A monoclonal antibody-based test system for detection of Entamoeba histolytica-specific coproantigen

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Background: Diagnosis of amebiasis based on stool microscopy or demonstration of anti-amebic antibodies has limitations. A diagnostic system based on demonstration of the parasite product in clinical specimens holds promise. Methods: Murine monoclonal antibodies were developed against an Entamoeba histolytica-specific coproantigen. A monoclonal antibody (MoAb) 3D10 was employed in a double-antibody sandwich microELISA system for the detection of amebic coproantigen in fecal specimens. The system was evaluated in three groups of subjects: 63 patients with intestinal amebae, 27 with non-amebic parasitosis, and 57 apparently healthy controls. Results: The MoAb 3D10 belonged to IgG, isotype and recognized three antigens, with mol. wt. 36, 25 and 17 kDa in the crude extract of E. histolytica (HM1-IMSS), and an amebic coproantigen with MW 36 kDa in the stool supernatant from patients with intestinal amebae. The coproantigen was detected in the stool eluates of 56 (89%) patients with intestinal amebae and in none of the stool eluates from other subjects, thereby giving this system a sensitivity of 89% and specificity of 100% for the detection of intestinal amebae. Conclusions: This monoclonal antibody recognizes an intact epitope on the E. histolytica-specific coproantigen. The validity of the MoAb-based microELISA system needs to be established. [Indian J Gastroenterol 1999;18:104-108]

Key words: Amebiasis, ELISA

Conventional laboratory diagnosis of intestinal amebiasis depends on demonstration of Entamoeba histolytica cysts or trophozoites in stool samples. Single stool examination detects 40%-50% of cases. The diagnostic efficacy can be enhanced to 60%-80% by examining samples on three consecutive days using appropriate staining and concentration procedures. These procedures are cumbersome and depend on the microscopist’s skill. Tests based on demonstration of anti-amebic antibodies are of limited value in endemic areas because the antibodies persist for years after successful eradication of the parasite.

Demonstration of parasite products in clinical specimens is an effective method of diagnosing current infection. Many attempts have been made to develop simple, rapid and accurate methods for detection of amebic antigens in stool samples. The sensitivity and specificity of an antigen detection system depend on the quality of antibodies used. It is important to use antibodies which directly identify amebic coproantigens in stool specimens from patients suffering from intestinal amebiasis.

We report the development of monoclonal antibodies against E. histolytica-specific coproantigen (EhCA) and their use in an enzyme-linked immunosorbent assay for the detection of this coproantigen in the stool samples of patients infected with E. histolytica/E. dispar.

Methods

Preparation of E. histolytica antigen

Axenic strain of E. histolytica HM1-IMSS was cultivated in TYIS-33 medium. E. histolytica trophozoites from the log phase growth were harvested, washed thrice with 0.15M phosphate buffered saline pH 7.2 (PBS-7.2) and disrupted by sonication in an ultrasonic disintegrator (MSE, London) at 23 KHz with six 30-second bursts. To the cell lysate, 0.5% Triton X-100 was added and the material was left at 4°C for 2 hours. This material was labelled as crude amebic extract (CAE) and a cocktail of protease inhibitors (phenylmethylsulfonyl fluoride [PMSF] 1 mM, N-[N-(3-transcarboxyirnone-2-carbonyl]-L-leucyl]agmatine [E-64] 20 μM and ethylene diaminetetraacetic acid disodium salt [EDTA] 2 mM) was added to it. The protein contents of the CAE were determined.

Preparation of anti-EhCA antibodies

Polyclonal antibodies (PoAb) to EhCA were raised as described previously. In brief, hyperimmune serum to the CAE was raised in rabbit and its IgG fraction was accomplished by DEAE cellulose chromatography. These antibodies were labelled as anti-CAE IgG antibodies. EhCA were affinity purified from stool supernatants from patients with intestinal amebiasis (Group A, subgroup I) by using anti-CAE IgG coupled to CNBr-activated sepharose 4B column. Antiserum to these EhCA was raised in rabbit and its IgG fraction was accomplished by DEAE cellulose chromatography. These antibodies were labelled as anti-EhCA IgG antibodies.

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Production of anti-EhCA monoclonal antibodies

Marine monoclonal antibodies (MoAb) were developed against EhCA as described by Groth and Scheidegger, 13 with a few modifications. Briefly, splenocytes from the Balb/C mice immunized with CAE were fused with mouse myeloma cell line P3-O14. All the wells with growing hybrids were screened by ELISA using purified EhCA. Cells from the positive wells were cloned by the limiting dilution method. Monoclonal so developed were again tested for MoAb in an ELISA system using purified EhCA. The relevant clone (3D10) was expanded as ascites in the peritoneal cavity of the pristain-primed Balb/C mice. The ascitic fluid was collected and centrifuged at 7500g for 20 minutes and supernatant so obtained to determine the immunoglobulin subclass of the MoAb by the Ouchterlony agarose immunoglobulin test using anti-mouse immunoglobulin isotype specific sera. The IgG fraction of the ascitic fluid was purified by protein-G sephrose-CL 4B affinity chromatography. The specificity of the MoAb was assessed by recognition of the antigens in CAE / total stool supernatant from patients with intestinal amebiasis / lysate of *Giardia lamblia* / lysate of *Leishmania donovani* resolved proteins in the western immunoblot system. 15

Clinical specimens

Stool samples from the following groups of subjects were examined by the formol-ether concentration method for the presence of EhCA.

- a) Patients with intestinal amebae (Group A) (n=63): This group was further subdivided into:
  - Subgroup I, including 43 patients with gastrointestinal symptoms like pain in abdomen, flatulence and/or diarrhea. Characteristic amebic ulcers were demonstrated by sigmoidoscopy. The sigmoidoscopic swabs were found to be positive for motile *E. histolytica* trophozoites with ingested erythrocytes. Their stool samples were positive for *E. histolytica* / *E. dispar* cysts on three consecutive days. They responded to anti-amebic chemotherapy.
  - Subgroup II, including 20 patients with gastrointestinal symptoms like pain in abdomen, flatulence and/or diarrhea. Their stool samples were positive for *E. histolytica* / *E. dispar* cysts on three consecutive days, but sigmoidoscopy could not be done in them. They responded to anti-amebic chemotherapy.

- b) Patients with non-amebic parasitosis (Group B) (n=27): This group included those patients whose stool samples contained *Giardia lamblia*, *Entamoeba coli*, *Endolimax nana*, or *Entamoeba hartmanni* cysts or *Ascaris lumbricoides* ova, but were negative for *E. histolytica* / *E. dispar* cysts on three consecutive days.

- c) Apparently healthy subjects (Group C) (n=57): This group comprised subjects who had no gastrointestinal symptoms and whose stools were negative for any pathogen on three consecutive days.

Preparation of specimens for demonstration of EhCA

Stool samples were collected and examined within two hours. They were emulsified in PBS-7.2 (approximately 1.0 g/mL) and centrifuged at 250g for 10 minutes. The cloudy, unfiltered supernatant was separated and labelled as "stool eluate". A cocktail of protease inhibitors (PMSF 1 mM, E-64 20 µM, EDTA 2 mM) was added to the eluates, which were stored at -70°C until tested for microELISA. The eluates were diluted 1:3 in PBS-7.2 containing 3% BSA and 10% normal rabbit serum before analysis.

MicroELISA for detection of EhCA

The double-antibody sandwich microELISA was performed as described by Wonsit et al 15 with a few modifications. Optimal concentrations of the capturing antibody, detecting antibody and antibody-enzyme conjugate were determined by the checker-board titration method. One hundred microliters of anti-EhCA IgG (11 mg/mL) diluted 1:1000 in 0.05M carbonate buffer pH 9.6 was coated on the wells of polystyrene microELISA plate (Costar, USA). After overnight incubation at 4°C, the plates were washed with 0.15M PBS-7.2 containing 0.05% Tween 20 (PBS-T) and the non-specific sites were blocked by adding 3% BSA to the wells. After washing with PBS-T, the wells were loaded with 100 µL of stool eluates from different groups of subjects and the plates were incubated at 37°C for 2 hours. The plates were washed thrice with PBS-T, and 100 µL of 1:20 diluted MoAb 3D10 (1.7 mg/mL) (diluted in PBS-7.2 containing 1% BSA, 10% normal rabbit serum and 10% stool suspension from parasitologically negative stool samples) was added. Plates were incubated at 37°C for 1 hour and then washed with PBS-T. One hundred microliters of 1:2000 diluted horse radish peroxidase conjugated anti-mouse IgG (NII, New Delhi) (diluted in PBS-7.2 containing 1% BSA, 10% normal rabbit serum and 10% stool suspension from parasitologically negative stool samples) was added to each well, followed by incubation at 37°C for 1 hour. The plates were washed and the reaction was developed by the addition of 100 µL of ortho-nitro phenylamine diamin (5 mg in 10 mL of 0.15M citrate phosphate buffer pH 5.0 and 5 µL of H₂O₂). The reaction was terminated by the addition of 50 µL of 6N H₂SO₄. Optical density (OD) was measured at 492 nm in an ELISA reader. The cut-off OD value was calculated from 10 randomly selected cases with intestinal amebae and 10 randomly selected apparently healthy subjects. Sensitivity and specificity of the assay were assessed.

Detection limit of microELISA system for EhCA

The detection limit of the microELISA system was found by loading varying concentrations of CAE in PBS or CAE spiked in parasitologically negative stool to the capturing antibody-coated wells of the ELISA plate instead of the stool eluates. The bound antigen was then detected as described above. The minimum concentration of CAE giving an OD value higher than the cut-off OD value was taken as the detection limit of the microELISA system.
Fig 1: Recognition of EhCA by MoAb 3D6. Lane A: CAE antigens reacted with anti-CAE antibodies; Lane B: Total stool supernatant reacted with MoAb 3D6. Arrow indicates the band. Molecular weight markers are indicated on left.

Results

Generation of MoAb

Six MoAb directed against EhCA were produced. MoAb 3D6 belonging to IgG1 subclass and recognizing a 36 kDa amebic coproantigen in total stool supernatant from patients with intestinal amebiae (Fig 1), was selected for further work. This MoAb recognized three amebic polypeptides with apparent MW of 36, 25 and 17 kDa in the "crude amebic lysate" and did not cross-react with lysates of G. lamblia and L. donovani (Fig 2).

Development and evaluation of PoAb-MoAb microELISA

OD values in the study groups are shown in Fig 3. Stool eluates from 41 (95%) of 43 Group A subgroup I patients and 15 (75%) of 20 Group A subgroup II patients had detectable levels of the amebic coproantigen. None of the 27 patients with non-amebic parasitosis and none of the 57 apparently healthy subjects had detectable amebic coproantigen in their stool eluates. These differences were significant (p<0.01; one-way ANOVA). By the critical difference method, the mean (SD) values in Group A samples (0.175 [0.065]) were significantly higher than those in Group B samples (0.024 [0.025]) (p<0.01) and those in Group C samples (0.032 [0.025]) (p<0.01).

The PoAb-MoAb micro-ELISA system had a sensitivity of 89% and specificity of 100% for the detection of intestinal amebiae. This microELISA system could detect < 156 ng of the CAE protein in PBS-7.2/well of the microELISA plate. In a 10% suspension of parasitologically negative stool, the assay was less sensitive and could detect <312 ng/well of the CAE proteins.

Discussion

Since the conventional methods of diagnosing intestinal amebiasis based on stool microscopy and demonstration of anti-amebic antibodies have limitations, the WHO recommended development of assay systems based on demonstration of parasite products in clinical specimens. Such systems will diagnose current infection. The presence of intact parasite (trophozoite or cyst) in clinical samples is not required, and only one test sample is required; hence these assays are convenient to both patient and diagnostician.

Several groups have reported ELISA systems employing polyclonal and monoclonal anti-amebic antibodies to detect amebic antigens in clinical specimens. Most often, the investigators were not able to identify the molecular species of the amebic coproantigen. An ideal assay sys-
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stem would be one which employs antibodies which are targeted against amebic antigens which are able to survive the action of gut proteases. In the present study we produced MoAbs against such coproantigens and made use of them in the detection of amebic coproantigen in an ELISA system.

The MoAb 3D10 recognized a distinct amebic coproantigen with MW of 36 kDa in the stool supernatants from patients with amebae and three antigens with MW of 36, 25 and 17 kDa in the crude amebic lysate in the western immunoblot system. The MoAb was specific for amebic coproantigen as it did not show cross reactivity with the crude lysates of G. lamblia and L. donovani. It is expected that one MoAb will recognize one epitope on the antigen and through the recognition of this epitope the entire antigen is recognized. The recognition of three antigens in the crude amebic lysate by MoAb 3D10 can be explained on the basis of presence of this epitope in these three amebic antigens. Alternatively, this could be because of the degradation of the amebic coproantigen with MW of 36 kDa by the parasite’s own proteases present in the crude amebic lysate. This possibility is remote because a cocktail of protease inhibitors was added to the CAE preparation.

A double-antibody sandwich microELISA system was developed and evaluated by employing anti-EhCA antibodies as the capture antibody and MoAb 3D10 as the detecting antibody for the detection of EhCA in stool samples. The reason for using these antibodies was that anti-EhCA antibodies would capture all the amebic coproantigens present in the stool samples of subjects with intestinal amebiasis while MoAb 3D10 will specifically detect the amebic coproantigen with MW of 36 kDa. Employing mean + 2 SD of the OD values in 10 randomly picked apparently healthy subjects as cut off, the microELISA system had a sensitivity of 89% and specificity of 100% for the detection of intestinal amebiasis. Stool eluates from 95% of patients in Group A, subgroup I and 75% of patients in Group A subgroup II were positive for the amebic coproantigen. None of the stool eluates from patients with non-amebic parasitosis and none of the apparently healthy subjects had detectable levels of the amebic coproantigen.

The assay missed seven cases with intestinal amebiasis (two from subgroup I, five from subgroup II). This could be because MoAbs are directed against a particular epitope of the antigen. It is possible that in these seven cases either the epitope recognized by the MoAb 3D10 was immunologically altered or was present at a concentration below the detection limit of this microELISA system. The false negative results could also be because of degradation of the antigen during storage, transportation, and freezing and thawing of stool samples. The epitope recognized by the MoAb might also be masked by local intestinal immune responses. The sensitivity and specificity of this assay system are in agreement with the other PoAb-MoAb based ELISA systems reported previously.3,8

The PoAb-MoAb microELISA system developed in this study could detect <156 ng of CAE/well in PBS-7.2 and <312 ng/well of CAE spiked in the parasitologically negative stool samples. The decrease in sensitivity when the amebic lysate was mixed with parasitologically negative stool sample could be because of interference by various stool-associated factors. These factors might act by desorbing the immunoreactants from the solid-phase surfaces. Such interfering factors could include a heat-labile protease with MW of 25 kDa.19 To circumvent this possibility, a cocktail of protease inhibitors was added to the stool eluates. This level of detection limit by the sandwich type ELISA system is in agreement with other reports.5

Antigen detection systems like ELISA have various advantages. Samples for testing are simple to prepare. Preservation of specimens to be utilized later is best accomplished by immediate freezing, though some loss of antigenicity has been reported on freezing.20 MoAb, while perhaps more delicate than PoAb, has the advantage of reagent uniformity, although it may not recognize as many epitopes as the latter does.

We therefore believe that the detection of E. histolytica-specific coproantigen in the stool samples of suspected cases of intestinal amebiasis by MoAb-based sandwich microELISA system holds promise. However, its practical validity will need to be established in properly selected cases.

References


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