Invited Review

Spore-Forming Microsporidian Encephalitozoon: Current Understanding of Infection and Prevention in Japan

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SUMMARY: Microsporida are spore-forming obligate intracellular parasites. They are known to cause opportunistic infections in humans through zoonotic, waterborne and foodborne transmission routes. This article reviews the present knowledge regarding microsporidian Encephalitozoon cuniculi infections in animals living in the human environment in Japan and discusses the basic measures required for the effective disinfection of Encephalitozoon. The article also discusses seroepidemiologic data from healthy people in order to shed light on the mechanisms of protective immunity and to identify potential strategies for preventive medicine.

1. Introduction

Microsporida are spore-forming, obligate, intracellular, eukaryotic parasites that lack mitochondria (1). In the late 1990s, Microsporida were removed from the Protozoa and classified as Fungi, based on molecular phylogenetic studies (2). The phylum Microsporida consists of nearly 150 genera with more than 1,000 species (3). Microsporida have been isolated from mammals, reptiles, fishes, birds, amphibians, and insects (4). However, only seven genera (Enterocytozoon, Encephalitozoon, Pleistophora, Trachipleistophora, Vitatorma, Brachiola, and Nosema) have been described as pathogens in humans (5). Reports from the World Health Organization (WHO), Geneva (6) and the National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, Maryland (7) recognized some of these to be emerging infectious agents: Enterocytozoon bieneusi, which causes persistent diarrhea, was found in 1985; Encephalitozoon hellem, which causes conjunctivitis and disseminated disease, was found in 1991; and Encephalitozoon cuniculi, which causes disseminated disease, was found in 1993.

Records of patients with microsporidian infections have increased worldwide with the increase in human immunodeficiency virus (HIV) infection (8). However, only two cases of human microsporidiosis have been reported in Japan. One involved an E. cuniculi infection in a 9-year-old boy, which resulted in encephalitis (9), and the other was a case of E. bieneusi infection, which caused diarrhea in an AIDS patient (10). In both cases, the background of infection was unclear, and epidemiologic studies were not performed.

Over the last 10 years, the number of rabbits diagnosed with encephalitozoonosis (microsporidiosis due to E. cuniculi) has been increasing in Japan, and E. cuniculi has also infected many squirrel monkeys (Saimiri sciureus) kept in Japan. E. cuniculi infection and its associated disease have therefore spread among animals living in the human environment, thus posing a potential threat to human health. This public health issue prompted us to perform systematic studies on Encephalitozoon infections.

This review deals mainly with our recent seroepidemiologic studies of E. cuniculi in rabbits, dogs, and monkeys, and in humans resident in enzootic districts. It also summarizes our present understanding of Encephalitozoon infections and their prevention, based on seroepidemiologic, immunosorologic, immunohistochemical, microbiologic and genetic research.

2. Natural infection in animals

2-1. Seroepidemiologic studies in rabbits

Encephalitozoonosis in rabbits frequently exists as a chronic, latent infection, and only a percentage of infected animals develop clinical disease (11). The clinical signs are almost entirely neurological, including torticollis and ataxia (11). However, it is very difficult to diagnose encephalitozoonosis based on clinical symptoms, since infections by other pathogens such as Pasteurella multocida, Listeria monocytogenes, and Toxoplasma gondii often cause similar symptoms (12). Serologic methods are therefore useful for diagnosing E. cuniculi infection or encephalitozoonosis (11) and also for conducting epidemiologic studies (13). We used two different enzyme-linked immunosorbent assays (ELISAs) to measure serum antibodies: sAg-ELISA, in which wells were coated with E. cuniculi soluble antigen (14), and spore-ELISA,

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levels were also observed in 14-40% of infected and seroconverted untreated rabbits. This was achieved by in vitro cultivation from experimentally infected animals, though the detection of DNA signals in other organs, such as the lung and kidney, was less consistent. Suter et al. (17) found that positive serology correlated well with the presence of the parasite, as isolation from brain tissue was usually asymptomatic. Table 1 shows that anti-E. cuniculi IgG antibodies were measured by sAg-ELISA (14) and antibody activities in 1:400-diluted sera were expressed as OD values.

The serologic survey performed by Igarashi et al. (13) also found anti-E. cuniculi IgG antibodies, not only in rabbits with neurologic signs but also in 22 (29.7%) of 74 healthy and singly kept rabbits and in 16 (43.2%) of 37 rabbits with other clinical symptoms, such as tumors and disorders of the digestive tract and respiratory system. This finding was unexpected, because, as mentioned above, E. cuniculi infection is usually asymptomatic. Table 1 shows that E. cuniculi latently and persistently parasitized various organs in healthy rabbits that demonstrated strongly positive results in the sAg-ELISA test for IgG antibodies. Microsporidian spores and DNA were detected in all of the brain specimens by post-mortem examination, though the detection of DNA signals in other organs, such as the lung and kidney, was less consistent. Suter et al. (17) found that positive serology correlated well with the presence of the parasite, as isolation from brain tissue was achieved by in vitro cultivation from experimentally infected and seroconverted untreated rabbits.

In Igarashi et al.'s survey (13), elevated IgM antibody levels were also observed in 14-40% of E. cuniculi IgG antibody-positive rabbits, including apparently healthy rabbits. The results of immunoblot tests for IgM antibodies using paired sera from individual rabbits appeared to produce three immunostaining patterns that differed in terms of the number and intensity of the bands. In the rabbits summarized in Table 1, rabbit nos. 1 and 2 exhibited patterns with insufficiently developed IgM, the pattern in rabbit no. 3 showed the initiation of IgM production, and rabbit nos. 4-10 showed patterns demonstrating IgM production. These patterns are shown in Figure 1. Some IgM antibodies decreased or disappeared over time, while other IgM antibodies did not disappear completely and remained detectable. It should be stressed that these rabbits had very high IgG antibody activities when the first sera or the second sera were tested (Table 1). Thus IgM
antibodies, which may contain persistent IgM as described by Cox et al. (18), may be detected during the course of the infection or with dissemination of the infection.

In an experimental study performed by Cox et al. (18), spores were excreted in large numbers in the urine from postinfection day 38 until postinfection day 63. However, few studies have analyzed urine samples from naturally infected rabbits, probably because of the difficulties associated with routine testing for microsporidian spores; e.g., rabbit urine usually contains large quantities of precipitable substances (18). We examined urine samples from 44 rabbits for microsporidian spores, and three samples were found to be positive by Cellufluor staining (a chemofluorescent agent for staining chitin) (Asakura, unpublished observation). However, only one sample was confirmed to contain *E. cuniculi* spores by polymerase chain reaction (PCR) followed by direct DNA sequencing (Asakura, unpublished results). The number of spores in the PCR-positive urine sample, of which the volume was approximately 1 ml, was estimated at 5 × 10^5. This urine was thought to be sufficiently potent to act as an infection source in a colony. Interestingly, the rabbit that produced this urine sample was young and apparently healthy.

During research into the detection of microsporidian spores in rabbit urine samples, we also detected urinary antibodies using spore-ELISA (19). However, urinary antibodies would be of limited use in diagnosing individual animals, because antibody activity is not always detectable in urine from seropositive rabbits. The impairment of glomerular filtration probably leads to the excretion of specific antibodies.

It seems probable that *E. cuniculi* is now widespread in commercial and pet rabbit populations. *E. cuniculi* infection has also been found in zoo rabbits and school rabbits in Japan (16,20). Breeders should therefore be required to supply *Encephalitozoon*-free pet rabbits. Antibodies should also be checked before the purchase of or experimentation with laboratory rabbits, because rabbits in research colonies in Japan are not maintained under specific-pathogen-free conditions, and natural infection of laboratory rabbits with *E. cuniculi* could affect the results of research (21).

### 2-2. Seroepidemiologic studies in dogs

*E. cuniculi* is a zoonotic parasite, and dogs, as well as rabbits, need to be monitored as a potential source of human *E. cuniculi* microsporidiosis (22,23). Experimentally infected dogs developed no clinical disease, but did demonstrate histopathologic lesions (11). We therefore performed a seroepidemiologic study to investigate whether or not dogs in Japan were infected with *E. cuniculi*. In the first survey in 2003, 159 apparently healthy dogs in the Kanto district were screened using sAg-ELISA for IgG antibodies (24). Sera from nine dogs exhibited relatively high ELISA values. The OD of one sample was 1.4, the ODs of eight samples ranged from 0.5–0.99, and the other samples had ODs <0.5. When the nine sera with relatively high values were checked by spore-ELISA, none of them reacted with the spore wall (SW) or the polar tube (PT) antigens. As a result, we concluded that all of the 159 dogs were seronegative. Similar negative results were obtained from a second survey in 2005, in which 311 dogs in Saitama Prefecture were tested by sAg-ELISA for IgG antibodies (N. Yamamoto, Saitama Institute of Public Health, personal communication). The examined sera were collected between 1995 and 2005, but their ELISA values were significantly lower than those from latently infected rabbits. These results indicated that *E. cuniculi* infection in dogs was not confirmed in the Kanto district. A similar finding was reported by Deplazes et al. (25), who surveyed 212 dogs in Switzerland and concluded that none of them were seropositive. Likewise, 1,104 dogs investigated in Norway were also seronegative (26).

In the case of *E. cuniculi* infection in dogs, both oral and transplacental routes of infection have been described (11). Many young puppies are thought to develop clinical disease after in utero infection from their asymptomatic dams, which may indicate that epidemiologic approaches should focus on puppies suffering from encephalitis-nephritis syndromes. In addition, care should be taken when importing dogs from canine encephalitozoonosis-prevalent countries.

### 2-3. Seroepidemiologic studies in monkeys

Many cases of *E. cuniculi* infection in squirrel monkeys have been found in the United States (27). *E. cuniculi* infection in squirrel monkeys in Japan was not investigated until 2003, when we tested two squirrel monkeys that had died of unknown causes. These two animals (one about 10 days old and the other a young male) produced positive laboratory tests for *E. cuniculi* (28). Ohta (29) histopathologically detected microsporidian spores in six of 37 investigated squirrel monkeys, using Brown-Hopps staining. Histopathologic findings based on hematoxylin and eosin (HE) staining indicated that tissue cysts (pseudocysts, which are structures containing cells of the parasite) were present in the cerebrum, cerebellum, kidney, liver, lung, heart, spleen, and adrenal gland. Gram-positive organisms were also seen in areas without lesions.

Ohta (29) carried out a seroepidemiologic survey of *E. cuniculi* infection in squirrel monkey colonies in Japan using sAg-ELISA, with peroxidase (PO)-labeled protein AG, which is capable of detecting squirrel monkey IgG, as the secondary reagent. Seropositive monkeys were detected in 11 of 12 colonies. Of a total of 425 monkeys, 143 (33.6%) were seropositive for anti-*E. cuniculi* IgG antibodies. Their tested sera were collected between 1998 and 2005. The results of analysis of the seroepidemiologic data suggested that intracolonial transmission in one colony had occurred via seropositive dams or via other monkeys, while the pathogen in another colony might have invaded before 1998.

Thus Ohta (29) found that many squirrel monkeys were seropositive for *E. cuniculi*, but apparently healthy. Newborn or young animals, such as the monkey about 10 days old mentioned above, can show severe symptoms (27,30), although adult monkeys tend to be asymptomatic. Asakura and Furuya (unpublished data) detected no microsporidian spores or DNA in the main organs (brain, lung, liver, spleen, and kidney) of 14 adult monkeys with high antibody activity, suggesting that seropositivity in most adult monkeys may be attributable to past infection.

Anti-*E. cuniculi* IgG antibody activity in squirrel monkeys was also confirmed by spore-ELISA (Asakura and Furuya, unpublished data). The highest titer in tested sera was estimated to be 1:102,400, which is similar to the antibody titers found in sera from rabbits with encephalitozoonosis. This result prompted us to investigate the seroepidemiology of *E. cuniculi* in other species of monkey. We obtained sera from 91 Japanese macaques (*Macaca fuscata*) from a colony in Japan. Sera from 46 of the 91 monkeys were collected between 1989 and 1999, and the others were collected in 2004. All these sera were negative for IgG antibodies (OD values of <0.5) (31) when examined by sAg-ELISA, in which protein AG-PO conjugate was employed to detect Japanese macaque IgG (32). It is therefore unlikely that *E. cuniculi* infection had occurred in this colony.
2-4. Immunohistochemical approach in monkeys

Postmortem diagnosis of animals with *E. cuniculi* infection has mainly involved histopathologic examination using modified Gram stains, such as Brown and Hopps modification (33). However, only heavily infected animals with evident histopathologic findings and granulomatous lesions were considered to be infected using this procedure, and HE staining did not make the detection of microsporidian parasites possible. Immunohistochemical methods, however, allow the identification of parasites in tissue sections, even in locations without severe cellular reactions (34). Guscetti et al. (30) detected *E. cuniculi* spores immunohistochemically in sections of brain tissue from emperor tamarins (*Saguinus imperator*) with lethal neonatal encephalitozoonosis, using a polyclonal rabbit antibody to *E. cuniculi* for specific staining. Images were visualized using a labeled streptavidin-biotin method. Park et al. (35) successfully stained infected rabbit brain sections with biotinylated rabbit anti-*E. cuniculi* IgG, which had been separated from a spontaneously infected rabbit with a high antibody titer using a protein A-sepharose 4B column, followed by a commercial avidin-labeled PO reagent. We purified anti-*E. cuniculi* antibodies by immunoadsorption from a commercial rabbit serum using preparative blots of purified *E. cuniculi* spore antigens. Ohta (29) used this purified antibody reagent to immunostain formalin-fixed and paraffin-embedded brain sections from spontaneously infected squirrel monkeys. Antigens in deparaffinized tissue sections were reactivated with trypsin. In Ohta’s study, PO-labeled anti-rabbit IgG antibodies were employed as the secondary antibody. The resultant reactants were visualized using diaminobenzidine substrate. Spores were immunostained, but the staining was blurry and uneven, indicating that the immunostaining procedure needed improving.

We developed an improved procedure by modifying the primary antibody, secondary antibody, chromogen, and reactivation of the antigen. Biotinylated and affinity-purified rabbit anti-*E. cuniculi* antibodies (BRAEC) were used as the primary antibody in place of unlabelled antibodies. Biotinylated goat anti-rabbit IgG antibodies (BGARIG, Zymed Laboratories, South San Francisco, Calif., USA) were used as the secondary antibody. HistoMark True Blue (KPL, Gaithersburg, Md., USA) was employed as the PO substrate. Formalin-fixed and paraffin-embedded brain tissue sections were deparaffinized and treated with L.A.B. solution (Polysciences Inc., Warrington, Pa., USA) to reactivate damaged antigens. When sections of infected squirrel monkey brain tissue were reactivated and reacted with BRAEC, followed by BGARIG and streptavidin-PO conjugate, the chromogen clearly stained spores in pseudocysts deep blue (Figure 2). We found that the addition of BGARIG to BRAEC amplified the biotin residues, thus improving the clarity of the spores. The minimum amount of BRAEC necessary for visualizing the spores was estimated to be 0.04 μg/ml. At a concentration of 4 μg/ml BRAEC, followed by BGARIG, streptavidin-PO, and HistoMark True Blue, no cross-reactions with *T. gondii* or *Neospora caninum* occurred (using commercial slides, VMRD, Inc., Pullman, Wash., USA). Our improved method can thus be used successfully for the immunologic staining of *E. cuniculi* organisms in squirrel monkey brain tissue sections. The same procedures would be expected to be applicable for the immunohistochemical diagnosis of *E. cuniculi* infection in other animal or human tissue sections.

2-5. Genetic analysis of *E. cuniculi* isolates

*E. cuniculi* isolates can be grouped into three types based on the number of 5′-GTGT-3′ repeats in the internal transcribed spacer (ITS) of the rRNA gene (36): these three genotypes are known as strain I (containing three GTGT repeats), strain II (two GTTT repeats), and strain III (four GTTT repeats). Strain I has been mainly isolated from rabbits and humans, strain II from mice and blue foxes, and strain III from domestic dogs and humans (37).

*E. cuniculi* has been isolated from many rabbits in our laboratory, and all isolates have so far been identified as ITS genotype I (i.e., as strain I) (Asakura, unpublished data). Also, the ITS region of *E. cuniculi* isolated from the organs of squirrel monkeys contained tetrarepeats of 5′-GTGT-3′, revealing that these isolates were ITS genotype III (28).

In addition to the three genotypes based on the number of GTTT repeats in the ITS of the rRNA, nucleotide sequence analysis of the polar tube protein (PTP) gene can also be used to divide *E. cuniculi* isolates into three genotypes in agreement with the results of analysis of the ITS of the rRNA gene (38). A set of primers (5′-GAGTTCCAGCTACTC-3′ and 5′-AGGAACCTCGGATGTTCC-3′) can generate a 363-bp product for genotypes I and II and a 285-bp product for genotype III by PCR (38). All the rabbit isolates we examined produced 363-bp PCR amplicons and were classified as PTP genotype I by sequence analysis (Asakura, unpublished data). Contrary to expectations, the squirrel monkey isolates also generated 363-bp PCR amplicons (28). The 363-bp sequence of the monkey isolates demonstrated 99% homology with the *E. cuniculi* genotype II PTP gene (GenBank accession no. AF310678) and had a nucleotide sequence GGTCC corresponding to nucleotides 596 to 600 of AF310678, which differentiates genotype I from genotype II by restriction digestion of PCR products with *SacI* (38). Three nucleotides (T at position 485, T at position 571, and A at position 701) in the gene sequence of AF310678 were replaced by C, C, and G, respectively.

Typing of *E. cuniculi* isolates based on analysis of the spore wall protein 1 (SWP-1) gene also was in agreement with the grouping results for the same isolates based on analysis of the ITS gene, when a set of primers (5′-ACTGACAAGTACC ACATC-3′ and 5′-TGGGACTCACAATTAGG-3′) was used to obtain PCR fragments (38). Moreover, genotype I and genotype III isolates could each be divided into two subgenotypes. We found that two-thirds of our rabbit isolates were genotype Ib while the remainder were genotype Ia, based on DNA sequencing (Asakura, unpublished data). The monkey isolates, however, did not belong to genotype I, II, or III, although their 333-bp PCR amplicons were related to a region of the SWP1 gene and had a structure including LSTTTSTEFTQANSEIGALTGRIF and glycine- and serine-rich repeats (28). The genotypes of seven representative *E. cuniculi* reference isolates identified at the National Center for Infectious Diseases, Centers for Disease Control and Prevention (Atlanta, Ga., USA) and three *E. cuniculi* isolates identified in our laboratory are shown in Table 2.

As summarized in Table 2, *E. cuniculi* SWP-1 genotype Ib1b has been isolated from dogs and humans. However, SWP-1 genotype Ib3 has been isolated only from humans (38). In 2002, Furuya (39) identified the *E. cuniculi* isolated from a patient with alveolar hydatid disease (AHD) as ITS genotype III (i.e., strain III). At that time, the SWP-1 gene was not used for genotyping, but recent analysis indicated that the isolate was of the same type as the isolates from squirrel monkeys (Asakura, unpublished data). The original host of...
this strain III is still unknown. *E. cuniculi* strain III has been isolated from emperor tamarins (30) and cotton-top tamarins who was accidentally infected with recently demonstrated in a healthy laboratory worker in France. Serum samples showed cross-reactivity with the SW of the IgM antibodies with PTs were considered to be typical or anti-SW antibody activities were detected. The reactivities as seen in Figure 3, though no significant anti-PT IgG or IgA (36.3%) of the 380 healthy persons, at titers of 1:50 -1,600, anti-PT antibodies. Seroepidemiologically analyzed using this enzyme immunoassay of host cells, as sporoplasm flows through the discharged spore structure with an extrusion that is essential for its invasion of the PT of these parasites. The PT is a typical microsporidian, but little cross-reactivity with *E. cuniculi*. Specific IgG antibodies against the PT of *E. cuniculi* isolates based on sequence analysis of amplicons in the spore wall protein 1 (SWP-1) region. *E. cuniculi* isolates based on DNA sequence analysis of the ITS, PTP and SWP-1 regions (41). Further information from different, spontaneously infected animals would help to shed light on the molecular epidemiology of *E. cuniculi* strain III.

3. Seroepidemiologic studies on *E. cuniculi* infection in humans

3-1. Detection of anti-*E. cuniculi* PT IgM antibodies

Specific IgG antibodies against the PT of *E. cuniculi* were recently demonstrated in a healthy laboratory worker in France who was accidentally infected with *E. cuniculi* (41). Positive serum samples showed cross-reactivity with the SW of *E. hellem* or Encephalitozoon intestinalis, but little cross-reactivity with the PT of these parasites. The PT is a typical microsporidian spore structure with an extrusion that is essential for its invasion of host cells, as sporoplasm flows through the discharged PT and into the host cell (42). We recently developed a spore-ELISA that can measure anti-*E. cuniculi* PT antibodies as well as anti-SW antibodies (15), as mentioned above. This method allows for the large-scale screening of human sera for anti-PT antibodies for seroepidemiologic analysis. Sera from 380 healthy persons (180 healthy residents and 200 blood donors in enzootic districts) and 78 HIV-infected persons were seroepidemiologically analyzed using this enzyme immunoassay (15), which is capable of measuring IgM, IgG, and IgA anti-PT antibodies.

Anti-PT IgM antibodies were detected in the sera of 138 (36.3%) of the 380 healthy persons, at titers of 1:50 -1,600, as seen in Figure 3, though no significant anti-PT IgG or IgA or anti-SW antibody activities were detected. The reactivities of the IgM antibodies with PTs were considered to be typical of IgM because of the low titers (≤1:1,600), but the IgM antibodies had stronger reactivities than rabbit anti-*E. cuniculi* PT IgG antibodies. A decreasing trend in positivity rates for anti-PT IgM was observed when subjects were grouped according to age: the positivity rate for anti-PT IgM was highest among people aged 19 years or younger (59% of healthy subjects), and the seropositivity rates decreased among the older subjects (*P* < 0.01). There was no significant difference in the positivity rates for anti-PT IgM antibodies between males and females. However, anti-*E. cuniculi* PT IgM antibodies were detected in only four of 51 HIV-positive individuals with CD4 cell counts <250/μL. In particular, anti-PT IgM was not detected in persons aged <30 years and with <250 CD4 cells/μL. Interestingly, a high positivity rate (44.4%) for anti-PT IgM was observed among the 27 HIV-positive persons with CD4 cell counts between 251 -900/μL. Furthermore, all these individuals were <30 years old.

3-2. Recognition site of anti-*E. cuniculi* PT IgM

*E. cuniculi* PT consists of three proteins: PTP1, PTP2, and PTP3 (43). PTP1 is the major component of the PT, and it is modified by O-linked mannose residues, to which concanavalin A can bind (44). Our latest study (45) indicated that the major activities of human sera with anti-PT IgM were predominantly towards *E. cuniculi* PTP1, which is an acidic protein with an apparent molecular weight of 52 kDa, as demonstrated by two-dimensional (2-D) immunoblot analysis followed by proteomic analysis. One-dimensional (1-D) immunoblot analysis also revealed that a band at 52 kDa, which was strongly reactive with mouse monoclonal antibody to the PT (14), was predominantly detected in all of the examined human sera with anti-PT IgM. NaOH treatment can remove glycoepitopes from blotted antigens (46). From the reactions of anti-PT IgM to the 52-kDa band after NaOH treatment...
treatment, 24 human sera with anti-PT IgM could roughly be grouped into three categories: sera containing antibodies against only a saccharic determinant (n = 3); sera containing antibodies against only a protein determinant (n = 11); sera showing dual recognition of saccharic and protein determinants (n = 10). Thus, two chemically different antigenic sites were identified on the 52-kDa protein by 1-D immunoblot analysis: a protein determinant and a saccharic determinant. In addition to PTP1, human sera also reacted with other protein spots such as those for PTP2 and zinc finger protein in 2-D immunoblot analysis (Table 3). Brosson et al. (47) recently identified 177 unique proteins, of which 25.6% had unknown functions, in a proteomic analysis of Encephalitozoon cuniculi. A recent report on intraocular microsporidiosis in a patient with idiopathic CD4+ T-lymphocytopenia proved the

Table 3. E. cuniculi proteins immunoreactive with human sera in two-dimensional (2-D) immunoblot analysis

<table>
<thead>
<tr>
<th>Protein identified</th>
<th>Immunoreactivity1) with Human 1</th>
<th>Immunoreactivity1) with Human 2</th>
<th>Immunoreactivity1) with Rabbit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polar tube protein 1 and its isoform</td>
<td>3+</td>
<td>3+</td>
<td>4+</td>
</tr>
<tr>
<td>Polar tube protein 2</td>
<td>1+</td>
<td>0</td>
<td>2+</td>
</tr>
<tr>
<td>Spore wall protein 1</td>
<td>0</td>
<td>0</td>
<td>2+</td>
</tr>
<tr>
<td>Translation elongation factor 1α</td>
<td>0</td>
<td>0</td>
<td>1+</td>
</tr>
<tr>
<td>Hypothetical protein</td>
<td>0</td>
<td>0</td>
<td>1+</td>
</tr>
<tr>
<td>6S proteasome ζ chain</td>
<td>0</td>
<td>0</td>
<td>1+</td>
</tr>
<tr>
<td>Similarity to HSP 70-related protein</td>
<td>±</td>
<td>0</td>
<td>±</td>
</tr>
<tr>
<td>Heat shock-related 70 kDa protein</td>
<td>0</td>
<td>0</td>
<td>±</td>
</tr>
<tr>
<td>Zinc finger protein</td>
<td>1+</td>
<td>±</td>
<td>0</td>
</tr>
<tr>
<td>Phosphomannomutase</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

1) Immunoreactivity was examined using 2-D immunoblotting using sera from two blood donors (H1 and H2) and a pool of sera from infected rabbits. Reactivity was scored as 0, ±, 1+, 2+, 3+ or 4+ (0, negative; ±, ambiguous; 1+ to 4+, degrees of positivity). The rabbit specimen was used as a positive control for 2-D immunoblot analysis. The results and methods are described in detail in reference 45.

3-3. Anti-PT IgM antibodies and human microsporidiosis

Regarding the specific immune responses to E. cuniculi infection, van Gool et al. (41) observed that anti-SW IgG production preceded that of anti-PT IgG. However, most of our subjects were negative for both anti-SW IgM and anti-SW IgG (15). Anti-PT IgM antibodies were detected at almost the same rate (36 - 37%) in serum samples collected from blood donors in 2005 and in serum samples collected from healthy residents in 2000 (15). Strong antigenicity and immunogenicity of E. cuniculi PT1 have also been reported (48). These findings suggest that our subjects may have been exposed to E. cuniculi, but that continuing and frequent E. cuniculi infections may prevent the IgM surge as an initial infection indicator, but not the secondary immune response. Van Gool et al. (49) demonstrated a high seroprevalence of E. intestinalis in immunocompetent subjects and inferred that the frequent detection of antibodies suggested a high rate of exposure in immunocompetent populations. Drinking water is a potential source of exposure, as E. bienesi, E. intestinalis, and Vittaforma corneae have all been detected in sewage, groundwater, or surface water sources (50). In relation to the Encephalitozoon seroprevalence in various human populations, Didier et al. (37) suggested the possibility of incidental exposure of humans, e.g., through food ingestion or insect stings. However, it is possible that anti-E. cuniculi PT IgM antibodies are cross-reactive with antibodies produced by other infectious agents such as fungi, since the primary response is usually of low specificity. E. cuniculi infection in immunocompromised patients results in disseminated disease that is clinically manifested by symptoms such as keratoconjunctivitis, hepatitis, and peritonitis (34). It should be emphasized that human microsporidiosis is predominantly associated with CD4+ T cell deficiency (51,52). A recent report on intraocular microsporidiosis in a patient with idiopathic CD4+ T lymphocytopenia proved the
infectivity of *E. cuniculi* in immunodeficient humans (53). However, no symptomatic cases of infection with *E. cuniculi* in immunocompetent people have been described (34), apart from the accidentally infected French individual who had severe keratoconjunctivitis (41). A Japanese child with microsporidiosis due to natural infection in 1958 is considered to be the only reported case caused by natural infection (9), but, unfortunately, the patient’s immune status was not recorded. A few cases of *E. cuniculi* infections have also been reported in transplant patients (54,55). Thus, except in some extremely rare situations, *E. cuniculi* is deemed unlikely to cause microsporidiosis in immunocompetent persons (34,56).

Almost all human *E. cuniculi* microsporidiosis cases have occurred in immunocompromised patients infected with HIV (34), suggesting that protective immunity plays an important role in preventing *E. cuniculi* infection. The in vitro infectivity of microsporidia was reduced by treatment with monoclonal and polyclonal antibodies to PT (57). However, as in other human opportunistic protozoal infections, microsporidian-specific antibodies alone may not be protective (52). In humans, cell-mediated immunity is said to be critical for protection against microsporidian organisms (52). More recently, Sak et al. (58) demonstrated that humoral antibodies enhanced the protective effect of CD4+ T lymphocytes using severe combined immunodeficient mice perorally infected with *E. cuniculi*, suggesting that the humoral part of the immune system may contribute to the defense against microsporidial infection. We have shown that the positivity rate for anti-PT IgM antibodies was significantly higher in healthy people than in HIV-positive persons (15), such that the CD4 cell level greatly affected the detection of anti-PT IgM antibodies. This observation leads us to suggest that circulating anti-PT IgM antibodies that are capable of strongly reacting with PTs extruded from germinal spores may play a part in protective immunity.

In this context, it is also of interest that a low level of anti-PT IgM antibody activity was detected in 30 (33%) of 91 Japanese macaques using spore-ELISA (31), where PO-labeled goat anti-monkey IgM (Fc-specific; Nordic Immunology, Tilburg, the Netherlands) was used for screening Japanese macaque IgM antibodies, though no results indicating the presence of sAg-ELISA-positive anti-*E. cuniculi* IgG antibodies were obtained, as mentioned above. *E. cuniculi* infection in Japanese macaques has not yet been reported, suggesting that the susceptibility of this species of monkey to *E. cuniculi* may differ from that of squirrel monkeys. If so, Japanese macaques would provide an animal model for studying the immunologic aspects of the presence of circulating anti-PT IgM antibodies and their role in protection.

### 3-4. Specificity of human antibodies

Regarding antibodies against *E. intestinalis* PT in immunocompetent persons such as Dutch blood donors and pregnant Dutch women (49), further study has demonstrated that the carbohydrate moieties of the microsporidian PTs are targeted by IgG in immunocompetent individuals (42). We demonstrated that anti-*E. cuniculi* PT IgM in human sera did not react with extruded PTs from germinated *E. hellem* or *E. intestinalis* (45), indicating that human anti-*E. cuniculi* PT IgM is species-specific. This finding was in agreement with those of Peek et al. (42), which showed that human sera containing anti-*E. intestinalis* PT IgG antibody activity did not immunostain extruded *E. cuniculi* PTs. Cross-reactivity between microsporidian spores and fungal spores might be considered likely because of their phylogenetic relationship (2).

However, apart from saccharic determinants, fungal sAg-ELISA tests, using wells coated with Laemmli sample buffer-extracted soluble antigens, showed that our mouse anti-PTP1 IgG monoclonal antibody failed to cross-react with soluble antigens from nine fungi (*Candida albicans*, *Fusarium moniliforme*, *Eurotium*, *Aspergillus fumigatus*, *Curvularia*, *Aureobasidium pullulans*, *Trichoderma sp.*, *Chaetomium globosum*, and *Rhizopus stolonifer*) (14).

Many reports on the seroprevalence of *E. cuniculi* in healthy people and patients with other diseases have been published (59-61). However, the reported rates of microsporidial seropositivity vary greatly depending on the serologic technique used, probably due to the use of antigens unsuitable for the measurement of specific antibodies and the use of secondary antibodies without differential specificities. For instance, a high rate of detection of IgG antibodies reacting with an *E. cuniculi* antigen band around 52 kDa was seen in patients with AHD using immunoblotting (59). However, a recent study revealed that none of the AHD sera that were immunoblot-positive and could be checked by spore-ELISA for IgG antibodies reacted with the PT antigen (Omura and Asakura, unpublished data), although the SW antigen reacted with some sera at low titers. Thus IgG antibodies that are only positive in immunoblot tests may be unexpectedly detected in many human sera, particularly from patients with polyclonal B cell activation, but they do not seem to have strict specificity for the parasitic antigen. Indicators of polyclonal B cell activation, such as autoantibodies to dsDNA and histones, can be found in *Echinococcus*-infected humans (62). Mathis et al. (8) noted that, despite immunoblot analysis that potentially increases the specificity of ELISA (63), it is unclear if the detection of antibodies to *E. cuniculi* reflects true interactions, antigen exposure without establishment of the parasite, cross-reactivity, or reactions due to polyclonal B cell stimulation, particularly in patients with tropical diseases.

### 4. Studies on inactivation of microsporidian *Encephalitozoon*

When considering methods for the prevention of *Encephalitozoon* infections, studies on the inactivation of microsporidian spores by heat treatment and chemical disinfectants are very important, since microsporidial spores are remarkably environmentally resistant and several epidemiologic studies have identified water contact as a risk factor for microsporidiosis (64). In vitro cell culture provides a useful approach to this subject, because *Encephalitozoon* spp. can be grown successfully in culture (65). For example, using MDCK cells, Waller (66) studied the sensitivity of *E. cuniculi* to various temperatures, disinfectants, and drugs. John et al. (67) and Huffman et al. (68) used RK-13 cells to assess the disinfection potential of UV light on *E. intestinalis* spores in water. We examined the sensitivity of *E. intestinalis* spores to high temperature, UV light, ozone, or chlorine, using monolayer cultures of BS-C-1 cells (69). Figure 4 shows a focus of spores that proliferated in BS-C-1 cells that were immunostained for visualization.

Although spores of *Encephalitozoon* spp. are highly resistant to environmental temperatures (70), we found that the exposure of 4 x 10^7 - 1 x 10^8 *E. intestinalis* spores to soft drink water at 85°C (which is the temperature used to sterilize commercial soft drinks) for 2 -6 min resulted in a 3 log_{10} or 99.9% reduction in the number of infective spores (69). When this in vitro assay using BS-C-1 cells was applied to examine
the effect of UV light irradiation, the results differed from those of the experiments done by John et al. (67), who used RK-13 cells and determined that the UV dose required for a 99.9% reduction in the number of infective *E. intestinalis* spores was 8.43 mJ/cm². In our assay using BS-C-1 cells, 99% of 5 × 10⁶ - 7.5 × 10⁷ *E. intestinalis* spores were inactivated with UV light exposure at 43.5 mJ/cm², but no further inactivation was achieved, even after longer exposure (69). It is possible that RK-13 and BS-C-1 cells could differ in their susceptibility to UV-irradiated spores that may be capable of transitory survival, though this is currently unknown.

Ozone also has the ability to inactivate environmentally resistant protozoa such as *Cryptosporidium parvum* oocysts (71). John et al. (72) demonstrated the successful disinfection of *E. intestinalis* in water by ozone. We therefore investigated the effects of ozone on the inactivation of *E. intestinalis* spores. Figure 5 depicts the time course of the inactivation of *E. intestinalis* spores using ozone at 0.1 ppm and 0.2 ppm. We found that 99.9% of *E. intestinalis* (3 × 10⁹ - 1.8 × 10⁴ spores) were inactivated by ozone at a dose of 0.2 ppm for 2 min, or a dose of 0.1 ppm for 5 min. Ozone was therefore able to inactivate >3 log₁₀ of *E. intestinalis* spores.

Johnson et al. (73) reported that spores of *E. intestinalis* were sensitive to chlorination, and they suggested that *Encephalitozoon* spp. might be inactivated by the level of chlorination that is readily attainable by most water utilities in the United States. Jordan et al. (74) demonstrated that the exposure of *E. cuniculi* to bleach reduced the infectivity of these organisms in tissue-culture model systems. We determined that *E. intestinalis* spores (7 × 10⁶) could be killed by sodium hypochlorite treatment, which is a general disinfectant for viruses and bacteria. When spores were exposed to a 0.01% solution (equivalent to 500 ppm free chlorine) for 10 min, followed by immediate neutralization of the chlorine by the addition of 1 M sodium thiosulfate, the resultant spores were found to be microscopically disfigured and were unable to survive on monolayers of BS-C-1 cells, indicating that hypochlorite solution could be used to disinfect *Encephalitozoon* spp.

5. Conclusions

*E. cuniculi* infections and encephalitozoonosis have been detected in many pet, school and zoo rabbits in Japan over the past 10 years. The parasite has also spread among squirrel monkeys. Infection has been confirmed by seroimmunologic, microbiologic, immunohistochemical and genetic methods, and others. *E. cuniculi* infection is usually asymptomatic, and sAg-ELISA for measuring IgG antibodies has therefore been useful for analyzing the prevalence in colonial as well as individually raised animals. Domestic dogs and Japanese macaques, however, were not found to be seropositive when they were screened for *E. cuniculi* infection using sAg-ELISA, partly followed by spore-ELISA.

In addition to the seroepidemiologic findings, information about the genotypes of *E. cuniculi* isolates from infected animals has been obtained using PCR followed by direct DNA sequencing. ITS or PTP genotype I was isolated from infected rabbits, and isolates were further grouped into Ia and Ib genotypes on the basis of SWP-1 gene analysis. Genotype Ib was found to be predominant over genotype Ia in our isolates. ITS genotype III was isolated from infected squirrel monkeys, but it differed from what is generally known as the “dog type”, suggesting that the genetic diversity of *E. cuniculi* may be wider than previously thought.

Anti-*E. cuniculi* PT IgM antibodies were demonstrated in 36% of healthy persons using spore-ELISA. The rate of the IgM antibody positivity in healthy persons was significantly higher than that in HIV-positive persons. The major antigenic site targeted by human anti-PT IgM was located on PTP1, and was analyzed using 2-D immunoblotting followed by proteomic analysis. These IgM antibodies were species-specific for *E. cuniculi*. Microsporidiosis due to *E. cuniculi* is too rare to be regarded as a problem in immunocompetent people, but *E. cuniculi* infection in humans appears to be opportunistic, and immunosuppressed persons should be considered to be at risk of infection. Studies on circulating anti-PT IgM antibodies might provide insight into protective immunity and aid advances in preventive medicine.

Infective *E. intestinalis* can be titrated using 24-well plates with monolayer sheets of BS-C-1 cells. This quantitative cell culture infectivity assay has been applied successfully to determine the inactivation of *E. intestinalis* by heat treatment, UV light irradiation, ozone, and chlorine, with significance for the prevention of *Encephalitozoon* infections.

Further research into the nature of *Encephalitozoon* infections is still required, and other microsporidia, such as *E. bieneusi* and *Brachiola*, also remain to be studied. Human cases of infection by various microsporidia have been reported from all over the world, but the natural reservoirs and modes of transmission remain to be investigated. Intestinal microsporidiosis is endemic in developing countries, and care should be taken to prevent its possible introduction from overseas. The studies reviewed here should provide a basis for
the further investigation of microsporidian infections in Japan.

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