exposing this 36-kDa antigen to 60 or 100°C (boiling) for 10 min before coating on to the wells of the ELISA plate. The effect of the proteolytic digestion was determined by treating wells coated with 36-kDa antigen with trypsin at a 1.0, 10, 100 or 1000 μg ml^{-1} concentration in pBS, pH 7.2, at 37°C for 2 h. The wells containing untreated antigen served as controls. The periodate modification of the 36-kDa amoebic antigen was accomplished by treating the antigen-coated wells with 0.025, 0.05, 0.1 or 0.2 M sodium metaperiodate in 20 mM sodium acetate buffer pH 4.5, in the dark at 4°C for 24 h. The antigen-coated wells treated with acetate buffer alone served as controls. The altered immunoreactivity of the treated antigen was assessed by incubating with MoAb $3D_{10}$ and then with anti-mouse-HRP conjugate. The reaction was developed with ortho-nitro-phenylene diamine (OPD) as substrate (5 mg OPD in 10 ml of 0.15 M citrate phosphate buffer, pH 5.0, and 5 μl H_2O_2) and the optical density (OD) was measured at 492 nm in an ELISA reader. The effect of heat, trypsin and sodium metaperiodate treatments was determined by comparing OD values of the treated wells with that of the untreated control wells. Each experiment was set up in triplicate and was repeated at least twice. The data were expressed as mean ± S.D. and were analyzed by one way ANOVA by critical difference formula.

2.6. Localization of the epitope recognized by the MoAb $3D_{10}$ on E. histolytica trophozoites and cysts

Localization of the epitope recognized by the MoAb $3D_{10}$ was investigated by studying the immunofluorescence pattern of the live trophozoites and fixed and permeabilized trophozoites of *E. histolytica* by flow cytometry. In addition, immunofluorescence patterns of cysts of *E. histolytica* (isolated from the confirmed cases of intestinal amoebiasis) following incubation with MoAb $3D_{10}$ were also investigated. Briefly, 10^6 trophozoites of *E. histolytica* were harvested and washed with PBS, pH 7.2. The cells were divided into two sets. The first set of cells was kept as such whereas the second set of cells was added to 70% chilled ethanol in PBS, pH 7.2, dropwise and slowly with shaking. The cells were kept at room temperature for 30 min, washed with PBS, pH 7.2, and labelled as fixed and permeabilized cells. The cells of both sets were incubated with 1:10-diluted MoAb $3D_{10}$, followed by incubation with 1:100-diluted FITC-conjugated anti-mouse antibodies (Becton and Dickinson, USA). The cells were suspended in 250 μl of PBS, pH 7.2, and the immunofluorescence was analyzed in CELL QUEST software of FACScan (Becton and Dickinson, USA). The *E. histolytica* trophozoites incubated with anti-CAE antibodies or with normal peritoneal fluid were employed as positive and negative controls, respectively. For studying the localization of the epitope on the cysts of *E. histolytica, E. histolytica* cysts were isolated from the stool samples from the confirmed cases of intestinal amoebiasis by the zinc sulfate flotation method [23]. The immunofluorescence procedure was the same as described for the live amoebic trophozoites. In order to check the cross reactivity of the MoAb $3D_{10}$, cysts of *Entamoeba coli* were included as controls.

2.7. Clinical samples

Serum samples were collected from patients with confirmed amoebic liver abscess, non-amoebic hepatic disorders, confirmed symptomatic intestinal amoebiasis, asymptomatic intestinal amoebiasis, non-amoebic intestinal disorders and apparently healthy subjects. The criteria for diagnosis of these subjects were as follows:

1. Confirmed amoebic liver abscess ($n = 34$). These patients had an enlarged tender liver and associated toxaemia, abscess demonstrated on ultrasound and aspiration of the abscess yielded 'anchovy sauce' pus which was either sterile on bacteriological examination or revealed *E. histolytica* trophozoites. They had an anti-amoebic antibodies titer of $>1:256$ as determined by an indirect haemagglutination (IHA) technique and there was clinical recovery after specific anti-amoebic treatment with metronidazole and emitine.
2. Symptomatic intestinal amoebiasis ($n = 6$). These patients had gastrointestinal symptoms like pain in abdomen, flatulence and/or diarrhea. None had dysentry but their stool samples revealed the presence of *E. histolytica* cysts by the formal ether concentration method [23]. All had anti-amoebic antibodies levels of $>1:256$ in their sera by the IHA technique. They responded to the anti-amoebic chemotherapy.
3. Non-amoebic hepatic disorders ($n = 8$). These included four patients with pyogenic liver abscess confirmed by the bacteriological examination of the pus and four with hydatid disease confirmed at laparotomy. None of these patients had detectable anti-amoebic antibodies in their sera.
4. Non-amoebic intestinal disorders ($n = 5$). This group included three patients with giardiasis having either *Giardia lamblia* cysts or trophozoites in their stool samples, one patient with *Entamoeba coli* and one patient with *Entamoeba hartmanni* in their stool samples but negative for *E. histolytica* cysts on three consecutive days of examination by the formal ether concentration method. None had anti-amoebic antibodies in their sera as determined by IHA.
5. Apparently healthy subjects ($n = 19$). These were adults of 20–30 years, resident in India since birth. They had

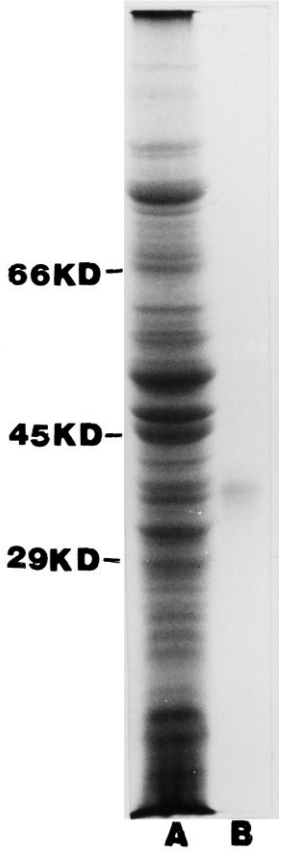

Fig. 1. SDS-PAGE of *E. histolytica* trophozoite antigens. Lane A: CAE proteins. Lane B: 36-kDa amoebic antigen. CAE and the partially purified 36-kDa amoebic antigen were run on 4% stacking and 10% separating gels and were stained with Coomassie blue. Molecular mass markers are indicated on the left.

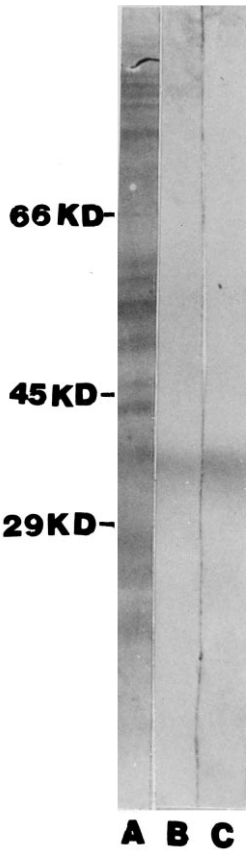

Fig. 2. Western immunoblots of the CAE antigens and the partially purified 36-kDa amoebic antigen. Antigens were run on 10% SDS-PAGE, transferred onto the nitrocellulose sheets and then, the strips were probed with the relevant antibodies. Lane A: CAE antigens reacted with anti-CAE antibodies. Lane B: partially purified 36-kDa amoebic antigen reacted with anti-CAE antibodies. Lane C: purified 36-kDa amoebic antigen reacted with MoAb $3D_{10}$. Molecular mass markers are indicated on the left.

no symptoms and the physical examination suggested that they were healthy. Repeated stool examination did not reveal trophozoites or cysts of *E. histolytica*. None had anti-amoebic antibodies in their serum samples.

6. Asymptomatic amoebic cyst passers ($n = 4$). These subjects did not have any symptom pertaining to the gastrointestinal tract and their stool examination revealed the presence of the *E. histolytica* cysts on three consecutive days of examination by the formal ether concentration method. None had anti-amoebic antibodies in their serum samples.

2.8. Recognition of 36-kDa protein by human serum

Each serum sample was tested against the 36-kDa antigen in an ELISA system [14]. Briefly, ELISA was performed in 96-well microtitration plates (Costar Corporation, USA) by coating wells with 100 μl of optimally diluted partially purified 36-kDa antigen (2.0 μg ml^{-1}) in 0.05 M carbonate buffer, pH 9.6. After overnight incubation at 4°C, the plates were washed with 0.15 M PBS, pH 7.2, containing Tween 20 (0.05%) (PBS-T) and the non-specific sites were blocked by adding 200 μl of 3% BSA in PBS, pH 7.2. To each well, 100 μl of 1:400-diluted patient serum was added and the plates were incubated at 37°C for 2 h. The wells were washed with PBS-T and 100 μl of 1:1000-diluted HRP-conjugated anti-human antibodies was added. The plates were again incubated at 37°C for 1 h. The plates were washed with PBS-T and the reaction was developed by adding 100 μl of OPD (5 mg in 10 ml of 0.15 M citrate phosphate buffer, pH 5.0, +5 μl H_2O_2) as the substrate. The reaction was terminated by adding 50 μl of 6 N H_2SO_4 and the OD was measured at 492 nm in an ELISA reader.

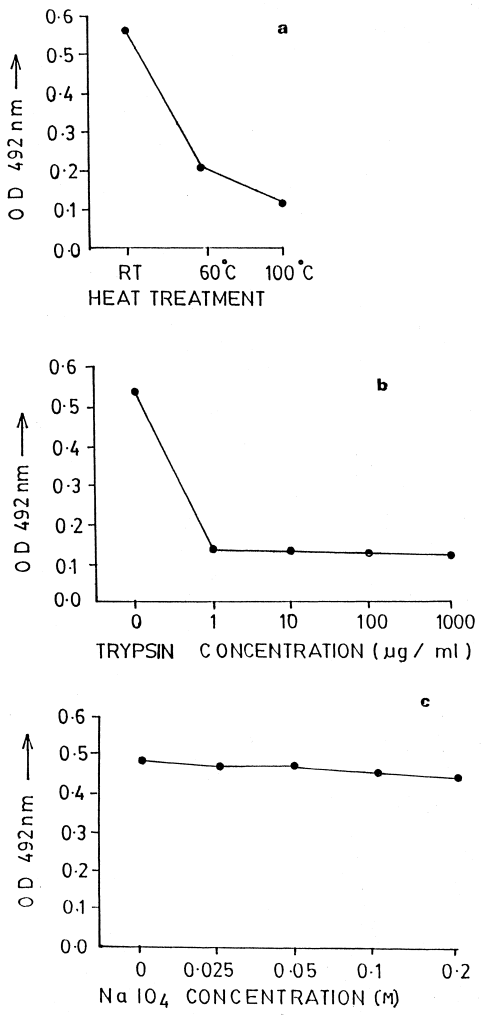

Fig. 3. Immunoreactivity of the partially purified 36-kDa molecule after (a) heat treatment, (b) proteolytic digestion with trypsin, (c) sodium metaperiodate oxidation. The partially purified antigen was subjected to the respective treatments and the altered immunoreactivity to the MoAb $3D_{10}$ was assessed in an ELISA system.

3. Results

3.1. Partial purification of 36-kDa antigen

The 36-kDa antigen was successfully eluted from the polyacrylamide gels. A single band at the 36-kDa position on SDS-PAGE revealed the purity and integrity of the protein during the purification process (Fig. 1). Immunoblotting of this partially purified 36-kDa antigen with MoAb $3D_{10}$ gave a discrete band at a position corresponding to a molecular mass of 36 kDa (Fig. 2).

3.2. Physico-immunochemical characterization of epitopic 36-kDa antigen

Heat treatment of 36-kDa antigen for 10 min at 60°C before coating on to the wells of the ELISA plate significantly reduced its immunoreactivity to MoAb $3D_{10}$ ($P < 0.01$) (Fig. 3a). Exposure of the antigen at 100°C for 10 min caused a further loss in its immunoreactivity. The proteolytic digestion of the 36-kDa antigen coated onto the wells of the ELISA plate with trypsin almost abolished its immunoreactivity with MoAb $3D_{10}$ ($P < 0.01$) (Fig. 3b). However, the immunoreactivity of 36-kDa amoebic antigen remained unaffected by sodium metaperiodate oxidation ($P > 0.5$) (Fig. 3c).

3.3. Localization of the epitope recognized by the MoAb $3D_{10}$ on E. histolytica trophozoites and the cysts

Interactions of the MoAb $3D_{10}$ with the live *E. histolytica* trophozoites gave a mean immunofluorescence intensity (MII) value of 127.5, which was significantly higher than the MII value of 41.6 obtained after incubation of live *E. histolytica* trophozoites with the normal peritoneal fluid. Interactions of the MoAb $3D_{10}$ with the fixed and permeabilized *E. histolytica* trophozoites resulted in a tremendous increase in the MII value (1205.8) as compared to the MII value with live *E. histolytica* (Fig. 4). Interaction of MoAb $3D_{10}$ with cysts of *E. histolytica* gave a MII value of 205.96 as compared to the control value of 32.09 and MII value of 39.19 with cysts of *Entamoeba coli* (Fig. 5).

3.4. Recognition of 36-kDa antigen by human serum

The mean ± S.D. value of the antibodies to the 36-kDa amoebic antigen in the serum (ELISA) from the healthy subjects was 0.080 ± 0.034 (Fig. 6). A cut off OD value for significance was calculated to be 0.182 mean+3 S.D. of the OD values observed with healthy subjects. The mean OD values of the serum from patients with non-amoebic hepatic disorders, non-amoebic intestinal disorders and the apparently healthy subjects were below the cut off OD value. Serum samples from 29 (85%) of the 34 cases of confirmed amoebic liver abscess were having OD values higher than the cut off OD value. The mean ± S.D. OD

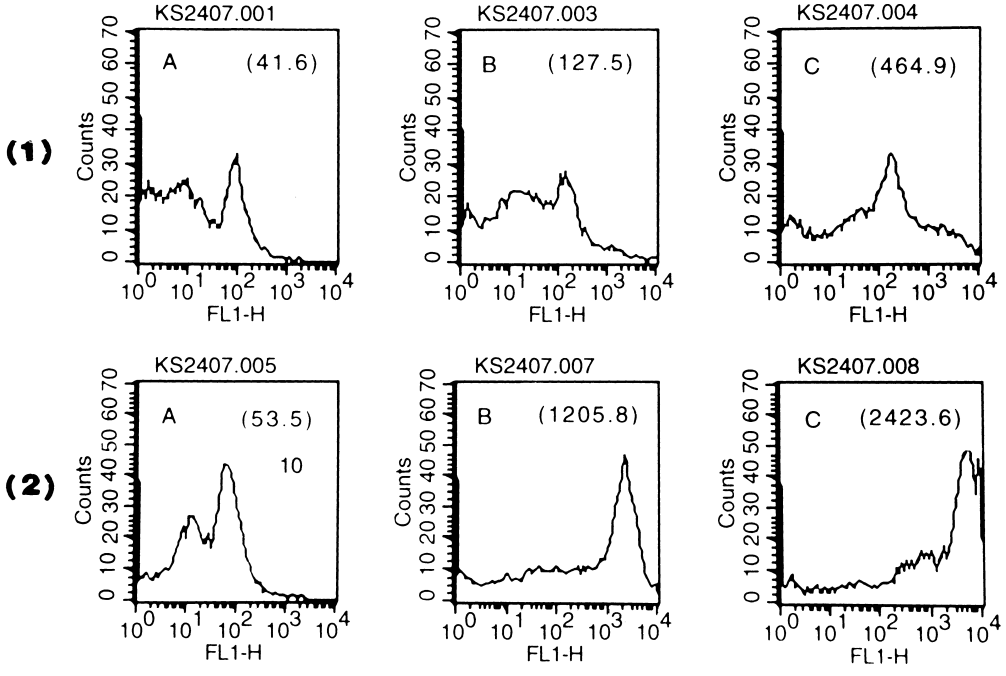

Fig. 4. Immunofluorescence patterns of *E. histolytica* trophozoites with MoAb $3D_{10}$. MII (given in parentheses at the top right corner of each box) with (A) normal peritoneal fluid, (B) MoAb $3D_{10}$, (C) anti-CAE antibodies. (1) Live *E. histolytica* trophozoites, (2) fixed and permeabilized *E. histolytica* trophozoites. Cells, after incubation with relevant antibodies, were incubated with FITC- labelled anti-mouse antibodies and the fluorescence was analyzed in CELL QUEST software of the FACScan.

value in these cases was 0.415 ± 0.400. The mean ± S.D. OD value obtained with serum samples from symptomatic intestinal amoebiasis was 0.314 ± 0.087 and five (83%) of the six patients were having OD values higher than the cut off OD value. Significantly, serum samples from all the four asymptomatic intestinal amoebic subjects had OD values below the cut off OD value.

4. Discussion

The involvement of various molecules of the parasite in the modulation of the disease process requires their recognition by the immune system of the host and development of the specific immune responses against them. Often, molecules expressed on the surface of the parasite play important roles in the interaction of the parasite and the host. A number of parasite components have been identified which mediate initial attachment, serve as virulence factors or interact in an immunomodulatory capacity with the host, though only a few have been characterized biochemically, immunologically and functionally [10–14]. Identification and characterization of more and more of the parasite antigens will help to understand the pathophysiology of the disease better and also pave the way for selecting potentially immunogenic amoebic proteins. Such data might also be useful in selecting either antigens for the serological diagnostic tests or DNA probes for the identification of the amoebae directly in the clinical samples like stool samples or abscess fluids. Recently, we reported the identification of a 36-kDa amoebic antigen by MoAbs. In the present study, we have partially purified this antigen and report the physico-chemical characterization of this antigen and its recognition by the sera from patients suffering from amoebiasis.

The elution of the 36-kDa protein from the polyacrylamide gels resulted in a pure, intact and immunoreactive antigen as evident by the SDS-PAGE and immunoblotting. The epitope recognized by the MoAb $3D_{10}$ was found to be protein in nature, it was heat sensitive and proteolytic digestion of the antigen almost abolished its immunoreactivity, whereas sodium metaperiodate did not.

Immunofluorescence studies showed the epitope to be localized both on the surface and in the cytoplasm with the majority of it in the cytoplasm of *E. histolytica* trophozoites. This is indicated by an increased mean immunofluorescence value when the trophozoites were fixed and permeabilized. It is possible that the cytoplasmic form is the precursor or the degradation product of the membrane form and the lower molecular mass bands recognized by the MoAb $3D_{10}$ in the CAE could well be the intermediates of the degradation process. The presence of two functionally different membrane bound and the cytoplasmic forms is also not ruled out. Alternatively, the fluorescence observed with the permeabilized trophozoites probably suggests the binding of the cytosolic protein to the antibody during its transit to the cell membrane. Another explanation could be that one or two out of these three amoebic antigens recognized by the MoAb $3D_{10}$ could be in the cytosol and remainders are on the surface of the

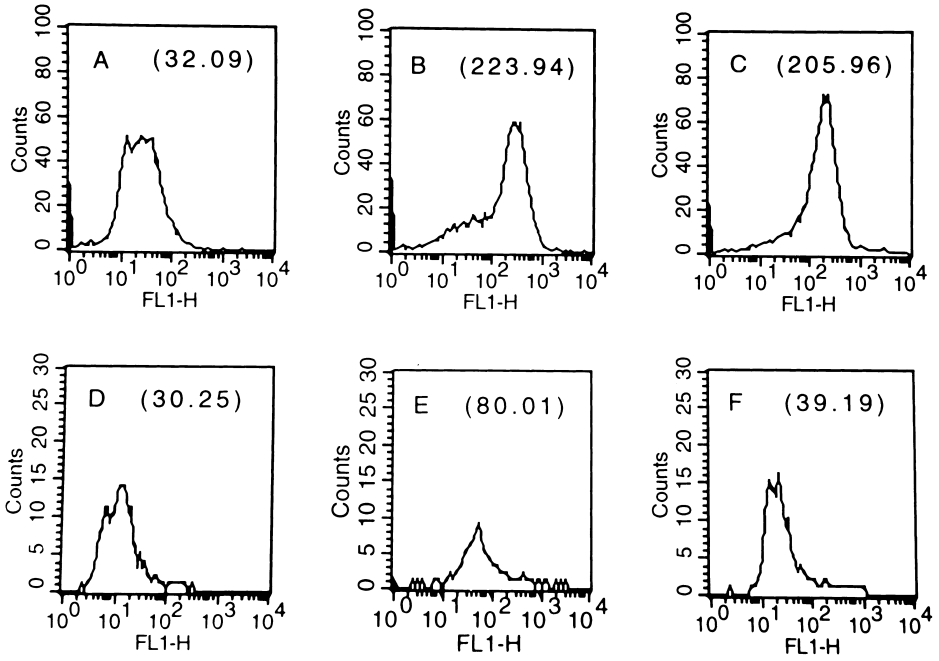

Fig. 5. Immunofluorescence patterns of the cysts of *E. histolytica* with MoAb $3D_{10}$. The upper panel shows MII values (given in parentheses at the top right corner of each box) of *E. histolytica* cysts after incubation with (A) normal peritoneal fluid, (B) anti-CAE antibodies, (C) MoAb $3D_{10}$. The lower panel shows the MII values of *Entamoeba coli* cysts after incubation with (D) normal peritoneal fluid, (E) anti-CAE antibodies, (F) MoAb $3D_{10}$. Cysts of *E. histolytica* and *Entamoeba coli* were separated from the stool samples by the zinc sulfate flotation technique, incubated with relevant antibodies followed by incubation with FITC-labelled anti-mouse antibodies and the fluorescence was analyzed in a CELL QUEST software of the FACScan.

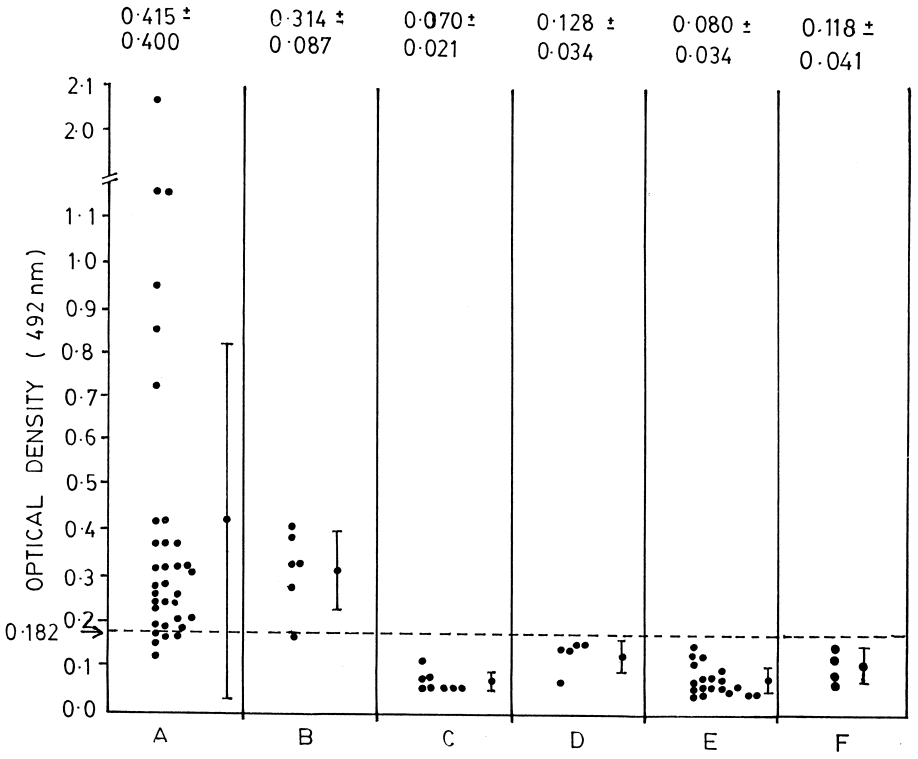

Fig. 6. Recognition of 36-kDa amoebic antigen in ELISA by the serum samples from (A) patients with amoebic liver abscess, (B) patients with symptomatic intestinal amoebiasis, (C) patients with non-amoebic hepatic disorders, (D) patients with non-amoebic intestinal disorders, (E) apparently healthy subjects and (F) asymptomatic cyst passers. The broken line represents the cut off OD value, bars represent S.D.

trophozoites. Further immunofluorescence studies with the cyst form of the parasite showed that this epitope is also present on cyst of *E. histolytica*. The absence of this epitope on the surface of *Entamoeba coli* indicated the specificity of this epitope for the cyst of *E. histolytica*. The fact that this epitope is also present on the cyst form of the parasite suggests that this is an important component of the cell architecture of the *E. histolytica*. Only a few amoebic antigens are reported to be present on both forms of *E. histolytica* [3,24]. This finding is important because this MoAb $3D_{10}$ can then be used directly in an immunofluorescence test to detect/identify the amoebic cysts directly in the stool samples from suspected cases of intestinal amoebiasis.

The 36-kDa amoebic antigen was recognized by 34 (85%) of the 40 patients with symptomatic amoebiasis and none of the patients with asymptomatic amoebiasis or those suffering from other intestinal or the hepatic disorders. This finding shows that specific antibodies against this antigen are generated during the course of the natural amoebic infection, making this molecule one of the important candidates for the serodiagnosis of amoebiasis. The lack of antibodies against this molecule in the subjects with asymptomatic intestinal amoebiasis suggests that this antigen might be expressed only on the surface of the amoebae with invasive potential.

In Summary, we have identified and partially purified a 36-kDa amoebic antigen by MoAb. The epitope recognized by the MoAb is a protein present both on the surface and in the cytoplasm of the parasite. The epitope is also present on the cysts of *E. histolytica*. The recognition of this 36-kDa protein of *E. histolytica* by sera from patients with amoebiasis renders this antigen potentially useful for the serodiagnosis of the amoebiasis and indicates a specific role for this molecule in invasive amoebiasis.

Acknowledgements

We thank Dr Kartar Singh of the Department of Gastroenterology, PGIMER, Chandigarh, India, for providing the clinical material. K.S. was a recipient of Junior and Senior Research Fellowships from the University Grants Commission, New Delhi, India.

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