

## *Encephalocytozoon intestinalis*-Specific Monoclonal Antibodies for Laboratory Diagnosis of Microsporidiosis

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**Two monoclonal antibodies which can be used for the unambiguous identification by fluorescence microscopy of *Encephalocytozoon intestinalis* spores in clinical specimens are described. Monoclonal antibody Si91 is specific for the extruded polar filament, and Si13 recognizes the surfaces of *E. intestinalis* spores. No cross-reaction with spores of *Encephalocytozoon 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 *Encephalocytozoon 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 mucocypidermoid cells NCI-H292 (ATCC-CCL 34) and rabbit kidney cells RK13 (ATCC-CCL 37). The monolayers were incubated at 36°C in 5% CO<sub>2</sub> 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). Spores could be harvested from monolayers in 75-cm<sup>2</sup> culture bottles over periods up to 2 weeks after seeding. Suspensions of parasites were sedimented by centrifugation (1000 × g, 2 min), washed, and stored in phosphate-buffered saline (PBS) at 4°C.

Parasite counts were performed by using 2- $\mu$ l droplets applied to 7-mm-wide wells on multiwell slides, stained according to the Uvitex2B method (11).

**MAbs.** BALB/c mice were immunized with 6 × 10<sup>7</sup> spores injected intraperitoneally without adjuvant. Identical injections were given on days 28 and 56 as booster and spleen cells were fused with Ag63X myeloma cells on day 59.

Hybridoma clones producing MAbs were selected by an indirect immunofluorescence assay using air-dried, formalin-fixed microdroplets 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 undiluted supernatants of hybridoma cultures 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).

**SDS-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 6 to 10% acrylamide. To the SDS sample buffer 3% dithiothreitol (DTT) was added, and extracts of 3 × 10<sup>8</sup> parasites were loaded on slab gels (slot, 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 supernatant diluted 1 to 10 in TPBS with 0.2% FCS. Immune reactions were detected with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dakopatt) diluted 1 to 1,000 in TPBS with 0.2% FCS and diaminobenzidine (0.4 mmol) in PBS with 0.1% hydrogen peroxide as the substrate.

**Immunoelectron microscopy.** Infected NCI cells were harvested from culture bottles by suspending the cells with 0.25% trypsin in PBS containing 1 mM disodium EDTA; after centrifugation at 40 × g for 10 min, the cell pellet was washed in PBS, pelleted again, and resuspended in 2% paraformaldehyde in Sørensen buffer (pH 7.4) (4 h, room temperature). After being washed in PBS, the material was resuspended in 12% gelatin in PBS, centrifuged, and, after being cooled, immersed in 2.3 M sucrose for 30 min and stored in liquid nitrogen. Sections 30  $\mu$ m thick were made with a cryomicrotome at -20°C, and after being washed in PBS these were incubated overnight with undiluted hybridoma supernatants Si91 and Si13. All incubations and washings of the sections were performed on a rotary shaker. Anti-mouse F(ab')<sub>2</sub> antibody fragments labeled with ultrasmall (1-nm diameter) gold particles (Aurion, Wageningen, The Netherlands) were used as the second antibody. In control experiments, sections were incubated with second antibodies alone. After an intensive washing, sections were silver enhanced according to Danscher's method (2); after dehydration and embedding in Epon 812 for electron microscopy, ultrathin sections were cut with a diamond knife (Drukker, Cuijk, The Netherlands), contrasted with uranyl acetate and lead citrate, and examined by using a JEOL 1200 EX/II (JEOL, Tokyo, Japan) electron microscope.

**Clinical samples.** Brush biopsy specimens and nasal swabs were smeared directly on microscope slides. Urine samples were sedimented 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 sedimented 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 Si13 with

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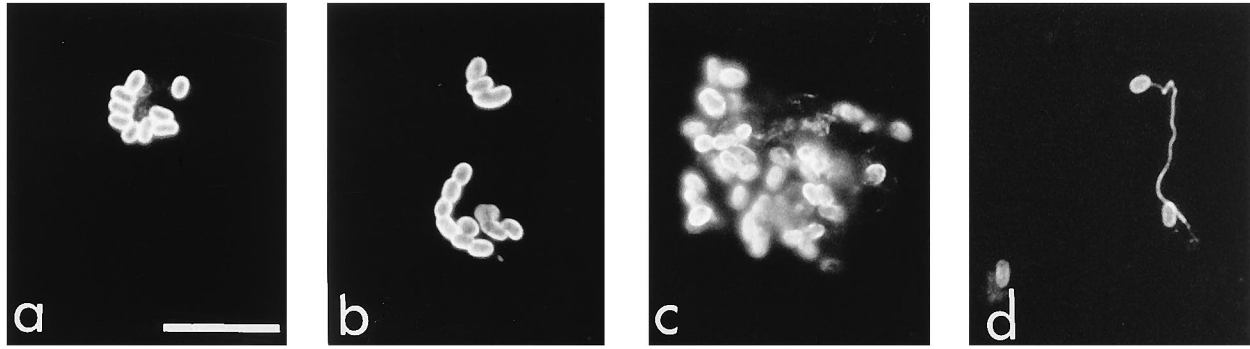


FIG. 1. Immunofluorescence patterns of *E. intestinalis* from in vitro culture after specific staining with MAb Si13 (a, b, and c) and spores from a urine sample sediment stained with the combination of Si13 and Si91 (d). With Si13, the surfaces of spores (a) and sporonts in chain formation (b) are stained. The parasites at various stages are stained within a disintegrating RK13 cell (c). Together, MAbs Si13 and Si91 clearly stain the surfaces of spores and the characteristic extruded polar filament in a patient's urine sample sediment (d). Bar = 10  $\mu$ m.

spores in stool specimens was improved by diluting the feces 1:2,000 in PBS with 1% DTT by using a whirl mixer; the material was sieved through a 70- $\mu$ m-pore-size disposable cell strainer and concentrated by centrifugation for 10 min at 2,000  $\times$  g. The spores in the sediment were provoked to discharge their polar filaments by a pH shock, in order to enable MAb Si91 to react with extruded polar filaments. This was achieved by resuspending the sediment in 0.025 N NaOH in PBS, a modification of the procedure described by Weidner and Baird (13).

## RESULTS

**Immunofluorescence assays.** Two MAbs were selected because of reactivity with *E. intestinalis* in the immunofluorescence tests. MAb Si13 (IgG3 subclass) reacted with mature spores as well as with sporonts in chain formation (Fig. 1a and b). Moreover, parasites at early stages of development within

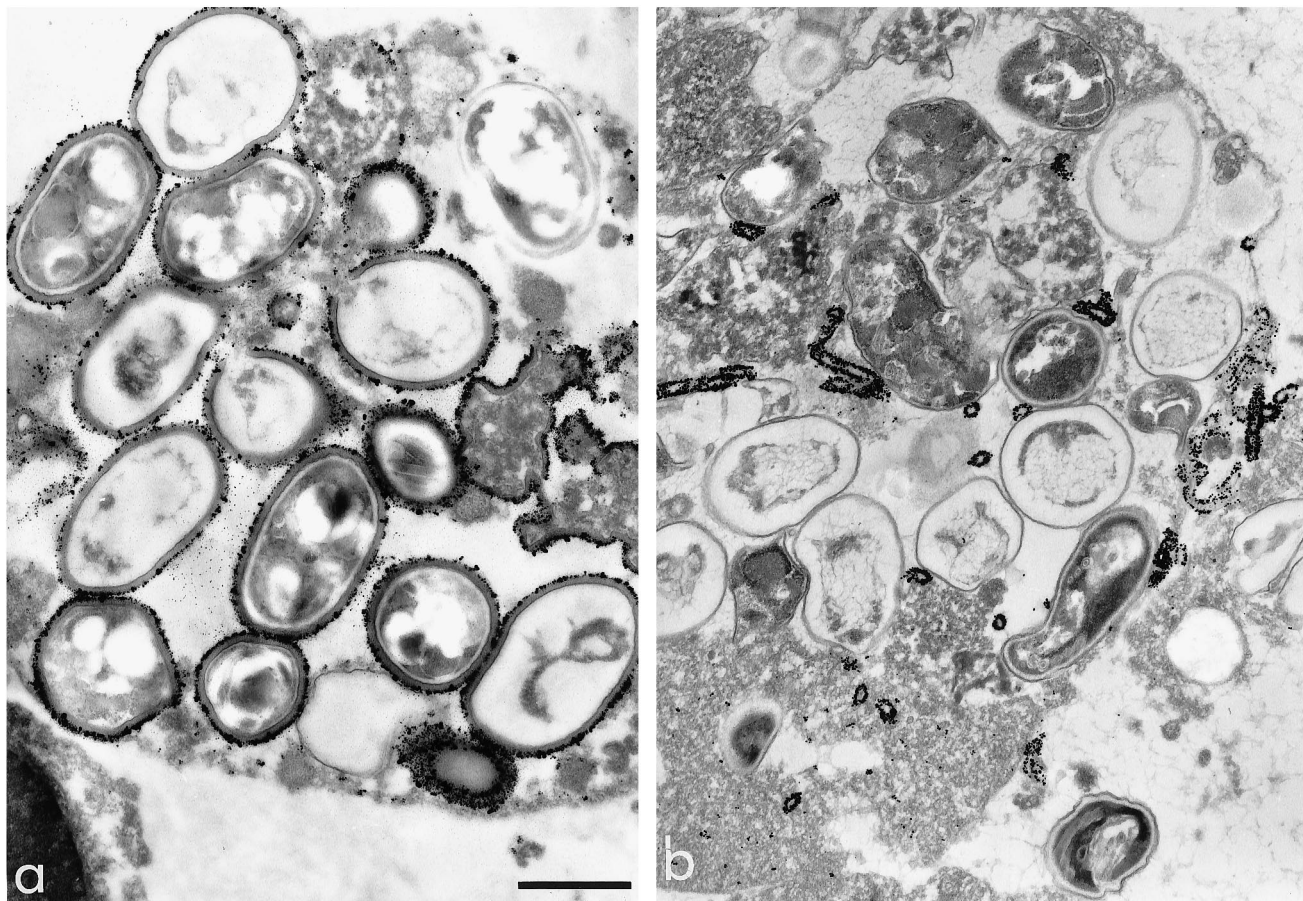


FIG. 2. Immunoelectron micrographs of *E. intestinalis* from in vitro culture in NCI cells after incubation with MAbs Si13 (a) and Si91 (b). With Si13 (a), the gold particles are uniformly distributed along the surfaces of mature spores and developing parasites, similar to the immunofluorescence assay result shown in Fig. 1c. The exclusive reactivity of MAb Si91 (b) with discharged polar filaments is clearly demonstrated by the distribution of gold particles along the surfaces of transversely sectioned and cross-sectioned polar filaments outside the spores. The filaments inside intact spores are not recognized by MAb Si91. Bar = 1  $\mu$ m.

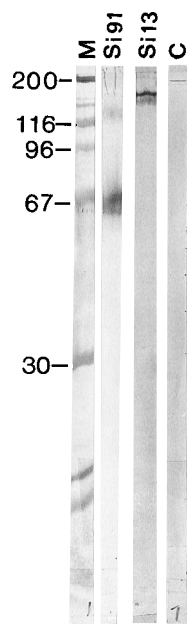


FIG. 3. Western blot (immunoblot) of SDS-PAGE-separated proteins of *E. intestinalis*. Lanes Si91 and Si13 show the reactions of the MAbs with the blotted parasitic proteins: Si91 with a diffuse band at 60 kDa and a minor reaction around 120 kDa and Si13 with a 130-kDa protein. Lane M shows the relative mobilities of molecular mass marker proteins stained with Indian ink; the corresponding molecular masses are indicated at the left in kilodaltons. In lane C, the background staining of a nonreactive MAb (IgG1 subclass) is demonstrated.

the cells from the culture monolayer showed positive reactions with this MAb (Fig. 1c).

MAb Si91 (IgG1 subclass) reacted exclusively with the extruded polar filaments of the mature spores. Si13 and Si91 showed no mutual competition in their binding to *E. intestinalis*: when the two MAbs were combined in one immunofluorescence test, both the walls of the spores and the extruded filaments were stained (Fig. 1d). With *Encephalitozoon hellem* suspensions as the antigen, no fluorescence of spores or filaments was observed after incubation with both MAbs.

**Immunoelectron microscopy.** The results of the immunofluorescence assays have been confirmed at the ultrastructural level. Incubations with Si13 resulted in homogeneous binding to the surfaces of both intra- and extracellularly located spores and parasites at various developmental stages (Fig. 2a).

MAb Si91 reacted exclusively with the surfaces of extruded polar filaments (Fig. 2b). No gold particles could be found on coiled filaments inside any of the numerous spores studied. In control experiments with second antibodies alone, no deposits of gold particles were observed.

**Immunoblotting.** Both MAbs Si13 and Si91 reacted with blots of SDS-PAGE-separated proteins (Fig. 3). Si13 reacted with a narrow, well-defined band at 130 kDa. Si91 showed two broad bands around 60 and 120 kDa.

No reactions were observed with similar blots of *E. hellem* proteins.

**Examination of clinical specimens.** With a combination of MAbs Si13 and Si91, spores of *E. intestinalis* were detected in urine, stool, and bronchial brush biopsy specimens, bronchoalveolar lavage fluid, and nasal swabs from a patient with electron microscopically confirmed disseminated *E. intestinalis* infection. In urine (Fig. 1d), bronchoalveolar lavage fluid, brush biopsy specimens, and nasal swabs, strong fluorescence of spores and extruded polar filaments was seen. Reactivity of

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 coiled 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 hertwigi*, 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 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 *Encephalitozoon* 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.

#### ACKNOWLEDGMENT

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