DEVELOPMENT OF MONOCLONAL ANTIBODIES SPECIFICALLY RECOGNIZING THE CYST STAGE OF ENTAMOEBA HISTOLYTICA

BRIGITTE WALDERICH, GERD-DIETER BURCHARD, JÜRGEN KNOBLOCH, AND LUDMILLA MÜLLER
Institut für Tropenmedizin, Forschungslaboratorien, Tübingen, Germany; Bernhard-Nocht-Institut für Tropenmedizin, Hamburg, Germany

Abstract. Protozoan cysts were isolated according to a two-step sucrose gradient procedure. Pure cysts of Entamoeba histolytica, in fixed and native states, were injected into BALB/c mice intraperitoneally for immunization. The spleens of these animals were used for fusion with AG8 mouse myeloma cells. Hybridomas were obtained and tested for the recognition of E. histolytica, E. dispar, E. coli, E. hartmanni, Endolimax nana, Jodamoeba bütschlii, and Giardia lamblia. Three monoclonal antibodies were identified that reacted only with cysts and trophozoites of E. histolytica. These can be used for differentiation and identification of E. histolytica in feces.

The identification of Entamoeba histolytica in feces is still a problem in routine diagnostics because E. histolytica has to be differentiated from a number of lumen-dwelling protozoa: E. coli, E. hartmanni, Jodamoeba bütschlii, Endolimax nana, Giardia lamblia, and most important, from E. dispar. Entamoeba dispar cannot be distinguished from E. histolytica by morphologic characteristics; however, there are differences between the two species that can be detected using enzymatic analysis, the polymerase chain reaction (PCR), specific DNA probes, or monoclonal antibodies (MAbs). The problem is that most of these tests cannot be performed in field studies because they require expensive technical support and chemicals. Two tests based on MAbs are available as capture ELISA kits that detect proteins expressed by E. histolytica trophozoites. However, it has not been shown that these tests are able to differentiate between the cyst stages of E. histolytica and E. dispar. This is an important point because the majority of the infected individuals carry cysts of E. histolytica or E. dispar.

It was our aim to produce MAbs that specifically recognize the cysts of E. histolytica to be used in a new method for the identification of E. histolytica and/or E. dispar directly in feces. Here we report the identification of three MAbs that specifically recognize E. histolytica cysts and could be used for the identification of E. histolytica.

MATERIALS AND METHODS

Isolates of E. histolytica/E. dispar. Patients visiting the outpatient department of the Institute for Tropical Medicine in Tübingen are routinely checked for cysts and trophozoites of E. histolytica/E. dispar with the methylthiolate-iodine-formaldehyde (MIF) concentration technique and by examination of unfixed stools. Furthermore, other parasitic infections and the presence of Salmonella sp., Yersinia sp., Shigella sp., and Campylobacter sp. are reported. Serum antibodies against E. histolytica were detected as described by Knobloch and Mannweiler and Knobloch. The medical history of the patients was obtained and unfixed stool samples were sent to our laboratory.

Isolation of protozoan cysts. Native human feces containing protozoan cysts as detected by the MIF concentration method were used for the experiments. Pure cysts were isolated as described by Walderich and others, using 1.5 M and 0.75 M sucrose gradients, followed by digestion with cellulase. The cyst fraction was washed with phosphate-buffered saline (PBS) by centrifugation at 300 × g for 5 min at 4°C and was stored at −20°C.

Cultivation and differentiation of E. histolytica and/or E. dispar. Native human feces were cultured in the medium of Robinson. As soon as sufficient growth had occurred, one culture flask containing approximately 5 × 10⁵ amebae was used for both the PCR and hexokinase enzyme determination. The amebae were harvested, washed with PBS by centrifugation at 300 × g at 4°C for 5 min, boiled for 10 min, and subjected to riboprinting-PCR using E. histolytica- and E. dispar-specific primers. The amplification product of 870 basepairs was detected by electrophoresis using 2% agarose gels and staining with ethidium bromide. Additionally, the hexokinase (EC 2.7.1.1) isoenzyme pattern was used to confirm the results of the PCR, as described by Sargeaunt. Three monoclonal antibodies were included as references for the PCR and hexokinase isoenzyme typing.

Immunization of mice. BALB/c mice (Charles River, Kisslegg, Germany) were immunized intraperitoneally with E. histolytica cysts that were used either in a native state or fixed in 4% paraformaldehyde in PBS. Twenty thousand cysts were injected at each immunization step: the first injection was done with cysts in Freund’s complete adjuvant, the next four injections were done with cysts in Freund’s incomplete adjuvant, and the last injection was done with cysts resuspended in PBS. The injections were done every two weeks.

Cell fusion and hybridoma culture. The cell fusions and hybridoma screening were performed according to the methods of Peters and Baumgarten. The immunized animals were killed two weeks after the last injection and the spleens were isolated under sterile conditions. The spleen cells were mixed with AG 8 mouse myeloma cells and were fused by adding polyethylene glycol 4000 (Gibco-BRL, Eggenstein, Germany) and seeded into microplates coated with feeder cells. The medium used for the fusions and for the feeder cells was RPMI 1640 (Gibco-BRL) enriched with 20% fetal calf serum, 2 mM glutamine, 10 ml/500 ml of hypoxanthine-aminopterin-thymidine supplement (Gibco-BRL), 100 U/500 ml of interferon-γ (Boehringer, Mannheim, Germany), and penicillin (100 U/ml)/streptomycin (10 μg/ml). The cells were incubated at 37°C in 5% CO₂ and a saturated humidity. After one week, the first hybridoma clones were
Cross-reactivity of cysts of other protozoan species with monoclonal antibodies (MAbs) directed against Entamoeba histolytica cysts in an indirect immunofluorescence test (IFT)

<table>
<thead>
<tr>
<th>Cyst fraction used in the IFT</th>
<th>Number of MAbs recognizing cysts</th>
<th>Cross-reactivity</th>
<th>Cyst fraction used in the IFT</th>
<th>Number of MAbs recognizing cysts</th>
<th>Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. histolytica 503</td>
<td>21</td>
<td>43%</td>
<td>E. histolytica 503</td>
<td>12</td>
<td>75%</td>
</tr>
<tr>
<td>E. coli</td>
<td>9</td>
<td>14%</td>
<td>E. coli</td>
<td>9</td>
<td>75%</td>
</tr>
<tr>
<td>E. hartmanni</td>
<td>3</td>
<td>14%</td>
<td>E. hartmanni</td>
<td>3</td>
<td>25%</td>
</tr>
<tr>
<td>Endolimax nana</td>
<td>9</td>
<td>43%</td>
<td>Endolimax nana</td>
<td>10</td>
<td>83%</td>
</tr>
<tr>
<td>Giardia lambia</td>
<td>4</td>
<td>19%</td>
<td>Giardia lambia</td>
<td>10</td>
<td>83%</td>
</tr>
<tr>
<td>E. dispar</td>
<td>4</td>
<td>19%</td>
<td>E. dispar</td>
<td>7</td>
<td>58%</td>
</tr>
<tr>
<td>E. histolytica specific</td>
<td>10</td>
<td>48%</td>
<td>E. histolytica specific</td>
<td>0</td>
<td>0%</td>
</tr>
</tbody>
</table>

* Fixed with 4% paraformaldehyde.

observed. The culture supernatant of the clones was tested for the presence of mouse immunoglobulin using an ELISA system (peroxidase-screening reagent; Boehringer).

**Hybridoma screening procedure.** The IgG present in the supernatants was isolated by protein-A affinity chromatography (Pharmacia, Freiburg, Germany) as recommended by the manufacturer. The isolated antibodies were checked for purity by electrophoresis on a sodium dodecyl sulfate–10% polyacrylamide gel, followed by silver staining. Pure IgG was used at concentrations of 0.01–0.1 mg/ml in indirect fluorescence tests and Western blots.

Cyst fractions (approximately 100 cysts per test) were incubated in 0.1% gelatin, 0.5% bovine serum albumin in PBS (PBG) for 30 min to block unspecific binding. The MAbs were added to the cyst sediment after centrifugation at 10,000 × g for 5 min. After 1 h of incubation at room temperature, the MAbs were removed by centrifugation in PBG three times at 10,000 × g for 5 min. Fluorescein isothiocyanate (FITC)–labeled α-mouse IgG antibody was added to the sediment in PBG at a dilution of 1:20, as recommended by the supplier (Dako, Hamburg, Germany). Incubation with the second antibody was done for 45 min at 4°C. After three washing steps with PBG, the sediment was examined with a Axioskop microscope supplied with an MC 80 camera (Zeiss, Oberkochen, Germany). Binding of the antibodies was determined microscopically with cysts of E. histolytica, E. dispar, E. coli, E. hartmanni, J. bütschlii, Endolimax nana, and G. lamblia, and trophozoites of E. histolytica and E. dispar.

**Determination of IgG subclasses.** The IgG subclasses of the MAbs were determined using a strip assay (Boehringer).

**Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting.** Electrophoresis and Western blotting were done according to methods of Lugtenberg and others and Towbin and others. Briefly, 0.5 mm–thick horizontal polyacrylamide gels were used as gradient (10–18%) or 10% gels. For silver staining and blotting, 5 μg of IgG or cyst or trophozoite lysate was used per 0.5-cm gel lanes. Electrophoresis was done under denaturing conditions at pH 8.8 with 9 mA (constant power) for 2.5 hr. Gels were stained with silver according the method of Morrissey. For immunostaining, the separated proteins were transferred onto a polyvinyl (PVDF) membrane (Roth, Karlsruhe, Germany) using the apparatus and the recommendations of Phase (Lübeck, Germany). The staining procedure was done after blocking unspecific binding with of 0.05% Tween 20, 5% milk powder, using 0.01 mg of MAb per lane. Bound MAbs were detected with α-mouse-IgG conjugated with alkaline phosphatase using 2,2′-azinobis (3-ethylbenzthiazoline sulfonylic acid) (ABTS) as a substrate.

**RESULTS**

**Isolation of pure E. histolytica cysts and production of MAbs.** Cysts from fecal samples were purified using the two-step gradient method as previously described. In parallel, E. histolytica and/or E. dispar was grown from feces according to the method of Robinson, and the trophozoites were differentiated by riboprinting-PCR and hexokinase isoenzyme typing. From 22 isolated and differentiated samples, five isolates were identified as E. histolytica, 16 as E. dispar, and one patient was infected with both of these organisms. Entamoeba histolytica without other protozoal cysts was found in sufficiently high numbers for several immunizations and screening of MAbs in only one fecal sample. Approximately 2 × 10⁶ cysts were recovered from this sample, designated isolate 503. The carrier of isolate 503 showed no symptoms specific for amebiasis; however, a high α-E. histolytica serum antibody level of more than 100 antibody units was detected by ELISA. The cysts of isolate 503 were used in the native and fixed states for the immunization of BALB/c mice. Mice nos. 1 and 2 (animals immunized with native cysts) produced 63 antibody-producing clones, and fusions 3 and 4 (animals immunized with fixed cysts) produced 24 antibody producing clones. Culture supernatants of these clones were tested for recognition of the antigen used for immunization and for cross-reaction screening with other protozoal cysts in an indirect immunofluorescence test (IFT).

**Screening of the MAbs by IFT.** Culture supernatants were first studied for their recognition of the cysts derived from isolate 503, which were used in either fixed or native states. Twenty-one MAbs of fusions 1 and 2 recognized the native cysts of isolate 503, which is 33% of the antibody-producing clones, and 12 MAbs (50%) obtained from fusion 4 bound to the fixed 503 cysts (Table 1). Supernatants that recognized their own cysts were collected and IgG was isolated from them by protein A-affinity chromatography. The MAbs were used for cross-reactivity binding tests with E. dispar, E. coli, E. hartmanni, Endolimax nana, J. bütschlii, and G. lamblia cysts (Table 1), which were enriched from human feces according to the two-step sucrose gradient method.
Monoclonal Antibodies Against Cysts of E. histolytica


cysts were used in either fixed or native states. Cysts of J. bütschlii showed fluorescence without incubating them with the MAbs and/or the second antibody. Because of this autofluorescence, their cross-reactivity with the MAbs could not be determined.

The MAbs obtained from fusions of cells from mice immunized with fixed cysts showed high cross-reactivity with the other protozoal cysts. For this reason, no MAb was found that specifically recognized fixed cysts of E. histolytica. However, MAbs 1.4D7, 1.7E1, 1.11D6, 1.11D10, 1.17C2, 1.17E4, 1.21G6, 2.1H7, 2.7E9, and 2.15C6 obtained against native E. histolytica 503 cysts showed no cross-reactivity with other protozoal cysts. These MAbs were also tested with fixed 503 cysts; however, no MAb recognized them.

The MAbs were subjected to IFTs with five different cyst isolates of E. histolytica and eight different cyst isolates of
**Table 2**
Recognition of different *Entamoeba histolytica* and *E. dispar* cyst isolates by monoclonal antibodies (MAbs) in an indirect immunofluorescence test

<table>
<thead>
<tr>
<th>MAbs</th>
<th><em>E. histolytica</em> cyst isolates (n = 5)</th>
<th><em>E. dispar</em> cyst isolates (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.71E1</td>
<td>4</td>
<td>2*</td>
</tr>
<tr>
<td>1.11D10</td>
<td>5</td>
<td>1*</td>
</tr>
<tr>
<td>1.17C2</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

* Very weak cross-reactivity.

*E. dispar.* Three MAbs (1.7E1, 1.11D10, and 1.17C2) were found to react specifically with *E. histolytica* cysts (Figure 1A–C and Table 2). Even when surrounded by fecal debris, which showed yellow autofluorescence, the cysts could be distinguished from the debris by their label (Figure 1A and B).

**Characterization of the specific MAbs.** All three MAbs were shown to be IgG1 antibodies by a strip assay. Their light chains reacted in the strip assay with \(\alpha\)-k light-chain antibodies, demonstrating that they are mouse-derived antibodies. These antibodies were used in Western blots to determine the molecular weight of the polypeptide that they recognized. Only one of the MAbs, 1.11D.10, recognized an 80-kD protein of cysts and trophozoites that was boiled in sodium dodecyl sulfate (Figure 2). This antibody also recognized trophozoites of *E. histolytica* strain HM-1:1MSS and trophozoites of the *E. histolytica* patient isolate 1111, which were cultured from feces of a patient who had a liver abscess (Figure 1D). This antibody also showed very weak recognition of *E. dispar* SAW 1734 trophozoites in the IFT; however, no recognition was found in Western blots of *E. dispar* SAW 1734 trophozoites. The other MAbs did not react with *E. histolytica* or *E. dispar* trophozoites.

**DISCUSSION**

Differentiation of *E. histolytica* from other lumen-dwelling protozoa, especially from the morphologically indistinguishable *E. dispar*, is still a problem in routine diagnostics, although a number of techniques have been recently established for the rapid identification of *E. histolytica.* It was the aim of our study to develop a simple and inexpensive method, without the necessity for *in vitro* cultivation of the amebae or a fecal PCR. Therefore, we attempted to isolate cyst-specific MAbs for the differentiation of *E. histolytica* in feces since the cyst stage is found most often in microscopic examination of the feces of patients. One of the difficulties in this project was to isolate pure cysts of *E. histolytica* for the immunization of mice. A new isolation method had to be established because published methods for the isolation of protozoan cysts and *in vitro* encystation were unsuccessful or yielded cyst suspensions containing fecal particles. The two-step gradient method followed by cellulose digestion yielded pure protozoan cysts. However, about half of the isolated cyst fractions contained other protozoan cysts in addition to *E. histolytica* cysts. It was difficult to isolate enough cysts from one patient for several immunizations and the screening procedure. Indeed, cysts from only one patient showing a monoinfection with *E. histolytica* were isolated in sufficient amounts to be used for production of MAbs. These cysts were used in the native and fixed states for immunization of mice because we intended to produce MAbs that could be used in fixed fecal samples and in native fecal samples. The results showed that all MAbs produced against fixed cysts from isolate 503 cross-reacted with the cysts of other protozoal species. None of them were specific for *E. histolytica* cysts. This may be due to the fixation procedure although the fixative and the time for fixation used was known to conserve antigens in a way that they can be used for immunolabeling. Cyst epitopes may also have similar structures in all protozoan cysts. This would also be an explanation for the high cross-reactivity of MAbs produced against native isolate 503 cysts. However, 10 antibodies were found to react only with *E. histolytica* cysts. Of these, three MAbs were tested with several *E. histolytica* and *E. dispar* cyst isolates and found to be useful for diagnostic purposes. Unfortunately, none of the MAbs recognized fixed *E. histolytica* cysts. Therefore, the MAbs can only be used with native fecal samples and not with preserved feces.

Our initial intention was to use fecal smears for the detection of cysts with FITC-conjugated MAbs. However, a disadvantage of this method is that laboratories in Third World Countries would need fluorescence microscopes for this test. This problem could be circumvented by coupling the MAbs to an enzyme and using a staining procedure that yielded an insoluble product. Based on the IFT experiments, we can expect that it is rather time-consuming to search for labeled cysts in smears containing only small numbers of them. This is a problem especially when a large number of patients have to be examined. Therefore, we are now working on the development of a capture-ELISA system with a polyclonal rabbit \(\alpha\)-cyst antibody to be used as the capture antibody. It could also be interesting to use the cyst MAbs combined with MAbs recognizing trophozoite proteins, such
as adhesin or serine-rich *E. histolytica* protein, which are now used in two commercially available kits.

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Authors' addresses: Brigitte Walderich, Jürgen Knobloch, and Ludger Maier for technical assistance.

Reprint requests: Brigitte Walderich, Institut für Tropenmedizin, Wilhelmstraße 27, 72074, Tübingen, Germany.

REFERENCES


