

Kinetic analysis of specific antibodies in serum and urine of experimentally *Encephalitozoon cuniculi*-infected rabbits

Oda, M.¹, Ueno, A.¹ Adilbish, A.¹, Li, Z.¹, Hernandez. H., O.,¹, Okada, T.¹, Furuya, K.²
and Igarashi, M.¹*

¹National Research Center for Protozoan Diseases, Obihiro University of Agriculture
and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

²Department of Parasitology, National Institute of Infectious Diseases, Tokyo 162-8640,
Japan

*Corresponding author: Makoto Igarashi, E-mail: makoto@obihiro.ac.jp

ABSTRACT

Encephalitozoonosis is a common infectious disease caused by *Encephalitozoon cuniculi* in rabbits. The purpose of this study was to examine the levels of antibody in urines of experimentally *E. cuniculi*-infected rabbits. Humoral immune response and spore excretion in urines were also examined. Rabbits were orally infected with *E. cuniculi*, and sera and urines were collected. Levels of serum IgG, IgM, IgA and urinary IgG were examined. The presences of spores in urines were examined by quantitative PCR. Specific IgG in sera started to be detected at day 20 after infection and kept high level during the course of experiment. Serum IgM started to be detected 1 week earlier than that of serum IgG and decreased to almost undetectable level by day 100 after infection. However, the level of IgM increased gradually again thereafter. Serum IgA was transiently detected between 20 and 70 days after infection and kept undetectable level thereafter. Specific IgG in urines started to be detected from 60 days after infection. The amount of spore DNA was correlated with the amount of IgG in urine. In conclusion, the detections of serum and urinary antibodies were useful for the prediction of stage of latent infection in encephalitozoonosis.

Keywords: encephalitozoonosis, experimental infection, urinary antibody

INTRODUCTION

Encephalitozoon cuniculi is an obligate intracellular parasite that belongs to the phylum Microsporidia. The parasite is distributed worldwide and is able to infect a variety of mammals, including rabbit, rodents, carnivores, monkeys and humans (Wasson and Peper, 2000; Mathis *et al.*, 2005). The major host for *E. cuniculi* is the rabbit and encephalitozoonosis is a common infectious disease in this species. Sero-positive rates in pet rabbits are usually high with 42% to 68% in populations (Halanova *et al.*, 2003; Dipineto *et al.*, 2008; Igarashi *et al.*, 2008; Jeklova *et al.*, 2010; Tee *et al.*, 2011). Infected rabbits excrete a lot of small spores in urine to environment. Therefore, this is important for the spread of infection to group-feeding rabbits and other animals including human.

E. cuniculi infection in rabbits usually exists as a chronic, latent infection, and only a percentage of infected animals develop the clinical disease (Snowden and

Shadduck, 1999). The clinical signs are almost entirely neurological, including torticollis and ataxia. In addition to neurological diseases, chronic renal disease due to *E. cuniculi* infection has also been well characterized in rabbits (Künzel and Joachim, 2010). The most commonly reported histological lesions that occur in spontaneous infections are granulomatous and non-suppurative encephalitis and nephritis (Flatt and Jackson, 1970; Testoni, 1974). In our previous study, we identified *E. cuniculi* specific IgG in urines from naturally infected rabbits (Furuya *et al.*, 2009). However, the kinetics and diagnostic potential of urinary antibody are not known. In healthy rabbits, proteins including antibody are scarcely excreted in urines because of filtration by kidneys (Smith and Hand, 1972). The presence of antibodies in urine of *E. cuniculi*-infected rabbits suggested that glomerular filtration might be impaired in connection with proliferation of the parasite in the kidney. Specific antibodies have been found in urines of human patients and animals with certain infections such as leishmaniasis (Kohanteb *et al.*, 1987; Sartori *et al.*, 1987; Solano-Gallego *et al.*, 2003).

In this study, we examine the humoral immune response, excretion of specific antibodies and spores in urine in experimentally infected rabbits and evaluate their usefulness for the information regarding the state of latent infection.

MATERIALS AND METHODS

Culture of *E. cuniculi*

E. cuniculi (Furuya *et al.*, 2001) were maintained in RK-13, rabbit's epithelial cells (ATCC CCL-37) grown in modified Eagles medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% fetal calf serum (FCS) at 37°C in 5% CO₂ incubator.

Experimental infection

Four female SPF rabbits (Kbl: Japan white species, thirteen week-old) were purchased from Clea, Tokyo, Japan. These rabbits were infected 10⁷ spores per orally. Two rabbits were euthanized at day 60 and day 200 after infection, respectively. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Obihiro University of Agriculture and Veterinary Medicine. Rabbits were sacrificed under pentobarbital anesthesia. The protocol was approved by the Committee on the Ethics of Animal Experiments of Obihiro University of Agriculture and Veterinary Medicine (Permit Number: 22-98). All efforts were made to minimize suffering.

Urine samples

Urine samples were collected from trays under cages. Collected urines were centrifuged at 2,000 ×g for 20 minutes. Urine supernatants were stored at -20°C until use. The pellets were re-suspended in 1 M Tris/citrate buffer, pH 2, to remove calcium carbonate. After washing twice with PBS, the pellets were stored at -20°C until use. In order to extract DNA, the pellets were suspended in proteinase K buffer (1% SDS, 12.5 mM EDTA, 150 mM NaCl, 100 mM Tris, pH 7.5, 10 μg/ml proteinase K) and incubated at 65°C for 3 hours. After phenol/chloroform extraction and ethanol precipitation, extracted samples were dissolved in TE (10 mM Tris, pH 7.5, 1 mM EDTA).

Enzyme-Linked ImmunoSorbent Assay

E. cuniculi spores were collected from culture supernatants. After disruption of host RK13 cells by suspending in PBS containing 0.5% Triton X-100 and 0.25% saponin, spores were washed with PBS three times and suspended in lysis buffer (4% SDS, 1.4 M mercaptoethanol, 0.5 M Tris pH 6.8). After heating at 95°C for 5 minutes, extracted soluble antigen was desalted by dialysis in PBS overnight, removed insoluble materials by centrifuge at 21,500 ×g for 5 minutes, and used as a soluble antigen for ELISA. The 96 flat-bottom wells of micro-titer plates (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight at 4°C with *E. cuniculi* soluble antigen at 2 µg/ml in 50 mM sodium carbonate buffer (pH 9.6). The plates were washed with PBS containing 0.05% Tween 20 (PBS-T), pH7.4. Non-specific binding sites were blocked with PBS containing 1% skimmed milk (PBS-M) for 1 hour at 37°C. The plates were then washed once with PBS-T and subsequently 100 µl of rabbit serum or urine of appropriate dilution in PBS-M was added and incubated at 37°C for 1 hour. Serum samples were diluted at the ratio of 1:500 for IgG and 1:50 for IgM and IgA detections. Urine samples were used without dilution. After incubation, the plates were washed six times with PBS-T. The bound antibodies were detected by incubating at 37°C for 1 hour with anti-rabbit IgG (GE Healthcare, Chicago, IL, USA), IgM (Southern Biotech, Birmingham, AL, USA), or IgA (Immunology consultants Laboratory, Atlanta, GA, USA)-conjugated with horseradish peroxidase diluted at a ratio of 1:2,000 in PBS-M. Finally the plates were washed six times with PBS-T and the bound peroxidase enzyme activity was revealed by adding 100 µl/well of ABTS substrate i.e., 3 mg 2,2'-Azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (Sigma), in ABTS buffer (0.1 M citric acid monohydrate, 0.2 M disodium hydrogen phosphate), pH4.0 and 3 µl H₂O₂ per 10 ml of buffer. Absorbances at 415 nm in each well were measured using a MTP-500 microplate reader (Corona Electrical, Hitachinaka, Japan).

Detection and quantification of spore DNA in urine

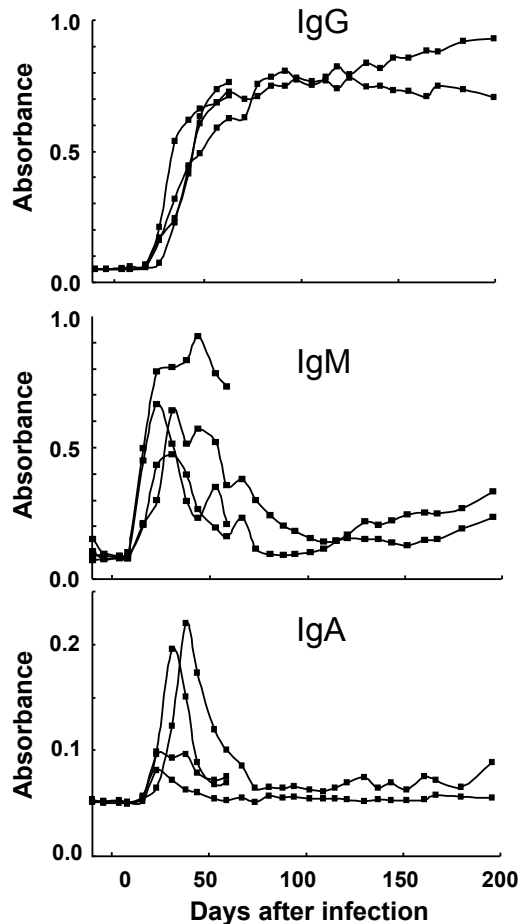
Quantitative PCR of DNA samples from urine pellets was performed using *E. cuniculi* small ribosomal DNA gene (accession No.L17072.1) as a target. Each reaction was carried out in a total volume of 20 µl, which consisted of Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA), 0.1 µM of primers, 5'-GAC GGC TCA GTG ATA GCA CG-3' and 5'-GCA GCT ACT GCT CAT CCC G-3', and 1 µl of template DNA. Cycling conditions were as follows; 10 min of initial denaturation at 95°C was followed by 37 cycles consisting of 95°C for 15 sec and 60°C for 1 min. Dissociation curve of PCR products was analyzed for each sample.

RESULTS

Four SPF rabbits were inoculated per orally with *E. cuniculi* spores and the levels of antibodies in both sera and urines were examined. The presences of spores in urines were also examined by quantitative PCR. The rabbits were sacrificed in pairs at 60 and 200 days after infection, respectively. During the course of experiment, none of the rabbits showed any clinical signs typical or atypical for encephalitozoonosis (data not shown).

After *E. cuniculi* infection, serum IgG titers of individual rabbits started to increase from day 20, reached high levels by day 60 after infection and maintained

thereafter. The timing of appearance and the level of titer were not significantly different among the rabbits (Fig. 1). The



appearances of serum IgM were one week earlier than that of IgG. Times of occurrence of the peaks were different among rabbits, days 23 and 44 after infection, respectively (Fig 1). The titers decreased to almost basal levels by day 100 after infection, however, increased slightly again until the end of experiment of day 200. The amounts of IgA in serum were significantly lower than those of IgG and IgM and the levels of titer were varied among the rabbits. IgA in sera were transiently detected between day 20 and 70 after infection and kept undetectable level thereafter (Fig. 1).

Fig. 1. Specific antibodies response detected in sera of *Encephalitozoon cuniculi* infected rabbits. Data are shown as absorbances of IgG, IgM or IgA isotypes at various days after infection.

The presence of specific IgG, IgM and IgA in urine samples was tested and only IgG antibody could be detected. The rabbits sacrificed at day 60 did not show IgG antibody excretion (data not shown). Therefore, only results from two rabbits sacrificed at day 200 were shown in Fig. 2. The titers of IgG antibody in urines were significantly different between two rabbits, started to increase from day 60 after infection and did not decrease until the end of experiment of day 200.

To detect and quantify *E. cuniculi* DNA from spores in urine samples, quantitative PCR was performed. The sensitivity was determined as low as 0.1 pg of purified DNA (data not shown). Results were shown in Fig. 2. None of the urine samples before infection showed positive (Fig. 2 and data not shown). Positive signals were observed in urine samples of one rabbit from day 35 to 115 after infection with high frequency. On the other hand, significantly lower levels of signal were observed in the other rabbit.

DISCUSSION

E. cuniculi spores mainly parasitized rabbit's brain and kidneys are excreted in urine to environment. Excreted spores are infectious to wide variety of animals including human through ingestion. Seroprevalence rates of *E. cuniculi* are usually high in pet rabbits and the potential zoonotic risk has been concerned.

In our previous study, we identified *E. cuniculi* specific IgG in urines from naturally infected rabbits (Furuya *et al.*, 2009). In this study, we examined the humoral immune response, levels of antibody and existence of spores excreted in urines of experimentally *E. cuniculi*-infected rabbits. During the experimental period, none of the rabbits showed any clinical signs, indicating mild infections. Infections were confirmed by the production and sustained level of serum IgG antibodies in individual rabbits. The level of serum IgM showed a peak of day 23 to 44 after infection. This result was similar to those described in previous reports (Cox, 1977; Kohanteb *et al.*, 1987). Although the amount of IgM decreased to almost basal level, the titer increased again thereafter in this study (Fig. 1). The presence of IgM antibodies was considered as an active infection even in asymptomatic rabbits (Jeklova *et al.*, 2010). Our data suggested that active infection occurred at secondary infectious sites after dissemination and stimulated secondary host humoral immune response. Long-term observation of IgM kinetics is required to evaluate its diagnostic potential for chronic infection. The amounts of specific IgA in sera were very low and transiently detected between day 20 and 70 after infection. In the obligate intracellular protozoan parasite, *Toxoplasma gondii* infection, the specific IgA in serum was reported as a good diagnostic marker for acute and congenital toxoplasmosis in human (Decoster *et al.*, 1988). It is interesting to know whether the same application would be useful for the diagnosis of acute and congenital encephalitozoonosis.

The specific IgG antibodies in urine samples started to be detected from day 60 after infection and the amounts were significantly different between two rabbits. The amount of IgG in urine was not correlated with the amount of antibodies in serum, but correlated with the amount of spores excreted in urine in this study. These results suggested that glomerular filtration might be impaired in connection with proliferation of the parasite in the kidney.

In conclusion, the detections of serum and urinary antibodies were useful for the prediction of stage of latent infection in encephalitozoonosis.

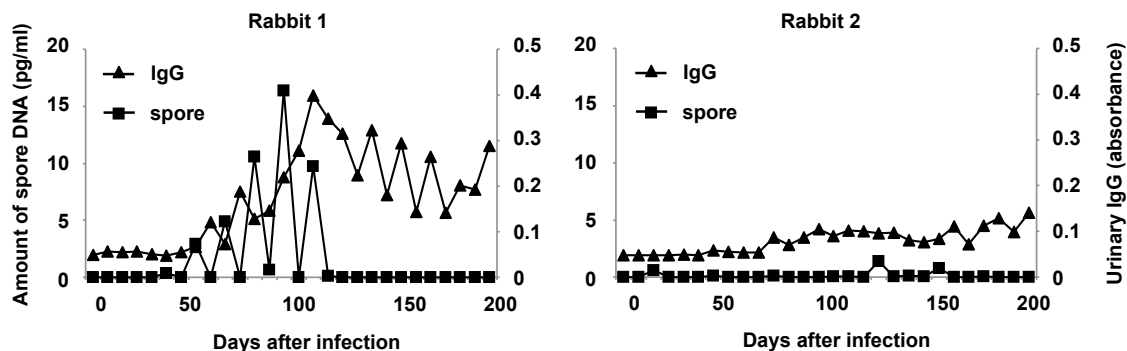


Fig. 2. Detection of specific IgG and spores in urines of *Encephalitozoon cuniculi* infected rabbits. The amounts of specific IgG antibodies are shown as absorbances at various days after infection. Results of quantitative PCR are expressed as the amount of DNA/ml of urine. The closed triangle and square indicate IgG and spore DNA, respectively.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUBMISSION DECLARATION AND VERIFICATION

The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

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