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Monoclonal Antibodies for Specific Detection of *Encephalitozoon cuniculi*

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Seven species-specific monoclonal antibodies (MAbs) were produced against *Encephalitozoon cuniculi* and characterized. The MAbs were immunoglobulin G, and when used for indirect microimmunofluorescence microscopy and Western immunoblot assays, they detected *E. cuniculi* originating from clinical samples. They did not cross-react with other *Encephalitozoon* species (*E. intestinalis* and *E. hellem*) or with a collection of gram-negative bacteria, yeast, and other parasites. The MAbs reacted primarily with 121-, 56-, 45-, 43-, and 41-kDa protein epitopes of *E. cuniculi*. These epitopes were demonstrated to be *E. cuniculi* species specific by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. We developed MAbs to strains of *E. cuniculi* that can be used successfully to distinguish *E. cuniculi* from other microsporidial species in cultures established from clinical specimens. These MAbs may provide a specific, simple, rapid, and low-cost tool for the identification and diagnosis of infections due to microsporidia.

Encephalitozoon cuniculi is a unicellular, obligately intracellular microsporidian species responsible for emerging opportunistic infections (5, 28, 34). Its genome sequence has been published recently (11). The infective stage of *E. cuniculi* is the mature spore, which contains a unique extrusion apparatus in the form of a coiled polar tubule for injecting infectious sporoplasms into host cells (7, 31). Spores are highly resistant in the environment because of the presence of a thick, chitin-containing wall (2). *E. cuniculi* causes hepatitis, peritonitis, encephalitis, intestinal infections, keratoconjunctivitis, sinusitis, rhinitis, respiratory infection, urinary tract infection, and disseminated infection in immunocompromised patients (13, 15, 20, 30, 33) and nonimmunocompromised patients (e.g., the elderly, travelers to the tropics, and residents of the tropics) (6, 8, 9).

Definitive identification of *E. cuniculi* currently depends on time-consuming and costly transmission electron microscopy to definitively identify spores in clinical samples. In addition, transmission electron microscopy may not be sensitive enough to detect small numbers of organisms. Serological studies for detecting microsporidium-specific antibodies are reliable for antemortem diagnosis in infected laboratory animals (24, 25). Mammalian microsporidian spores do stain with Gram stain, Giemsa stain, Calcofluor, and concentrated trichrome stain (26), but because these organisms are very small, they are difficult to distinguish from bacteria and small yeasts. A few *E. cuniculi* isolates were recovered from various clinical specimens (27), but PCR assays are currently available in only a few laboratories (20). Identification of microsporidian agents at the species level is important because several new drug therapies are effective in treating infections caused by some, but not all, microsporidia (1). Certain therapeutic agents (e.g., fumagillin, albendazole) are effective in treating urogenital and respiratory infections caused by *E. cuniculi* (8).

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We (14) and others (4, 19) previously demonstrated the usefulness of monoclonal antibodies (MAbs) for the rapid and specific detection of microsporidia, including *Encephalitozoon hellem* (14) and *E. cuniculi* (4, 19). It would be advantageous to have species-specific monoclonal antibodies available for the diagnosis of microsporidiosis. In this study, our goal was to develop a diagnostic reagent for the routine identification of *E. cuniculi* in microsporidian-positive clinical specimens. Here, we describe the characteristics and specificities of seven species-specific monoclonal antibodies that we produced against *E. cuniculi*.

MATERIALS AND METHODS

Sources of *Encephalitozoon* spp. The geographic sources of the *Encephalitozoon* sp. strains used in the study are listed in Table 1. *Encephalitozoon* isolates were cocultured with MRC5 cells (human fetal lung fibroblasts) in minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum and 1% glutamine, and the culture medium was replaced every week. Culture media from all T-150 flasks, containing extruded spores and unattached host cells infected with developmental stages of the parasite, were centrifuged at 1,500 × *g* for 20 min at 4°C, and the supernatant was aspirated. The pellets were put back into the same culture flasks. This facilitated infection of a maximum number of host cells with the respective parasites (>70%), as revealed by using DiffQuik and Weber 2R staining according to the manufacturer's recommendations, followed by microscopic examination (Axioskop 20; Carl Zeiss, Gottingen, Germany) at a magnification of ×1,000. Therefore, spores that were extruded into the culture supernatants from all parasites were harvested by centrifugation as described above, sonicated three times (for 1 min each time), and centrifuged over 25% sucrose at 7,500 × *g* for 30 min. The parasites were washed twice with sterile phosphate-buffered saline (PBS [pH 7.2]) to remove the sucrose and were then suspended either in sterile deionized water, for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), or in PBS, for microimmunofluorescence (MIF).

MIF. The MIF assay (16) was used to screen hybridoma clones and to determine the specificities of the MAbs. Antigens were placed on 24-well microscope slides with a pen nib. The antigens were fixed in acetone for 10 min at room temperature, air dried, and incubated with supernatants in a humidified chamber at 37°C for 30 min. After two washes in PBS (5 min each) and rinsing in sterile distilled water for 1 min, the slides were air dried at 37°C. Following incubation at 37°C for 30 min with dechlorotriazinyl amino fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG) plus IgM (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) diluted 1:200 in PBS with 0.2% Evans blue (Sigma Chemical Co.), the slides were washed as described above and mounted with Fluoprep (BioMerieux, Marcy l'Etoile, France) before being observed under an epifluorescence microscope (Axioskop 20; Carl Zeiss) at a magnification of ×400. Serum samples from healthy, uninoculated mice were used as negative controls.

TABLE 1. Strains of *Encephalitozoon* spp. used for screening and determination of MAb specificity

Species	Strain	Genotype	Karyotype	Source
Encephalitozoon intestinalis				The Netherlands
Encephalitozoon cuniculi				The Netherlands
Encephalitozoon hellem	EU1	1	A	North America
	ASM1	1	A	The Netherlands
	PV4	1	B	Italy
	PV6	1	B	Italy
	PV7	1	B	Italy
	PV8	1	B	Italy
	PV9	1	B	Italy
	PV10	1	B	Italy
	PV11	1	B	Italy
	PV12	1	B	Italy
	PV93	1	B	Italy
	PV94	1	B	Italy
	PV95	1	B	Italy

Production of MAbs. For production of MAbs (10), 6-week-old female BALB/c mice were inoculated intraperitoneally six times with 10^7 *E. cuniculi* organisms in 0.5 ml of PBS without adjuvant, at 7-day intervals. One week after the final intraperitoneal inoculation, the mice were injected once through the tail vein with 10^6 spores suspended in 0.1 ml of PBS. Serum samples from the mice were screened by a MIF assay, and the antibody titer was 1:1,600. Three days later, spleen cells from the mice were fused with SP2/0-Ag14 myeloma cells (10:1) by using 50% polyethylene glycol 6000 (Sigma Chemical Co.). Fusion cells were grown in hybridoma medium (Seromed, Berlin, Germany) with 17% heat-inactivated fetal bovine serum (Gibco BRL, Eggenstein, Germany) and hypoxanthine-aminopterin-thymidine selective medium (Sigma Chemical Co.) at 37°C under a humidified atmosphere supplemented with 5% CO_2. The supernatants were screened for antibodies to the antigens of *E. cuniculi*, *Encephalitozoon intestinalis*, *E. hellem* EU1, and *E. hellem* PV6 by MIF, and positive hybridomas were subcloned three times by limited dilution. Isotypes of MAbs were determined by using ImmunoType Mouse IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3 assays (Sigma Chemical Co.) according to the manufacturer's recommendations. The specificities of the MAbs were tested by Western immunoblotting and MIF.

SDS-PAGE and Western immunoblotting. Antigens were suspended in an equal volume of sample buffer (0.0625 M Tris hydrochloride [pH 8.0], 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue) (12) and separated electrophoretically in 10% resolving gels with 5% stacking gels at a constant current of 8 to 10 mA per gel for 3 to 4 h in running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) in a Mini Protean II apparatus (Bio-Rad, Richmond, Calif.). Prestained SDS-PAGE standards (low range [112, 81, 49.9, 36.2, 29.2, and 21.3 kDa]; Bio-Rad) were used as a reference. The separated antigens were transferred to 0.45-μm-pore-size nitrocellulose membranes (Hybond-C; Amersham, Little Chalfont, Buckinghamshire, United Kingdom) at 100 V for 1 h at 4°C in an electrophoretic transfer cell (Mini Trans-Blot; Bio-Rad). After transfer, the nitrocellulose membranes were incubated overnight in PBS with 5% nonfat dry milk to block nonspecific binding sites. After three 10-min washes in PBS, the membranes were air dried, cut into strips, incubated with MAbs diluted to 1:100 or higher in PBS containing 5% nonfat dry milk at room temperature for 1 h, and washed twice (10 min each time). After incubation at room temperature for 1 h with peroxidase-conjugated goat anti-mouse IgG $F(ab')_2$ fragment (heavy and light chains) (AffiniPure; Jackson ImmunoResearch) diluted 1:200 in PBS with 5% nonfat dry milk and three washes in PBS as described above, color was developed with a coloring buffer containing 0.015% 4-chloro-1-naphthol and 0.015% hydrogen peroxide in 16.7% methanol in PBS.

Cross-reactivity studies. The reactivities of the MAbs were assessed by MIF. Blind testing of MAbs by MIF was carried out with *E. cuniculi*, *E. intestinalis*, 13 strains of *E. hellem* isolates from patients in Italy, The Netherlands, and North America, *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Shigella dysenteriae*, *Proteus vulgaris*, *Salmonella enterica*, and *Aspergillus fumigatus*.

RESULTS

MAb production. Antisera from immunized BALB/c mice were screened for *E. cuniculi*-specific antibody responses 7 days after each injection before a final booster. All mice began to produce antibodies after the second intraperitoneal injection. The highest antibody response was raised after the sixth injection and produced a titer of 1:1,600. One mouse (whose spleen was three times bigger than those of nonimmunized mice) was then selected for the fusion protocol. A total of 1,248 wells were seeded with fused SP2/O myeloma cells. On the 5th day after fusion, viable clones were observed in about 80% of the wells. The supernatants began to be screened with whole spores of *E. cuniculi*, *E. hellem* EU1, *E. hellem* PV6, and *E. intestinalis* on the 10th and 15th days after fusion. After the second screening, 191 antibody secretion hybridomas still reacted against spores of *E. cuniculi*. Seventy-three hybridomas were selected for subcloning by limiting dilution. After cloning procedures by limiting dilution were performed three times, seven stable hybridomas were selected on the basis of their reactivities with the four antigens and were selected against the second large panel of *Encephalitozoon* spp. (Table 2). The seven MAbs were expanded in culture.

MIF reactivities and isotyping of MAbs. The immunoglobulin class and subclass of each of the seven MAbs are presented in Table 2. Seven MAbs of subclasses IgG1, IgG2a, and IgG3 (Table 2), produced from subcloned hybridomas, were examined for their reactivities with *Encephalitozoon* strains. These MAbs were strongly reactive with *E. cuniculi* strains but displayed no cross-reaction with *E. intestinalis* or with any strains of *E. hellem*. The reactivity of each hybridoma is presented in Table 2. The polyclonal antisera generated more background than MAbs in acetone-fixed antigens with a MIF stain.

SDS-PAGE. The protein profile of *E. cuniculi* could be divided into three major groups of bands: (i) high-molecular-mass bands greater than 100 kDa, (ii) intermediate-molecular-mass bands of 30 to 80 kDa, and (iii) low-molecular-mass bands of less than 30 kDa. Although the SDS-PAGE profiles of the different species of *Encephalitozoon* were not the same, 121-, 56-, 45-, 43-, and 41-kDa protein bands appeared to be common to the *E. cuniculi* isolates under study, and they were also the most prominent bands in the SDS-PAGE profiles.

Western immunoblotting. Immunoblotting demonstrated that polyclonal antisera raised against *E. cuniculi* displayed many bands (Fig. 1, lane 1). Seven MAbs were directed against epitopes of the four previously identified intermediate-molecular-mass proteins and one high-molecular-mass protein (Fig.

TABLE 2. Reactivities of MAb panel elicited by *E. cuniculi* with different *Encephalitozoon* sp. antigens

MAb	Class	Specificity (kDa)	Reactivity[a] with antigens of:			
			E. cuniculi	*E. intestinalis*	*E. hellem*	
					PV6	EU1
EC8C12	IgG3	56	+	−	−	−
EC14E10	IgG2a	56	+	−	−	−
EC14D10	IgG1	56	+	−	−	−
EC11C5	IgG3	45	+	−	−	−
EC10G4	IgG3	43	+	−	−	−
EC7D5	IgG3	41	+	−	−	−
EC10E7	IgG3	121	+	−	−	−

[a] +, positive reactivity; −, no reactivity.

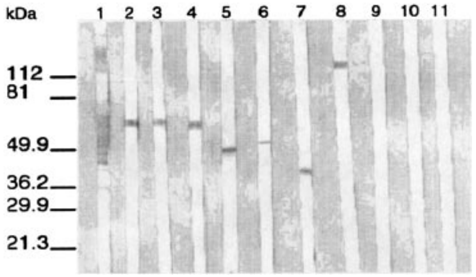

FIG. 1. Western immunoblotting of seven representative MAbs with *E. cuniculi* and other *Encephalitozoon* species. Lanes 1 to 8, immunoblotting of murine polyclonal antisera raised against *E. cuniculi* and MAbs EC8C12, EC14E10, EC11D10, EC10G4, EC11C5, EC7D5, and EC10E7, respectively, with *E. cuniculi*; lanes 9 to 11, immunoblotting of MAb EC812 with *E. intestinalis*, *E. hellem* PV6, and *E. hellem* EU1, respectively. Molecular mass markers are shown on the left.

1, lanes 2 to 8). Of the seven MAbs, three showed reactivity with a 56-kDa protein, one each showed reactivity with a 45-, a 43-, and a 41-kDa protein, and one showed reactivity with a high-molecular-mass protein (121 kDa), but they did not react with any antigens from the other *Encephalitozoon* species, *E. hellem* and *E. intestinalis* (Fig. 1, lanes 9 to 11). In Western blots of *E. cuniculi* antigens that had been digested with proteinase K, the MAbs failed to react with protein epitopes. However, heating of the antigens (100°C for 10 min) before immunoblotting did not affect the reactivities of the MAbs (Fig. 2).

Cross-reactivity studies. Blind testing of bacteria was performed with MAbs from supernatants of hybridomas. The MAbs reacted with all the strains of *E. cuniculi* tested but did not react with the other *Encephalitozoon* species or with other bacteria and yeasts used in the study.

DISCUSSION

Microsporidian infections are difficult to diagnose, primarily because the organisms are difficult to distinguish from bacteria and small yeasts in clinical samples. Giemsa stain (17, 18) and a modified trichrome stain using chromotrope 2R (29) have been used to detect microsporidia in clinical samples, but with some difficulties. Giesma-stained microsporidia are blue and display a purple-blue nucleus which distinguishes them from bacteria. It is difficult, however, to find microsporidia in clinical samples in which most other organisms also stain blue. The modified trichrome (chromotrope 2R) staining method described by Weber et al. (29) has the advantage that most bacteria counterstain light green, leaving the microsporidia pink. However, microsporidia may be missed if the parasite burden is low or if microsporidia are mixed with mucus. In addition, small yeast and sporulated bacteria in stool also stain pink, which can complicate the interpretation of smears. A Calcofluor staining method utilizing Uvitex 2B (Ciba-Geigy) or a fluorescent brightener also may be useful for detecting microsporidia (26). The microsporidia display relatively thick rings of fluorescence. However, the anterior regions appear concave, because yeasts also stain with Calcofluor.

The use of microsporidian-specific antibodies in MIF procedures appears to overcome some of these difficulties. Recently, polyclonal antisera produced against *E. cuniculi* and *E. hellem* were used to diagnose ocular and systemic *E. hellem* infections (21, 22), and a polyclonal antiserum raised against *E. cuniculi* in rabbits was used to detect cross-reacting *Enterocytozoon bieneusi* organisms in deparaffinized tissue sections (32) and in stool (35). We also raised MAbs against *E. hellem* (14). Currently, identification of human microsporidia at the species level depends on the use of time-consuming electron microscopy and molecular techniques (23, 28). Electron microscopy, however, cannot distinguish *E. cuniculi* from *E. hellem* in formalin-fixed tissue sections (20, 23). Because *E. cuniculi* and *E. hellem* are morphologically identical, staining and culture methods do not allow for their differentiation. The MAbs and polyclonal antibodies provided different advantages in MIF staining; we therefore used MIF and Western blotting for species identification.

In this report, we describe the production of seven MAbs that may be used as specific diagnostic reagents to identify *E. cuniculi* in cultures established from clinical samples from patients with microsporidiosis. The MAbs were characterized by using both MIF and Western blotting techniques. They detected acetone-fixed, culture-derived *Encephalitozoon* species. We found that the polyclonal antisera generated more background in acetone-fixed antigens with the MIF stain. The degree of background depended on the dilution of antiserum, as also described by Weiss et al. (32). The results clearly showed that the MAbs reacted with isolates of *E. cuniculi* at titers of 1:100 or greater yet showed no reactivity with either *E. hellem* or *E. intestinalis* in the MIF test. The murine polyclonal antisera detected many bands by Western blotting. The MAbs produced specific banding patterns by Western blotting with isolates of *E. cuniculi*. Three of the seven MAbs reacted with proteins of 58 kDa. Four of the seven MAbs reacted with 121-, 45-, 43-, and 41-kDa protein bands, respectively, in immunoblotting with *E. cuniculi*. This reactivity appeared to be specific for *E. cuniculi*. Finally, we have shown that these MAbs failed to react with spores of *E. intestinalis* and *E. hellem*, as well as with bacteria, yeasts, and other parasites, thus emphasizing the specificity of these MAbs for definitive clinical diagnosis.

Diagnosis of microsporidiosis at the species level is important, because certain therapeutic agents are effective in treating infections caused by some, but not all, microsporidian species. For example, systemic infections caused by *E. cuniculi* can be successfully treated with fumagillin and albendazole (3, 22). Hence, quick and definitive identification of *E. cuniculi*, pref-

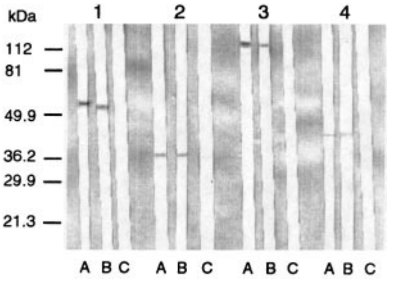

FIG. 2. Western immunoblotting of *E. cuniculi* antigens treated in different ways with MAbs. Lanes: A, native antigens without treatment; B, antigens heated at 100°C for 10 min; C, antigens treated with proteinase K at 1.5 mg/ml at 37°C for 2 h. Numbers 1 to 4 above lanes correspond to MAbs EC8C12, EC7D5, EC10E7, and EC11C5, respectively. Molecular mass markers are shown on the left.

erably by MIF and Western blotting using our MAbs described here, will be greatly advantageous in the successful diagnosis and treatment of microsporidiosis due to *E. cuniculi*.

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