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**Encephalocystozoon intestinalis**: Specific Monoclonal Antibodies for Laboratory Diagnosis of Microsporidiosis


**Departments of Medical Microbiology and Pathology, University Hospital Nijmegen, 6500 HB Nijmegen, and Department of Medical Microbiology, Academic Medical Centre, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands**

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Two monoclonal antibodies which can be used for the unambiguous identification by fluorescence microscopy of *Encephalocystozoon intestinalis* spores in clinical specimens are described. Monoclonal antibody S191 is specific for the extruded polar filament, and S113 recognizes the surfaces of *E. intestinalis* spores. No cross-reaction with spores of *Encephalocystozoon hellem* was observed. Immunogold electron microscopy confirmed the specific reactivities of both antibodies. Combined in an indirect immunofluorescence assay, these antibodies are used to identify spores in feces. Although there was some cross-reaction with fecal bacteria and fungi, the typical morphology of the extruded polar filaments enabled proper identification of the *E. intestinalis* spores. Parasites could also be demonstrated to be present in urine, nasal swabs, lung brush biopsy specimens, and bronchoalveolar lavage fluid from a patient with disseminated infection with *E. intestinalis*. The use of these monoclonal antibodies facilitates the detection and species determination of *E. intestinalis* in clinical specimens.

Microsporidiosis is frequently found in patients with AIDS. The laboratory diagnosis of this infection no longer depends on the availability of biopsy specimens from affected tissues; it can also be made by identifying spores in feces, urine, and other body fluids. The spores of microsporidial species cannot easily be identified by classical staining methods because of their small size and lack of characteristic morphological details by light microscopy. Methods for identification have recently been improved by modifications of sample preparation (9) and staining (4, 6, 8, 11) methods. Moreover, specific staining of spores with polyclonal antibodies has been introduced, but clinical application is hampered by high levels of background staining and undesired cross-reactions (1, 15). The present study aimed at the selection and description of *Encephalocystozoon intestinalis*-specific monoclonal antibodies (MAbs) and the development of a methodology for their diagnostic application.

Recently, the reclassification of the species *Septata intestinalis* to *E. intestinalis* has been proposed (3). In this paper, the latter name has been adopted.

**MATERIALS AND METHODS**

**Parasites.** *E. intestinalis* was isolated from feces by the Transwell method (10). Parasites were seeded on two types of monolayers: human lung mucoepidermoid cells NCI-H292 (ATCC-CCL 34) and rabbit kidney cells RK13 (ATCC-CCL 37). The monolayers were incubated at 30°C in 5% CO2 in Dulbecco minimum essential medium (catalog no. 074-02100; Gibco) with 10% fetal calf serum (FCS) and 1% nonessential amino acids (catalog no. 16810-49; Flow). Sperms could be harvested from monolayers in 75-cm2 culture bottles over periods up to 2 weeks after seeding. Suspensions of parasites were sedimented by centrifugation (1800 g, 2 min), washed, and stored in phosphate-buffered saline (PBS) at 4°C.

**Parasite extracts** were performed by using 2-μl droplets applied to 7-mm wells on multwell slides, stained according to the Uvitex2B method (11).

**MAbs.** BALB/c mice were immunized with 6 × 107 spores injected intraperitoneally without adjuvant. Identical injections were given on days 28 and 56 and after booster and spleen cells were fused with Ag8/12 myeloma cells on day 59.

**SIDS-PAGE and Immunoblots.** *E. intestinalis* proteins were solubilized and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using the Laemmli buffer system (5) and gradient gels of 10% acrylamide. To the SIDS sample buffer 3% dithiothreitol (DTT) was added, and extracts of 3 × 108 parasites were loaded on slab gels (4%, 14 cm wide; gels, 0.7 mm thick). After electrophoresis at a constant current of 50 mA, proteins were transferred to polyvinylidene difluoride membranes in a Bio-Rad Transblot System at a constant voltage of 100 V for 2 h. Molecular weight markers were run in separate lanes, and these were stained with 0.1% Indian ink in PBS with 0.02% Tween 20 (TPBS). Blots saturated with 5% FCS in PBS were incubated with antibody-containing hybridoma supernatants diluted 1 to 10 in TPBS with 0.2% FCS. Hybridoma clones producing MABs were selected by an indirect immunofluorescence assay using air-dried, formalin-fixed microsporidia of *E. intestinalis* suspensions. These suspensions contained free mature spores and parasites at various developmental stages but also parasites within intact cells of the NCI or RK13 monolayers. Positive reactions of unclotted supernatants of hybridoma clones were visualized with fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G (IgG) (Cappel) (diluted 1:160) by fluorescence microscopy. The IgG isotype of MABs was determined with a dipstick isotyping kit according to the instructions of the manufacturer (GIBCO BRL).

**Immunoelectron microscopy.** Infected NCI cells were harvested from culture bottles by suspending the cells with 0.25% trypsin in PBS containing 1 mM diiodoindolmyelidite (EDTA); after centrifugation at 40 × g for 10 min, the cell pellet was washed in PBS, pelleted again, and resuspended in 2% paraformaldehyde in Sörensen's buffer (pH 7.4) (4°C, room temperature). After being washed in PBS, the material was resuspended in 12% gelatin in PBS, centrifuged, and, after being cooled, immersed in 7.3 M sucrose for 30 min and stored in liquid nitrogen. Sections 30 μm thick were made with a cryotome at −20°C, and after being washed in PBS these were incubated with undiluted supernatants of hybridoma supernatants diluted 1 to 10 in TPBS with 0.2% FCS. Immunoreactions were detected with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dako) diluted 1:10,000 in TPBS with 0.2% FCS and diaminobenzidine (0.4 mmol) in PBS with 0.1% hydrogen peroxide as the substrate.

**Clinical samples.** Bronchoalveolar lavage fluid and nasal swabs were smeared directly on microscope slides. Urine samples were smeared at 2,000 × g for 10 min, treated with 50 mg of acetylcysteine per ml, resuspended in PBS, and applied to slides. Bronchoalveolar lavage fluid was resuspended at 2,000 × g for 10 min, resuspended in PBS, and applied to slides. All slides were fixed in formalin or methanol before incubation with the MABs. Reactivity of S113 with these monoclonal antibodies facilitates the detection and species determination of *E. intestinalis* in clinical specimens.
RESULTS

Moreover, parallel with early stages of development within the lower third of the stress lines, several well-defined projections in the form of flattened, rather narrow, and relatively thin filaments were observed in adjacent to the Immune fluorescent assay. Two filaments were selected for

In addition, a modulation of the fluorescence detected by Western and Immunofluorescence was performed. The presence of a single white square in which the MAP17 and MAP18 were detected was highlighted. In contrast, the MAP13 and MAP12 were not observed in the same projection.
both MAbs with spores in untreated feces was poor but could be improved as described in Materials and Methods. In unprepared stool samples, stored at 4°C for periods up to 6 weeks, spores were provoked to discharge polar filaments. Positive immunofluorescence results with both MAbs were achieved with these stool samples. After 3 months of storage in a refrigerator, spores in feces lost their reactivity with MAb Si13 and their capability to discharge the polar filament.

Cross-reactions with bacteria and fungi are seen but do not interfere with proper diagnosis because of the typical morphology of the E. intestinalis spores with extruded filaments.

**DISCUSSION**

Reactions of MAb Si13 with intracellular parasites at various developmental stages, sporonts, and mature spores indicate the presence of a common antigen on the surfaces of parasites at all stages. The target protein has a relative mobility of 130 kDa, as was demonstrated on immunoblots of SDS-PAGE-separated total parasite extracts.

MAb Si91 reacted exclusively with the polar filaments after extrusion from the spores, not with the coated filament inside the maturing spore. This suggests a modification in the conformation of the proteins involved. In a report on polar tube protein from spores of Glugea hertzogii, the polar tube protein has been described as being resistant to dissociation in 3% SDS but sensitive to reduction in 1% DTT (12). The E. intestinalis polar tube protein in this study was characterized by the presence of three diffuse bands in immunoblots with the filament-specific MAb Si91. The eventual incomplete solubilization in SDS might explain the appearance of the antigenic protein as multiple vague bands after SDS-PAGE separation. In contrast to what Weidner found (12), the addition of DTT in the sample buffer for SDS-PAGE in our experiments had no effect on the results obtained with polar tube protein in the immunoblots. With all clinical specimens other than feces examined in this study, both MAbs Si13 and Si91 showed brilliant fluorescence without any pretreatment of the samples. Spores could not be found easily, even if present in small numbers.

The application of these MAbs as tools for detecting E. intestinalis in feces requires pretreatment of the samples. The reactivity of MAb Si13 was improved by adequate washing of the spores with the reducing agent DTT, and specific demonstration of the polar filament with MAb Si91 was facilitated by provocation of extrusion of this organelle. This capability of discharging the polar filament, however, was abolished in feces kept for 3 months. Apparently the capability to discharge the filament is not indefinitely resistant to environmental conditions in feces. The reactivity of MAb Si13 with its epitope on the surface protein also deteriorates during storage of the sample in a refrigerator. Examination of stool specimens should therefore preferably take place within days after sample collection.

Extensive cross-reactivity among different microsporidian species has been observed with polyclonal sera from rabbits immunized with a single microsporidian species (7), implicating common antigens among species. The use of such antibodies for diagnostic purposes has been suggested by several authors (14, 15). The MAbs described in this report are exclusively specific for E. intestinalis. We did not observe cross-reactions with the closely related E. hellem.

An advantage of this high degree of species specificity, however, is the easy species differentiation of Enterocytozoon spores.

It can be concluded that the MAbs described in this report...
have great potential for the demonstration and species determination of *E. intestinalis* in clinical samples.

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**REFERENCES**


