MONOCLONAL ANTIBODY-BASED ELISA FOR THE DETECTION OF CIRCULATING ENTAMOEBA HISTOLYTICA ANTIGEN IN HEPATIC AMEBIASIS IN HAMSTERS (MESOCRICETUS AURATUS)

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Abstract. Circulating amebic antigens were determined by using a sandwich ELISA with specific monoclonal antibody in the sera of 35 group I hamsters, 7 of which were sacrificed at intervals after hepatic inoculation with 500,000 axenically grown HM:1:1MSS strain of E. histolytica trophozoites, 7 group II infected hamsters in which metronidazole treatment was given and 18 group III uninfected controls. Amebic antigenemia was demonstrated in 5 of 7 (71.4%), 6 of 7 (85.7%), 7 of 7 (100%), 7 of 7 (100%) and 7 of 7 (100%) of group I hamsters on days 5, 10, 15, 20 and 30 of infections respectively, whereas 6 of 7 (85.7%) of group II hamsters were weakly positive, one was negative and all 18 group III hamsters were negative. The sensitivity of the assay was 100% after the animals were infected 15 days onwards. The level of antigenemia in hamsters of group I with abscess was significantly higher than those of the same group without abscesses (p < 0.05). Absence or reduction of antigenemia after treatment could be interpreted to mean a positive test of cure and favorable therapeutic response. The MAb-PAb-based ELISA for the detection of circulating E. histolytica represents a simple and sensitive diagnostic test for invasive amebiasis in hamsters. Application of this test in amebic liver abscess patients should be of diagnostic value for indication of present infection or test of cure after successful treatment.

INTRODUCTION

Amebiasis, a disease caused by Entamoeba histolytica with or without clinical symptoms, occurs throughout the world, especially in tropical and subtropical climates and in places with poor sanitary conditions due to fecal contamination of water and food or direct fecal-oral contact. Serological tests such as immunoelectrophoresis (IEP), cellulose acetate membrane precipitin (CAP) test and enzyme-linked immunosorbent assay (ELISA) to detect antibodies to E. histolytica are particularly useful for the diagnosis of extraintestinal amebiasis such as amebic liver abscesses (Tharavanij and Chaiyumpa, 1969; Thammapalerd et al, 1981; Samrejrongroj and Tharavanij, 1985; Nicholls et al, 1994). In such cases stool examination is often negative and positive antibody titers may persist for months or years after successful treatment. Therefore, it is difficult to differentiate between present and past infections (Sepulveda and Martinez-Palomo, 1982). Detection of circulating amebic antigens would be better correlated with present infection. Circulat-

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Amebic and culture conditions
CIRCULATING ANTIGENS OF _E. histolytica_

The HM1 : IMSS strain of _E. histolytica_ was maintained axenically with twice weekly sub-culture in screw-capped tubes in TPS-1 medium in the presence of 10% heat-inactivated bovine serum and vitamin mixture according to the technique described by Diamond (1968).

**Preparation of _E. histolytica_ antigen for animal inoculation**

Log-phase (72 hours) cultures of trophozoites were harvested by chilling on ice for 5 minutes, followed by centrifugation at 350 g for 5 minutes, counted on a hemacytometer, resuspended in a volume of complete TPS-1 medium to give a final concentration of 2.5 x 10^7 trophozoites per 100 μl. Tubes containing amebae were kept on ice for no longer than 10 minutes before inoculation.

**Experimental animals**

Male syrian golden hamsters (Mesocricetus auratus), 50-60 days old were kindly supplied by the animal house of the Faculty of Tropical Medicine, Mahidol University and the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand. BALB/c mice were kindly supplied by AFRIMS and adult New Zealand white rabbits aged 2 to 3 months were purchased from the Faculty of Veterinary Medicine, Chulalongkorn University.

**Surgical and hepatic inoculation procedure**

The hamsters were fasted for 18 hours and received intraperitoneal injections of sodium pentobarbital solution (42 mg/kg body weight). Their abdominal cavities were opened by longitudinal midline incision of approximately 3 cm in length, the liver was exposed, and the inoculum was slowly injected into the left lobe of the exposed liver. After inoculation, care was taken to preclude leakage and hemorrhage of the inoculum by blotting the area with sterile cotton swabs. The abdominal wall and skin were severed separately with 0-3 normal catgut. Aseptic precautions were observed throughout each operation which took about 10 minutes to complete. After operation, the animals were housed and fed on commercial mouse diet, vegetables and water ad libitum until sacrificed. Sixty male hamsters were divided into three groups. Group I comprising 35 animals, each animal was inoculated intrahepatically with 500,000 trophozoites of _E. histolytica_ in 0.2 ml of sterile phosphate buffered saline (PBS) solution, pH 7.2 using disposable tuberculin syringes attached to a 1 cm 26 gauge needle. The abdominal wall and skin were severed separately with 0-3 sterile normal catgut. Group II comprising 7 animals, they were inoculated with the same dose as those animals in Group I and 20 days post inoculation, they were treated with metronidazole (200 mg/kg body weight) by oral gavage for 6 consecutive days and examined 10 days after the last dose of treatment. Group III comprising 18 age-matched control animals, 15 of which were similarly given 0.2 ml of sterile PBS, while 3 were not given any injection.

**Sera**

Blood was collected from the heart of the hamsters prior to intrahepatic inoculation with _E. histolytica_ and on days 5, 10, 15, 20 and 30 days post-inoculation with _E. histolytica_ group I animals, 36 days of group II animals and 30 days of group III animals, respectively. Sera were separated and protease inhibitors were added at a final concentration of 1 mM of phenyl methyl-sulphonyl-fluoride (PMSF) and 1 mM of n-1-p-osyl-L-lysine chloromethyl ketone (TLCK), the sera aliquoted in 1 ml portion and kept frozen at -78°C until tested.

**Preparation of rabbit anti- _E. histolytica_ polyclonal antibodies (PAb)**

Two healthy rabbits were immunized intramuscularly five times with 0.5 ml of crude amebic whole cell lysate equivalent approximately to 5 x 10^9 trophozoites of the HM1:IMSS strain of _E. histolytica_ emulsified with an equal volume of Freund's complete adjuvant initially and Freund's incomplete adjuvant subsequently at an interval of two to three weeks. The rabbits were bled by cardiac puncture 9 days after the last immunizing dose, and the serum IFA antibody titers against HM1:IMSS were both 1:640. Sera were aliquoted and kept at -20°C until used. Serum IgG was purified by affinity chromatography on protein A Sepharose CL-4B according to the technique of Seppala _et al_ (1981). The purified polyclonal IgG (PlgG) at the concentration of 5 μg/ml gave IFA antibody...
titer of 1:1,280. The PIGG was aliquoted, lyophilized and stored at -78°C until used.

**Anti-E. histolytica MAb**

Murine MAb's were raised against the HM1:IMSS strain of *E. histolytica* in Sp2/0 myeloma cells according to the method of Galfre and Milstein, 1982 (Thannhauser and Tharavanij, 1991). Several antibody-secreting hybridoma clones were obtained, one in particular, and IgG1 specific Eh208C2-2 MAb was selected for this study. The IgG fraction of the ascites (MLgG) was purified by protein A Sepharose CL-4B affinity chromatography as described above and used in a sandwich ELISA system for the detection of circulating antigens in the sera of hamsters with hepatic amebiasis, infected hamsters after treatment and healthy hamsters. The protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin (Sigma Chemical Company, St. Louis, USA) as standard.

**MAb-PAb-based ELISA for the detection of *E. histolytica* antigen in the sera**

The technique used followed antibody sandwich ELISA previously described by Wonsit et al. (1992). Briefly, a 96-well polystyrene micro ELISA plate (Dynatech) was coated with 100 µl of 10 µg/ml of Eh208C2-2 MLgG at 37°C for 1 hour and further at 4°C overnight. The unbound components were washed twice with distilled water each for three minutes. The nonreactive sites were blocked with PBST containing 5% nonfat dry milk (Carnation) at 37°C for 1 hour. After washing 4 times with PBST, 100 µl of sera from infected, control and drug-treated hamsters at various days post inoculation of *E. histolytica* was added to each well followed by incubation at 37°C for 2 hours. The plate was again washed with PBST and 100 µl of 10 µg/ml of PIGG added, followed by incubation at 37°C for 1 hour. After washing, 100 µl of goat alkaline phosphatase (AP)-conjugated anti-rabbit IgG (Zymed) at 1:1,000 dilution in PBST-1% BSA was added to each well, followed by incubation at 37°C for 1 hour. The plate was washed and 100 µl of substrate solution (p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8) added. The enzyme-substrate color reaction was allowed to develop at room temperature and then stop with 3N NaOH after 40 minutes. The optical density (OD) of each well was measured at 405 nm with an ELISA plate reader (Titertek Multiskan, MCC/340, Flow Laboratories).

The optimal dilutions of rabbit antisera and conjugate were determined using a checkerboard titration. For each test, a negative, a positive and a PBST controls were included. Each serum sample was tested in duplicate and the mean OD value calculated.

**Statistical analysis**

Continuous variables were compared using the nonparametric Kruskal-Wallis method. All p values less than 0.05 were considered to indicate statistical significance.

**RESULTS**

**MAb-PAb-based ELISA for the detection of circulating *E. histolytica* antigens**

The cut-off OD value of 0.257 was based on the mean ±2SD of reactivity in normal hamsters. Therefore, the test sample was considered positive if its OD value was equal to or above 0.257.

Amebic antigenemia was demonstrated in 5 of 7 (71.4%), 6 of 7 (85.7%), 7 of 7 (100%), 7 of 7 (100%) and 7 of 7 (100%) of group I hamsters on days 5, 10, 15, 20 and 30 of infections respectively, whereas 6 of 7 (85.7%) of group II hamsters were weakly positive and one (14.3%) was negative and all 18 of group III hamsters were negative (Fig. 1).

![Fig. 1-Scatter diagram of the optical density (OD) using MAb-PAb-based ELISA for the detection of circulating *E. histolytica* antigens of 3 groups of hamsters at various days post inoculation. Group I, 15 animals intrahepatically infected with 5 x 10^6 trophozoites of *E. histolytica* Group II, 7 infected and treatment group and. Group III, 18 age-matched control group.](image-url)
In group I hamsters, abscesses were demonstrated either by gross pathology or by H & E staining in 3 (42.8%), 2 (28.6%), 4 (57.1%) and 3 (42.8%) hamsters on days 5, 10, 15, 20 and 30 respectively. No obvious abscess was seen in group II hamsters receiving metronidazole treatment. Livers of hamsters in group III were all normal. The level of antigenemia in hamsters of group I with abscess was higher than hamsters of the same group without abscess (p < 0.05) (Fig 2A, 2B). Such difference was not correlated with days post inoculation (p > 0.05).

DISCUSSION

The golden hamster (Mesocricetus auratus) is the animal of choice for the study of hepatic amebiasis and for the detection of circulating amebic antigens in our laboratory whereby liver tissue sections containing E. histolytica trophozoites were recognized by MAAb-based IFA and MAAb-based IPx tests (Sherchand et al, 1994). Wide necrotic areas develop in the liver after trophozoites are inoculated right into the organ. These lesions respond well to the standard antiamebic therapy with metronidazole (Flagyl) 200 mg/kg body weight.

A number of tests has been employed to detect parasitic antigens other than amebic antigens in sera of infected humans and murines; however, publications on the detection of circulating amebic antigens in human sera and pus, and in experimental amebiasis are scant. Detection of antigen in human liver pus and sera using specific antiserum raised in rabbits against axenic amebic antigen and CIEP by Mahajan and Ganguly (1980) gave 92% (115/125) and 27% (24/89) positivity respectively in patients with amebic liver abscess. Yinayak et al (1986) could detect amebic antigen in circulating immune complexes (CIC) in 93% of the confirmed cases of amebic liver abscess using polyclonal antibodies and ELISA. Onyemelikwe and Onyewotu (1981) reported the presence of immune complex activity in 58% (7 of 12) of amebic liver abscess patients in Nigeria in which all these patients were positive by amebic gel diffusion test. However, they did not show that specific amebic antigens are present in those intact immune complexes.

The present study demonstrated that the double antibody sandwich ELISA is potentially useful for amebic antigen detection in sera of hepatic hamsters experimentally infected with E. histolytica trophozoites with the sensitivity of 100% after 15 days onwards of infection. Circulating amebic antigens were detected as early as day 5 throughout the experimental period of 30 days post inoculation in infected group I hamsters and remained positive thereafter, whereas 6 of 7 (85.7%) of drug treated group II hamsters were weakly positive and one (14.3%) was negative and all 18 of age-matched control group II hamsters were negative. In group II of infected and drug-treated animals, 20 days post inoculation, drug were given orally for 6 consecutive days and 70 days after the last dose of treatment sera were tested. If drug was given earlier than 20 days post inoculation with E. histolytica or if follow up for more than 30 days were carried out, it is speculated that the assay would become negative in all metronidazole-treated hamsters. If this turns out to be the case, this assay may be used as a test of cure and will have practical
value in distinguishing between present and past infection in patients with invasive amebiasis. In addition, attempts to develop a more sensitive and simple antigen detection system, such as a dot-blot ELISA using nitrocellulose paper should be evaluated.

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REFERENCES


