

Mouse Monoclonal Immunoglobulin E Antibodies Specific for the Microsporidian *Encephalitozoon cuniculi* Polar Tube Protein 1

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Abstract

The microsporidian *Encephalitozoon cuniculi* is a spore-forming, obligate, intracellular parasitic pathogen with a unique organelle called a polar tube, the extrusion of which is essential for invading a host cell. The polar tube consists of three proteins: polar tube protein 1 (PTP1), PTP2, and PTP3. We established three mouse monoclonal antibodies (MAb1, MAb2, and MAb4) against *E. cuniculi* PTP1. An enzyme-linked immunosorbent assay (ELISA) indicated that all three MAbs reacted with the outer surface of extruded polar tubes and that they also strongly bound to an intracellular antigen in infected host cells. Two-dimensional (2-D) immunoblot analysis showed that MAb1 and MAb2 recognized PTP1 spots at 52 kDa and some spotty smears at molecular weights of less than 50 kDa, whereas MAb4 recognized only PTP1 spots at 52 kDa. Interestingly, all three MAbs were of the immunoglobulin (Ig) E class, suggesting that, in addition to the highly immunogenic or antigenic nature, the PTP1 antigen may have the potential to induce specific IgE antibody production in mice. These antibodies may be useful in the study of allergenic PTP1 as well in the purification and detection of the PTP1 antigen.

Introduction

ENCEPHALITOZOON CUNICULI is a microsporidian parasitic pathogen belonging to the phylum Microsporidia.⁽¹⁾ It infects various animals, including humans, and is distributed worldwide.⁽²⁾ The polar tube (PT) is a unique and specialized organelle of Microsporidia that is essential for host cell invasion.⁽³⁾ We recently detected specific anti-*E. cuniculi* PT IgM antibodies in sera from healthy and human immunodeficiency virus-infected persons in Japan.⁽⁴⁾ PTP1 could react with these antibodies.⁽⁵⁾ This finding and others⁽⁶⁾ suggest that the PTP1 antigen may be highly immunogenic in humans and animals.

Many monoclonal antibodies specific for *E. cuniculi* antigens have been reported.^(7–11) However, most of them are against sporal antigens, since anti-spore antibodies are considered to provide a simple tool for identification and diagnosis of infections due to microsporidia. There are quite a few published reports on the production of monoclonal antibodies against the PT antigen. Moreover, Ec102, mouse anti-*E. cuniculi* PT monoclonal antibodies that were reported

by Delbac et al.,⁽⁸⁾ recognized both PTP1 and PTP2.^(12,13) Our interest was to develop monoclonal antibodies capable of discriminating only the PTP1 antigen, since such reagents may aid in analyzing the antigenic determinants reactive to human or animal antibodies. We established three mouse monoclonal antibodies (MAb1, MAb2, and MAb4) against *E. cuniculi* PTP1. One of them, MAb2, has already been used for confirming the location of the PTP1 band in one-dimensional (1-D) immunoblot analysis.⁽⁵⁾ The objective of this study was to define the antibody reactivity in ELISA tests and 2-D immunoblot analysis, and to determine their Ig class. We discuss recognition sites and specificity of the MAbs and the potential immunogenicity and allergenicity of the PTP1.

Materials and Methods

Preparation of purified spores of *E. cuniculi*

E. cuniculi spores of strain HF, which was classified as genotype 1,⁽¹⁴⁾ were collected from culture supernatants of infected BS-C-1 cells (ATCC CCL-26, American Tissue Cul-

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ture Collection, Manassas, VA) or RK-13 cells (ATCC CCL-37) and purified using Percoll gradient centrifugation followed by Ultrafree-MC 5.0 μ m membrane filtration (Millipore, Bedford, MA), as described previously.⁽⁵⁾ Purified *E. cuniculi* spores were suspended in an appropriate volume of Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) containing 5% 2-mercaptoethanol (Bio-Rad) and heated to 95°C for 5 min. After centrifugation at about 7800 g for 5 min, pellets were treated twice more with the same buffer, and the three supernatants were pooled. The protein fraction of these pooled supernatants was desalted using a NAP-5 column (GE Healthcare Bio-Sciences, Uppsala, Sweden). Soluble microsporidial antigen propagated in BS-C-1 cells was used for immunization, and that from spores propagated using RK-13 cells, for various immunological tests.

Mouse monoclonal antibodies

Monoclonal antibodies were produced from hybridomas using an established technique.⁽¹⁵⁾ Intraperitoneal immunization of three female BALB/c mice was performed using 50 μ g of the soluble microsporidial antigen emulsified with an equal volume of Freund's complete adjuvant (Wako Pure Chemicals, Osaka, Japan). The mice were boosted in the same manner two weeks later and a sample of blood taken to check antibodies. All three immunized mice tested positive for specific antibodies, and the mouse giving the highest response was further boosted intravenously with 10 μ g of the soluble antigen 4 weeks after the second injection. Three days later, the spleen was harvested from the mouse for cell fusion.

Fusion of spleen cells with a non-producing mouse myeloma cell line, p3U1, was performed in a ratio of 4:1 with 50% polyethylene glycol 1500 (Roche Diagnostics, Mannheim, Germany), according to Galfre et al.⁽¹⁵⁾ with slight modifications. After fusion, cells were distributed into microtiter plates and cultured in hypoxanthine-aminopterin-thymidine medium (Sigma Chemical, St. Louis, MO) containing 10% fetal calf serum for hybridoma selection.

When hybridoma cells proliferated, culture supernatants were periodically screened for antibody detection. Cells from wells that revealed fairly high antibody activity were cloned twice by the limited dilution technique in the presence of a mouse thymocyte feeder layer. Positive clones were expanded in *in vitro* culture using RPMI medium (Sigma) supplemented with 10% fetal calf serum, penicillin, and streptomycin. These were injected intraperitoneally into BALB/c mice to produce ascitic fluid containing highly concentrated antibodies.

ELISA using wells coated with *E. cuniculi* soluble antigen (*Ec* sAg-ELISA)

The soluble microsporidial antigen was coated onto a 96-well, flat-bottom microtiter plate (high-binding polystyrene; Corning, Corning, NY) by adding 100 μ L of 2 μ g/mL protein solution in 50 mM Tris-HCl buffer (pH 8.0). Plates were incubated for 48 hours at 4°C and then washed three times with phosphate-buffered saline (PBS, pH 7.2). Coated wells were treated with blocking buffer (SuperBlock, Pierce, Rockford, IL) to block non-specific sites and then washed twice with PBS and air-dried. The plates were kept at 4°C until use.

In tests, the primary samples and the enzyme-labeled secondary antibody were incubated for 1 h at room temperature, and bound enzyme was activated by adding the ABTS

substrate (Zymed Laboratories, San Diego, CA). After stopping the enzymatic activity with addition of 1% sodium dodecyl sulfate solution, the absorbance was measured by a microplate reader (Benchmark, Bio-Rad) at 415 nm. As the secondary antibody, goat anti-mouse IgG+M+A (H+L, Zymed Laboratories) coupled with horseradish peroxidase (HRP) was used for screening at a dilution of 1:3000. This antibody is also specific for light chains, so it was effective for screening and could also detect IgE, as described in a previous report.⁽⁵⁾ PBS containing 0.05% Tween-20 (PBS-T) was used for washing and dilution.

Sera from symptomatic rabbits with natural *E. cuniculi* infection were added at a 1:400 dilution to every test as positive controls, followed by a 1:3000 dilution of protein A-HRP conjugate (Kirkegaard & Perry Laboratories, Gaithersburg, MD), as described previously.⁽⁵⁾

ELISA using wells coated with germinated spores of *E. cuniculi* (*Ec* spore-ELISA)

The *Ec* spore-ELISA is a synonym for the microplate enzyme immunostaining assay.⁽⁴⁾ According to the method described previously,⁽⁴⁾ germinated spores were coated onto a 96-well flat-bottom microtiter plate. The procedure for detecting anti-PT antibodies was essentially the same as the *Ec* sAg-ELISA, except for the substrate for bound HRP. Evidence of HRP bound to antibodies was visualized using the substrate aminoethyl carbazole (Zymed Laboratories).

ELISA using wells coated with germinated spores of *Encephalitozoon hellem* (*Eh* spore-ELISA) or *Encephalitozoon intestinalis* (*Ei* spore-ELISA)

E. hellem spores (ATCC # 50451) and *E. intestinalis* spores (ATCC # 50507), purchased from ATCC, were propagated by *in vitro* culture using BS-C-1 cells. Germinated spore-coated, 96-well flat-bottom microtiter plates were prepared using the same method as germinated *E. cuniculi* spores and tested for cross-reactivity of monoclonal antibodies using the ELISA procedures described above. As positive controls, cross-reactive rabbit sera were used, followed by a 1:3000 dilution of protein A-HRP conjugate.

ELISA using wells coated with fungal soluble antigens (*fungal* sAg-ELISA)

For this study, we prepared the following nine purified fungal spore specimens: *Candida albicans*, *Fusarium moniforme*, *Eurotium*, *Aspergillus fumigatus*, *Curvularis*, *Aureobasidium pullulans*, *Trichoderma* sp., *Chaetomium globosum*, and *Rhizopus stolonifer*. Using the same method as that for preparing the microsporidial soluble antigen, the fungal soluble antigens were extracted from each spore specimen, coated on microtiter plate wells at 2 μ g/mL protein solution, and tested using ELISA binding reactions for each monoclonal antibody under the same conditions as those for *Ec* sAg-ELISA. In each fungal ELISA test, human sera showing high antibody activities were placed in coated wells as positive controls, followed by a 1:3000 dilution of the secondary reagent, anti-human IgG (H+L) labeled with HRP (Wako Pure Chemicals).

Determination of the Ig class of monoclonal antibodies produced

The Ig class of monoclonal antibodies was determined by their reactivity to the secondary antibodies or reagents in the

Ec sAg-ELISA and Ec spore-ELISA tests. The following HRP-labeled secondary antibodies (or reagents) were employed: goat anti-mouse IgG+M+A, protein A, protein A/G (Prozyme, San Leandro, CA), goat anti-mouse IgE (ϵ -chain specific, Bethyl Laboratories, Montgomery, TX), goat anti-mouse IgG (γ -chain specific, Zymed), goat anti-mouse IgA (α -chain specific, Zymed), goat anti-mouse IgM (μ -chain specific, Zymed), and goat anti-mouse (light chain (Bethyl Laboratories). HRP conjugates of goat anti-mouse IgG+M+A and protein A were used at dilutions noted above. Reactivities of monoclonal antibodies for HRP conjugates of protein A/G, goat anti-mouse IgE, goat anti-mouse IgG, goat anti-mouse IgA, goat anti-mouse IgM, and goat anti-mouse κ light chain were tested using specified dilutions.

Titration of monoclonal antibodies

Titration of monoclonal antibodies contained in culture supernatants following the second cloning, and also in ascitic fluids, was performed after determination of the Ig class of monoclonal antibodies had been established using the Ec sAg-ELISA and Ec spore-ELISA tests. Coated wells were allowed to react with two-fold serial dilutions, starting from 1:2 for culture supernatants and from 1:100 for ascitic fluids. Goat anti-mouse IgE conjugated with HRP was used as the secondary antibody at a dilution of 1:5000. Antibody titers in the Ec sAg-ELISA test were expressed as the reciprocal of the final culture supernatant or ascitic fluid dilution, which gave significant color absorbance. Antibody titers in the Ec spore-ELISA test are expressed as described in the previous paper.⁽⁴⁾

2-D immunoblot analysis

Reactivities of monoclonal antibodies in Ec spore ELISA-positive culture supernatants following the second cloning were examined by 2-D immunoblotting, as described previously.⁽⁵⁾ Briefly, 2-D polyacrylamide gel electrophoresis (PAGE) was performed using approximately 50 μ g of spore protein, which was prepared under reducing conditions of 1, 4-dithiothreitol (DTT). Isoelectric focusing was performed using commercially available 7 cm, pH 3-10 Immobiline DryStrip gels (Amersham Biosciences, Uppsala, Sweden). Separation in the second dimension was performed using a mini-gel (10 $\times$ 10 cm) electrophoresis system with 12.5% Ready gels (BioCraft, Tokyo, Japan). Spore proteins separated by 2-D PAGE were transferred onto PVDF membranes (ProBlot membranes, Applied Biosystems, Foster City, CA). Blotted membranes were subjected to immunoblot analysis.

The secondary antibody for detecting monoclonal antibodies bound in the 2-D immunoblot analysis was alkaline phosphatase (ALP)-labeled rabbit anti-mouse IgG (H+L specific, Zymed Laboratories), at a 1:1000 dilution, which could easily detect IgE antibodies because of its specificity for the L chain. ALP signals were detected using a BCIP/NBT phosphatase substrate kit (Kirkegaard & Perry Laboratories). As a positive control, we used a pool of 11 sera from symptomatic rabbits with natural *E. cuniculi* infection, at a dilution of 1:400. In addition, mouse anti-*Entamoeba histolytica* monoclonal antibody (Biogenesis, Bournemouth, United Kingdom) and a pool of 11 sera at the same dilution from healthy rabbits, which did not show a band pattern in immunoblot analysis, were used as negative controls. To detect rabbit IgG,

ALP-labeled protein A (Zymed Laboratories) was used at a dilution of 1:1000 as the secondary reagent.

Results

Using the spleen from a mouse immunized with a soluble microsporidial antigen extracted from *E. cuniculi* spores, three different lines of hybridoma were established. Screening using Ec sAg-ELISA followed by Ec spore-ELISA produced three new monoclonal antibodies, MAb1, MAb2, and MAb4. Culture supernatants following the second cloning and the ascitic fluids produced titers of 2^7 to 2^8 and titers of 100×2^6 to 100×2^{10} , respectively, in the Ec sAg-ELISA test. Each MAb reacted with filaments extruded from germinated spores (Fig. 1) in the Ec spore-ELISA. However, these MAb-immunostained filaments were apparently thinner than filaments visualized using naturally infected rabbit sera (data not shown) regardless of their antibody concentrations. Titers of anti-PT antibody in the culture supernatants and ascitic fluids by Ec spore-ELISA were similar to those estimated by Ec sAg-ELISA. These MAbs also reacted with an intracellular antigen in infected RK-13 cells (Fig. 1), but not in normal RK-13 cells, normal BS-C-1 cells, *E. hellem*-infected BS-C-1 cells, or *E. intestinalis*-infected BS-C-1 cells (data not shown). None of the three MAbs responded to extruded PTs in the Eh spore-ELISA test or the Ei spore-ELISA test (data not shown), and were negative in the fungal sAg-ELISA tests (data not shown).

All three MAbs generated clearly positive signals in 2-D immunoblots, showing some acidic PTP1 spots with slightly different isoelectric points at 52 kDa and also acidic spots at molecular weights of higher than 75 kDa (Fig. 2). The acidic spots above 75 kDa appeared to be aggregates of PTP1. MAb1 and MAb2 also generated additional positive signals in the acidic region below 50 kDa, which formed some spotty smears (data not shown), but MAb4 did not (Fig. 2). None of the MAbs reacted with a basic spot that corresponded to PTP2 at 30-35 kDa, which were identified by LC-MS/MS analysis, as reported in a previous paper.⁽⁵⁾ IgG antibodies in a pool of sera from rabbits infected naturally with *E. cuniculi* showed multiple spots in 2-D blots, including PTP1, PTP2, and spotty smears below 50 kDa (data not shown). Neither pooled normal rabbit sera nor mouse anti-*E. histolytica* monoclonal antibody reacted with 2-D blots (data not shown).

HRP-labeled goat anti-mouse IgE, goat anti-mouse IgG+M+A, and goat anti-mouse κ light chain exhibited specific binding to MAb1, MAb2, and MAb4 in culture supernatants (Fig. 1) or ascitic fluids in Ec sAg-ELISA and Ec spore-ELISA experiments, but HRP-labeled protein A, protein A/G, goat anti-mouse IgG, goat anti-mouse IgA, and goat anti-mouse IgM did not. Therefore, all three MAbs belong to the IgE class and have the κ light chain.

Discussion

In a previous study, MAb2 labeled the 52 kDa PTP1 band in 1-D immunoblot analysis.⁽⁵⁾ In this study, we determined the immunoreactivity, specificity, and Ig class of three anti-PTP1 monoclonal antibodies, including MAb2, using various ELISAs and 2-D immunoblotting.

The three MAbs specifically reacted with the outer surface of the PT extruded from *E. cuniculi* germinated spores (Fig. 1). The antibodies also reacted with an intracellular antigen

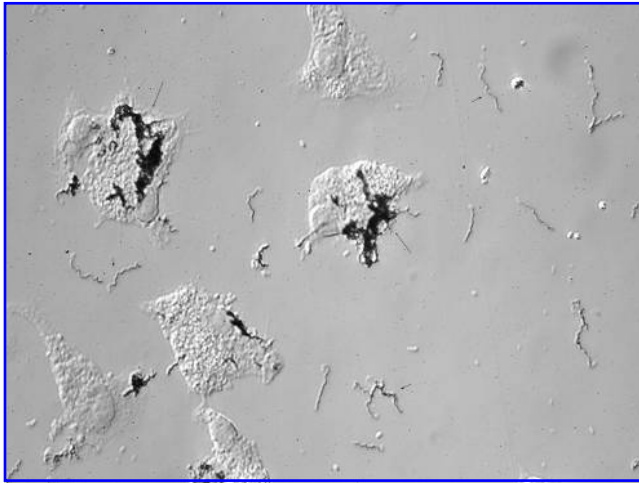


FIG. 1. Immunostaining of an intracellular antigen and extruded filaments (PTs) with mouse monoclonal antibody MAb4. Infected cells and germinated spores coated onto a plate were reacted with a 1:10 dilution of MAb4 culture supernatant (followed by HRP-labeled anti-mouse IgE). Note the positive signals (intracellular antigen) inside the infected cells (arrows). Some immunostained filaments (short-sized arrows) are also seen outside the infected cells.

in *E. cuniculi*-infected cells, but not in normal host cells or cells infected with other *Encephalitozoon* species, suggesting that the intracellular antigen was a specific product of *in vitro* propagation. If this antigen becomes an intracellular marker of *E. cuniculi* infection of host cells, our MAbs may provide an alternative immunoassay for detecting infected host cells or specific antibodies. This point requires further work for verification.

The antibodies reacted with two spots in 2-D immunoblots that corresponded to PTP1 at 52 kDa (Fig. 2), as identified by N-terminal amino acid sequencing in a previous study.⁽⁵⁾ MAb1 and MAb2 also generated identical spotty smears below 50 kDa (data not shown), whereas MAb4 showed a different pattern of epitope recognition (Fig. 2). Thus, there may be two different epitopes in PTP1 molecules. Intramolecular disulfide bridges are present in PTP1 molecules.⁽¹³⁾ Immunoreactive spotty smears below 50 kDa can result from cleavage of the S-S bonding in the PTP1 molecules, since they specifically react with MAb1 or MAb2 capable of recognizing the 52 kDa PTP1 antigen. However, the detectable amount would be small, as these smears were not seen in 1-D immunoblot analysis using the same antibodies.⁽⁵⁾

E. cuniculi PTP1 is modified by O-linked mannosylation, and concanavalin A binds to these O-linked mannose residues.⁽¹⁶⁾ Concanavalin A binding to PTP1 blotted onto a PVDF membrane was successfully eliminated by treatment with 0.05 M NaOH at 40°C for 16 h, indicating the presence of a saccharic determinant and a proteinic determinant on the 52 kDa band in 1-D immunoblot analysis.⁽⁵⁾ Culture supernatants containing MAb1, MAb2, or MAb4 similarly reacted with the 52 kDa bands regardless of NaOH treatment (data not shown), indicating they react with protein epitopes. Ec spore-, Eh spore-, and Ei spore-ELISA showed that the MAbs exhibited species-specific reactivity, and fungal sAg-ELISAs showed that the MAbs did not cross-react with the

fungal antigens, even though Microsporidia are phylogenetically related to fungi.⁽¹⁷⁾

These MAbs showed stronger reactions to the 52 kDa PTP1 spots in the 2-D immunoblots (Fig. 2) than extruded filaments (Fig. 1), indicating that solubilized PTP1 has a strong antigenicity. In fact, our BALB/c mice immunized with the soluble microsporidial antigen and complete adjuvant, raised anti-52 kDa PTP1 antibody levels easily (data not shown). Four tandem repeats that exist in the chemical structure of *E. cuniculi* PTP1⁽¹²⁾ may influence the antigenicity and immunogenicity of the PTP1 molecules. Carbohydrate residues in the PTP1 molecules may also affect immunogenicity, as suggested in other studies.⁽¹⁸⁾

E. cuniculi components are not in general viewed as having allergenicity, the capacity to induce IgE antibody production. Minimal T-helper cell type 2 (Th2) cytokine production has been observed during murine *E. cuniculi* infection.⁽¹⁹⁾ The mRNA for interleukin (IL)-4, a prominent Th2 cytokine, was undetectable in the splenocytes of the infected mice, although an increase in the mRNA for IL-10, another Th2 cytokine, was seen. Similarly, no circulating IL-4 was detected in the sera of infected mice. IL-4 from Th2 subsets is central in mediating IgE production.⁽²⁰⁾ Nevertheless, in this study, we could establish three specific IgE monoclonal antibodies, which came from mouse spleen cells immunized with the soluble microsporidial antigen and complete adjuvant. Intraperitoneal inoculation of mice with such an immunogen may induce IgE antibody production. Tetanus toxoid may be cited as an example of a substance that has not been widely appreciated as an allergen, but is nevertheless capable of inducing IgE antibodies.⁽²¹⁾

To the best of our knowledge, this is the first report of the production of anti-*E. cuniculi* monoclonal antibodies of the

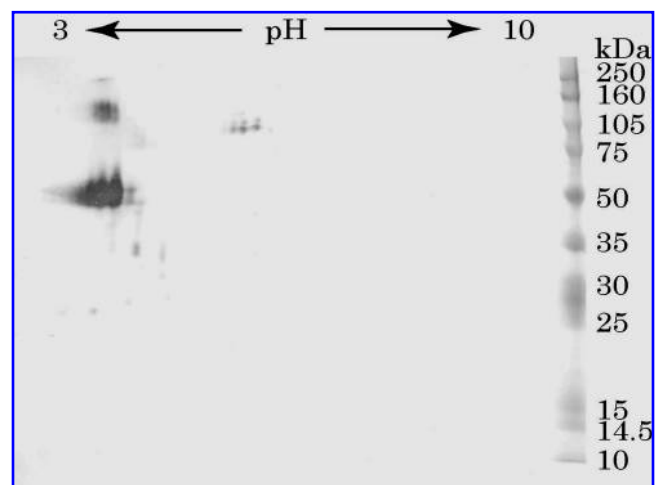


FIG. 2. 2-D immunoblot analysis. *E. cuniculi* spore antigens were fractionated by 2-D PAGE and transferred to a PVDF membrane. The blotted membrane was used for immunostaining and was incubated with MAb4. Note some acidic protein spots predominantly immunostained at 52 kDa, of which two correspond to PTP1 spots capable of being stained with BODIPY FL-X or Coomassie Blue reagent, which were identified by N-terminal amino acid sequencing as reported in a previous paper.⁽⁵⁾

IgE class using a hybridoma technique, although several other IgG or IgM antibodies have been reported.^(7–11) Receptors for IgE exist on mast cells, basophils, and eosinophils.⁽²²⁾ Our IgE monoclonal antibodies may provide a tool for the experimental approach to allergic or hypersensitive aspects in microsporidial infection or defense against microsporidial infection, as well as for developing them to be used diagnostically.

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References

- Canning EU, and Varva J: Phylum Microsporidia. In: The Illustrated Guide to the Protozoa, 2nd ed. Lee JJ, Leedale GF, and Bradbury P (Eds). Society of Protozoologists, Lawrence, KS, 2000, pp. 39–126.
- Mathis A, Weber R, and Deplazes P: Zoonotic potential of the Microsporidia. *Clin Microbiol Rev* 2005;18:423–445.
- Xu Y, and Weiss LM: The microsporidian polar tube: a highly specialized invasion organelle. *Int J Parasitol* 2005;35:941–953.
- Omura M, Furuya K, Kudo S, Sugiura W, and Azuma H: Detecting immunoglobulin M antibodies against microsporidian *Encephalitozoon cuniculi* polar tubes in sera from healthy and human immunodeficiency virus-infected persons in Japan. *Clin Vaccine Immunol* 2007;14:168–172.
- Furuya K, Omura M, Kudo S, Sugiura W, and Azuma H: Recognition profiles of microsporidian *Encephalitozoon cuniculi* polar tube protein 1 with human immunoglobulin M antibodies. *Parasite Immunol* 2008;30:13–21.
- Keohane EM, and Weiss LM: The structure, function, and composition of the microsporidian polar tube. In: The Microsporidia and Microsporidiosis. Wittner M, and Weiss LM (Eds). ASM Press, Washington, DC, 1999, pp. 196–224.
- Enriquez FJ, Ditrich O, Palting JD, and Smith K: Simple diagnosis of *Encephalitozoon* sp. microsporidial infections a panspecific antiexospore monoclonal antibody. *J Clin Microbiol* 1997;35:724–729.
- Delbac F, Duffieux F, David D, Metenier G, and Vivares CP: Immunocytochemical identification of spore proteins in two microsporidia, with emphasis on extrusion apparatus. *J Eukaryot Microbiol* 1998; 45: 224–231.
- Lujan HD, Conrad JT, Clark CG, Touz MC, Delbac F, Vivares CP, and Nash TE: Detection of microsporidia spore-specific antigens by monoclonal antibodies. *Hybridoma* 1998;17:237–243.
- Sak B, Sakova K, and Ditrich O: Effects of a novel anti-exospore monoclonal antibody on microsporidial development in vitro. *Parasitol Res* 2004;92:74–80.
- Mo L, and Drancourt M: Monoclonal antibodies for specific detection of *Encephalitozoon cuniculi*. *Clin Diag Lab Immunol* 2004;11: 1060–1063.
- Delbac F, Peuvrel I, Metenier G, Peyretailade E, and Vivares CP: Microsporidian invasion apparatus: identification of a novel polar tube protein and evidence for clustering of *ptp1* and *ptp2* genes in three *Encephalitozoon* species. *Infect Immun* 2001;69:1016–1024.
- Peuvrel I, Peyret P, Méténier G, Vivares CP, and Delbac F: The microsporidian polar tube: evidence for a third polar tube protein (PTP3) in *Encephalitozoon cuniculi*. *Mol Biochem Parasitol* 2002;122:69–80.
- Asakura T, Nakamura S, Ohta M, Une Y, and Furuya K: Genetically unique microsporidian *Encephalitozoon cuniculi* strain III isolated from squirrel monkeys. *Parasitol Int* 2006;55:159–162.
- Galfre G, Howe SC, Milstein C, Bucher GW, and Howard JC: Antibodies to major histocompatibility antigens produced by hybrid cell lines. *Nature* 1977;266:550–552.
- Xu Y, Takvorian PM, Cali A, Orr G, and Weiss LM: Glycosylation of the major polar tube protein of *Encephalitozoon hellem*, a microsporidian parasite that infects humans. *Infect Immun* 2004;72:6341–6350.
- Thomarat F, Vivares CP, and Gouy M: Phylogenetic analysis of the complete genome sequence of *Encephalitozoon cuniculi* supports the fungal origin of microsporidia and reveals a high frequency of fast-evolving genes. *J Mol Evol* 2004;59:780–791.
- Peek R, Delbac F, Speijer D, Polonais V, Greve S, Wentink-Bonnema E, Ringrose J, and van Gool T: Carbohydrate moieties of microsporidian polar proteins are targeted by immunoglobulin G in immunocompetent individuals. *Infect Immun* 2005;73:7906–7913.
- Khan IA, and Moretto M: Role of gamma interferon in cellular immune response against murine *Encephalitozoon cuniculi* infection. *Infect Immun* 1999;67:1887–1893.
- Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, and Coffman RL: Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 1986;136:2348–2357.
- Aalbers RC: Structural features of allergenic molecules. In: Allergy and Asthma in Modern Society: A Scientific Approach. Crameri (Ed). Chem Immunol Allergy, vol. 91. Karger, Basel, 2006, pp. 134–146.
- Adamczewski M, and Kinet JP: The high-affinity receptor for immunoglobulin E. In: Lymphocyte Activation. Samelson LE (Ed). Chem Immunol, vol 59. Karger, Basel, 1994, pp. 173–190.

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