Establishment of an alveolar echinococcosis rat model with a single hepatic lesion and serological analysis.

単発性肝病変を有する多包虫症ラットモデルの作製と血清学的解析

The United Graduate School of Veterinary Sceince

Yamaguchi University

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March 2017

Doctor's Thesis

The United Graduate School of Veterinary Science
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GENERAL INTRODUCTION

Human alveolar echinococcosis (AE) is caused by an infection by *Echinococcus multilocularis*. *E. multilocularis* is a parasite that resides in the intestine of definitive host animals. These animals are infected because of the ingestion of infected rodents, which act as intermediate hosts for the parasite (Pawlowski et al., 2001). The parasite produces eggs in the intestine of the definitive host, and proglottides containing the eggs are excreted with its feces. Upon ingestion by a rodent, these eggs hatch in its stomach and intestine to release oncospheres that are later activated in the small intestine. Oncospheres penetrate mucosa and enter circulation through blood vessels. Metacestodes are predominantly formed in the rodent liver (Thompson, 1995). Although typically not an intermediated host, human beings can acquire an *E. multilocularis* infection through accidental ingestion of the eggs. As a result, AE lesions are formed in the liver, as in the case of rodents. Upon infection, the AE lesion grows in size during a long asymptomatic period and eventually occupies the host liver. Lack of appropriate treatment may result

in liver failure and even death (Pawlowaki et al., 2001; Nunnari et al., 2012).

Detection of AE lesions at an early stage, when the lesion can be completely resected, can play a crucial role in its treatment. However, mainly owing to the long incubation period, early detection of this disease is difficult (Aoki et al., 2015). For the diagnosis of human AE, imaging-based diagnostic methods such as computed tomography (CT), and magnetic resonance imaging (MRI), and serodiagnosis, have been applied. However, it is difficult to diagnose human AE by imaging-based methods because the characteristics of human AE, as identified by CT or MRI, are similar to those of hepatic malignant tumor or calcium deposit (Etlik et al., 2005). Therefore, serodiagnosis plays an equally important role in the detection of human AE.

In a previous study, serodiagnosis using certain antigens was attempted, and its effectiveness was confirmed (Pawlowaki et al., 2001). Serodiagnosis using crude antigens from the whole parasite or protoscolex is the most sensitive, and is used even today. However, low specificity for the antigen makes AE diagnosis difficult in areas where other Taeniidae infections are

prevalent (Lightowlers and Gottstein, 1995). Further, serology using ezrin-radixin-moesin-like protein such as EmII/3 and Em18 has been shown to be useful for the specific serodiagnosis, too. Serodiagnosis using Em18 is highly specific, and its sensitivity was found to be approximately 90-100% (Lightowlers and Gottstein, 1995; Pawlowaki et al., 2001). Therefore, specific antigens, including EmII/3 and Em18, are used for the diagnosis of human AE. Furthermore, the benefits of the follow-up of recovery after a surgical or drug treatment have been examined, and the findings have been brought into clinical use (Gottstein et al., 1989; Ma et al., 1997; Fujimoto et al., 2005; Ishikawa et al., 2009; Tappe et al., 2009 and 2010; Sako et al., 2011). Levels of antibodies against the crude antigen and Em2 or Em18 antigens have typically been shown to decrease after surgical operations over a long period of 0.5-1 year (Lanier et al., 1987; Gottstein et al., 1989; Ishikawa et al., 2009; Sako et al., 2011). Recently, however, a rapid decline in the antibody response against recombinant Em18 (RecEm18) has been reported in some human cases following resection of AE lesions (Akabane et al., 2012; Ito, 2013). In these reports, the antibody response against RecEm18

decreased within a few days after surgery. In the clinical field, it is important to confirm the effectiveness of surgical or drug treatment at the earliest. Information about the changes in antibody levels immediately after treatment can facilitate improvements in the medical follow-up. Therefore, further analysis using animal models is required to test whether a rapid decline in antibody levels is a reproducible phenomenon.

Human AE research with animal models have been performed mainly on rodents. This involves two types of study models: the egg model and the homogenate model. The egg model relies on the introduction of *E. multilocularis* eggs in the rodent by ingestion, similar to that in the case of human AE (Matsumoto et al., 1998 and 2010). However, parasite eggs are dangerous to humans. As a result, high bio-safety level facilities are required to use this model for studies. In the homogenate model, on the other hand, a homogenate of larvae is introduced into the abdominal cavity, liver, and portal vein (Liance et al., 1984; Ohnishi, 1984; Nakao et al., 1990; Nakaya et al., 2006). This route of infection is, thus, different from that in the case of human AE. However, use of this model renders high bio-safety level facilities unnecessary. In

most AE patients, a single AE lesion is formed in the liver, and the enlargement of the lesion is very slow (Pawlowaki et al., 2001). However, in the current animal models, multiple lesions are formed in the liver and lungs, which enlarge faster than that in human AE. Thus, the characteristics of the lesions formed during studies using either the egg model or the homogenate model are different from those of human AE.

The present study describes a new animal model, in which an AE lesion is formed in one arbitrary region in the liver without metastasis or abdominal dissemination. Further, it also discusses the usefulness of the model in evaluating a follow-up serodiagnosis in human AE.

Chapter I

Echinococcus multilocularis: Single hepatic lesion experimentally established without metastasis in rats.

ABSTRACT

I herein describe the establishment of single hepatic lesions of *Echinococcus multilocularis* in rats. A 3-mm incision was made on the liver with a surgical knife, and one small round vesicle of *E. multilocularis* (between 1 x 1 mm and < 2 x 2 mm in diameter) was transplanted into the incision and covered with absorbable hemostat gauze. The presence and growth of the transplanted vesicle was monitored for 12 weeks using magnetic resonance imaging (MRI). Hepatic lesions, the metacestode of this parasite, were confirmed in 12 of 17 infected rats (70.6%) by MRI and macroscopic examinations. The average size of the metacestodes with brood capsules at 12 weeks after the experimental transplantation of a single vesicle was 6.1 ± 2.5 mm x 4.4 ± 1.5 mm. The smallest size of the metacestodes detected by MRI was

approximately 3 x 3 mm. This new approach of establishing single hepatic metacestodes of *E. multilocularis* in experimental animals is expected to be useful for analyzing the immune-pathological mechanisms of hepatic AE.

INTRODUCTION

Hepatic alveolar echinococcosis (AE), which is caused by the metacestode of *Echinococcus multilocularis*, develops space-occupying lesions on/in the liver and is often misdiagnosed as hepatic cancer. Most recently, a group of researchers observed an unexpected very rapid decline (within one week after surgery) in the antibody titer following curative surgery for hepatic AE (Akabane et al., 2012; Ito, 2013). Therefore, I became interested in conducting further analyses of unexpected rapid declines in the antibody titer after surgery in cases of hepatic AE. For this purposes, however, an experimental animal model of single AE lesions on the liver must be established.

There have been several reports of methods establishing hepatic AE lesions in animal models, including the injection of

homogenized metacestode tissue into the portal vein in rats (Ohnishi, 1984) and mice (Nakao et al., 1990; Nakaya et al., 2006). However, this approach is not suitable for my purpose of establishing single metacestodes of *E. multilocularis* on the liver. Gottstein et al. (2002) tried to inject a single vesicle of *E. multilocularis* into a point of the liver of mice using a hypodermic needle. However, the metacestode formed lesions in the abdominal cavity in the majority of mice.

In my preliminary study, I attempted to transplant single vesicle into rat livers with poor success, similar to the findings of Gottstein et al. (2002). Ultimately, I succeeded in preventing the parasite from leaking from the liver into the abdominal cavity by covering the vesicle with absorbable hemostat gauze and monitored the growth of the metacestodes by MRI until 12 weeks after infection. In this study, I report the technical procedure for establishing an experimental animal model with single hepatic metacestodes of *E. multilocularis* that can be monitored by MRI.

MATERIAL AND METHODS

All experiments were conducted in compliance with the guidelines of the Animal Care and Use Committee of Tottori University.

Animals

Four- to five-week old specific pathogen-free female SD rats (SLC, Hamamatsu, Japan) were used in the present study. The rats were acclimated to an air-conditioned animal room for infected experimental animals (containment room with a biosafety level of 2) in the Experimental Animal Facility at Tottori University (lights on from 7 a.m. to 7 p.m.; temperature, 25 ± 3°C). All animals had access to food and water in sipper bottles *ad libitum*.

Metacestodes of *E. multilocularis*

The metacestodes of *E. multilocularis* isolated from *Myodes rufocanus bedfordiae* that was captured and found naturally infected with the parasite in Ebetsu, Hokkaido, were maintained in BALB/c mice at Asahikawa Medical University and used in this

study. The metacestodes were divided into small pieces with a surgical knife and kept in a dish with sterile phosphate-buffered saline. Tiny vesicles (Fig. 1a) were isolated by tearing the metacestodes with forceps and collected with a transfer pipette under a stereomicroscope.

Transplantation of single vesicle from the metacestodes of *E. multilocularis*

Each rat (N = 17) was anesthetized with isoflurane (1-3 %) using a vaporizer and fixed in the dorsal position. After opening the abdominal cavity, the left caudal lobe of the liver was exposed, and a 3-mm incision was created on the liver with a surgical knife. Single vesicle measuring 1.0 x 1.0 mm – < 2.0 x 2.0 mm in diameter was transplanted into the incision and covered with absorbable hemostat gauze (Surgicel; Ethicon, Inc., OH, USA) (Figs. 1b and 1c). After confirming the hemostasis of the incision, the liver was returned to the abdominal cavity, and the abdominal incision was closed. The same procedure was performed in uninfected rats without transplantation of the vesicle, these rats served as controls.

MRI analysis

MRI observations of the abdominal cavity in the rats were carried out at 2, 4, 6, 8, 10 and 12 weeks after surgical treatment. The rats were anesthetized according to the method described above. MRI was performed using a 0.3 T AIRIS Vento instrument (HITACHI, Inc., Tokyo, Japan). The following parameters were employed: field of view, 180 mm; slice thickness, 2.5 mm; interval, 3.0 mm. The parameters of slice thickness and interval are shortest width in the MRI instrument. T1-weighted (T1W) and T2-weighted (T2W) images were obtained under imaging conditions with repetition/echo time = 380/15 and 4,000/100 msec, respectively.

Necropsy and histological examination

The rats were killed under isoflurane anesthesia via exsanguination by cutting the common carotid artery 12 weeks after transplantation, and the abdominal and thoracic cavities of the rats were observed. The liver was removed and fixed with neutral-buffered 10% formalin, then embedded in paraffin.

Five-μm-thick sections of the lesions were stained with hematoxylin and eosin or periodic-acid-Schiff (PAS), followed by hematoxylin counterstaining (Nakaya et al., 1997).

RESULTS

Macroscopy of metacestodes of *E. multilocularis*: Figure 2a shows the macroscopic view of a well-developed translucent and whitish metacestode located in the left lobe of the liver. In 14 of the 17 transplanted rats, the metacestode was observed at the site of transplantation. The largest metacestode was approximately 11 x 8 mm in size and the smallest metacestode was approximately 3 x 3 mm in size. In one of the remaining three rats, the metacestode was established at the outer edge of the left lobe and the size was approximately 6 x 4 mm. In two other rats, no metacestodes were detected. In 12 of the 17 infected rats (70.6%), the metacestodes were enlarged with exogenous budding, and the average size was 6.1 ± 2.5 mm x 4.4 ± 1.5 mm (major axis and minor axis, mean ± standard deviation). Approximately 1 x 1-mm single broken vesicle that did not differentiate into metacestodes were found in the two other rats. No metacestodes

were found on the lungs or in the thoracic or abdominal cavity. In control rats, the metacestodes were not observed.

Morphological observations of metacestodes of *E. multilocularis* on MRI: The detection rate of a single metacestode of *E. multilocularis* by MRI was 86.7% (13 of the 15 rats; macroscopically detected) just before necropsy at 12 weeks of infection. The grown metacestodes were visualized as hyperintense on T2WI and isointense or hypointense on T1WI with clear boundaries after 2 weeks of infection (Fig. 3). The size and position of the metacestodes were correctly visualized by MRI. Two metacestodes approximately 1 x 1 mm in size were not visualized by MRI. The smallest metacestode detectable by MRI was approximately 3 x 3 mm.

Histological examination of metacestodes of *E. multilocularis* : At 12 weeks after transplantation of the single vesicle, enlarged metacestodes with exogenous budding and vesicles surrounded by inflammatory cells (leukocyte layer, fibroblast layer and epithelioid cell layer) were observed (Fig. 2b). Budding vesicles

consisted of a laminated layer with a PAS-positive layer and a germinal layer (Fig. 2c). Protoscolices and germinal cells was poor, and calcareous corpuscles were not observed within 12 weeks. Only one rat harbored metacestodes with protoscoleces. In two rats, small vesicles approximately 1 x 1 mm in size were crushed, and the host cells were invaded in the vesicles.

DISCUSSION

I herein described a new experimental method for establishing single hepatic metacestode of *E. multilocularis* without metastasis. Several reports have proposed different methods to establish secondary metacestodes of *E. multilocularis* in laboratory animals: injection of the parasite homogenates into the portal vein, which can be used to establish massive hepatic and lung metacestodes (Ohnishi, 1984; Nakao et al., 1990; Nakaya et al., 2006), injection of homogenates into the abdominal cavity, which can be used to establish massive abdominal metacestodes (Kizaki et al., 1993), injection of homogenates into the brain, which can be used to establish massive metacestodes in the brain (Asanuma et al., 2003), and the tail vein, which can be

used to establish metacestodes in the lungs (Asanuma et al., 2006) and transplantation of single vesicle in the liver (Gottstein et al., 2002). Among these studies, the approach by Gottstein et al. (2002) was expected to be the most rational method of establishing single secondary metacestodes of this parasite in the liver. However, in their study, single metacestodes in the liver were formed in only two of five mice and other three mice have meatcestodes in the peritoneal cavity. In the present study, I devised a method to prevent the transplanted vesicle from leaking out into the abdominal cavity by placing absorbable hemostat gauze on the vesicles and then monitored the transplanted vesicles for differentiation and development into metacestodes by MRI throughout the experiment.

Developed metacestodes with brood capsules were found at the site of transplantation in 13 of the 17 rats, and one metacestode was found at the outer edge of the left lobe in one rat. In the other four rats, no developed metacestodes were detected. The success rate of the present model was 12 of 17 rats. Regarding the failure to establish metacestodes, there are two possibilities: 1) the transplanted vesicle was damaged and

punctured at transplantation, and 2) the condition of the transplanted vesicle was inactive. Gottstein et al. (2002) reported that punctured vesicles either do not grow or grow very slowly (Gottstein et al., 2002). A similar observation stressing the importance of intact vesicles for the successful establishment of secondary echinococcal metacestodes has been reported using *E. granulosus* in mice (Casado et al., 1992; Zhang et al., 2005). In the present study, two rats had no metacestodes and two rats had 1 x 1-mm small vesicles that did not differentiate into metacestodes. These findings strongly suggest that some vesicles used for transplantation were damaged and not intact. I expect that the failure to establish metacestodes at the site of transplantation in minor cases could be prevented if cultured vesicles were used and the quality and viability of the vesicles were assessed under a microscope just before transplantation.

Although the development of germinal cells and protoscoleces were poor at 12 weeks of infection (Fig. 2c), this finding is expected in rats. Rats are good experimental animals for studies of AE, however, the development of metacestodes takes much longer than that observed in mice. It has been reported that

metacestodes with a large volume and weight mass (500-700 g) with full protoscolices develops in rats within one to two years of secondary infection (Ito et al., 1996; Yamano et al., 2014). In egg infection, humans and some mice strains, C57BL/6 and C57BL/10, are also low susceptible intermediate hosts, with poor germinal cells and protoscoleces in the AE lesions (Nakaya et al., 1997; Pawlowski et al., 2001; Matsumoto et al., 2010). The present histological findings, the poor germinal cells or protoscoleces, of metacestodes were similar to the previous studies.

Some studies have reported MRI observations of AE (Balci et al., 2000; Kodama et al., 2003; Etlik et al., 2005; Yoshida et al., 2010; Yapici et al., 2011). However, these papers demonstrated relatively large lesions by MRI and there are no reports discussing the minimum size of lesions detectable by MRI. In the present study, the metacestodes were hyperintense on T2WI and isointense or hypointense on T1WI. The rate of detection by MRI was 86.7% (13/15 rats) at 12 weeks after transplantation. Two rats with small vesicles measuring approximately 1 x 1 mm in size were not detected by MRI. The smallest size of metacestodes detectable by MRI was approximately 3 x 3 mm in size. It is clear

that metacestodes measuring 3 x 3 mm in size or larger can be measured exactly by MRI in the present animal model.

Most recently, a group of researchers analyzed the antibody response against recombinant Em18, the most specific and sensitive diagnostic marker protein for detecting active AE cases (Ito et al., 2007), during the perioperative period in curative hepatic AE cases (PNM Stage 1 and PNM Stage 2 by Kern et al. (2006)) in Japan and found that the antibody response declined immediately, even during the perioperative period of surgery and became negative within half a year (Akabane et al., 2012; Ito, 2013). Although it has been reported that the antibody response to *E. multilocularis* declines after surgery (Gottstein et al., 1991; Ishikawa et al., 2009; Tappe et al., 2009, 2010), no previous studies have attempted to observe the antibody responses during the perioperative period or within six months of surgery. The antibody response during the perioperative period has not been clearly elucidated. Therefore, I am keen to establish an experimental animal model with single hepatic metacestode of *E. multilocularis* as a single hepatic AE model.

Using the present method, it is easy to produce rats with

single hepatic metacestodes of *E. multilocularis* on the liver. The lesions created in this rat model can be resected curatively. Therefore, this rat model can be used to analyze rapid declined in the antibody response after surgery and may be useful for evaluating the efficacy of new noninvasive treatments.

FIGURE

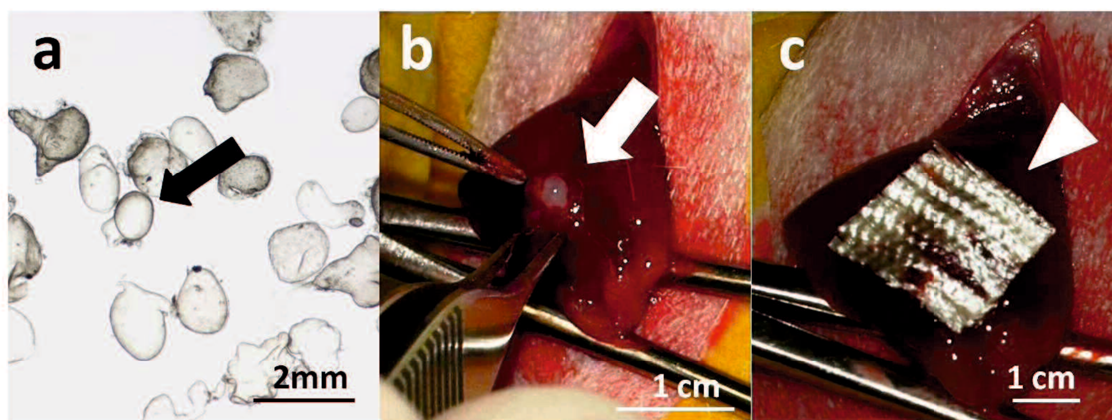

FIGURE 1. a: Transplantation of a single vesicle of *E. multilocularis*. A vesicle (arrow) isolated from metacestodes of *E. multilocularis* grown in BALB/c mouse. b: A single vesicle (arrow) transplanted into the incision on the liver. c: A single vesicle was covered with Surgicel (arrowhead).

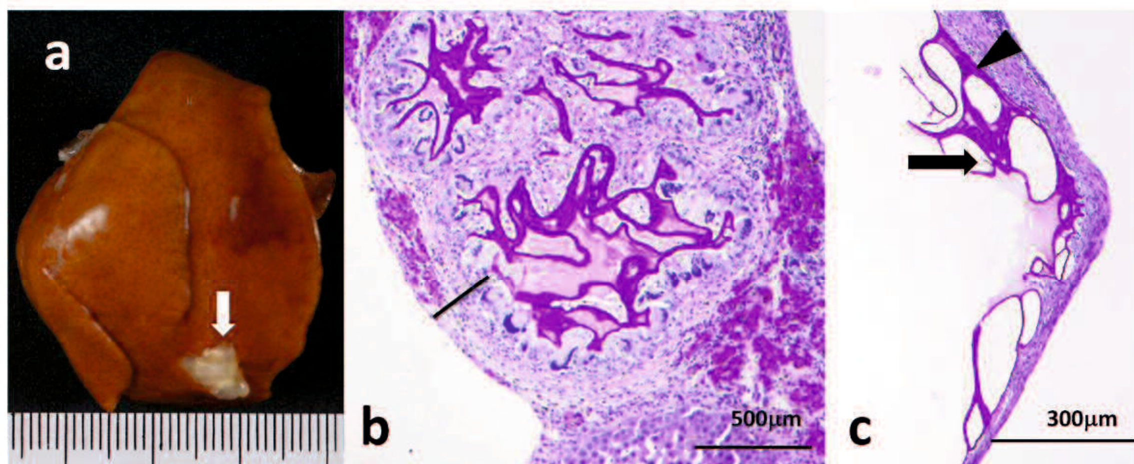

FIGURE 2. a: Single enlarged metacestode of *E. multilocularis* (arrow) on the liver. Macroscopic figure of the metacestode on the left lobe of the liver. Histological figure of PAS stain (b and c). b: The vesicle is surrounded by host tissue (black line). c: A PAS positive laminate layer (arrowhead) and a poor germinal layer (arrow) is observed.

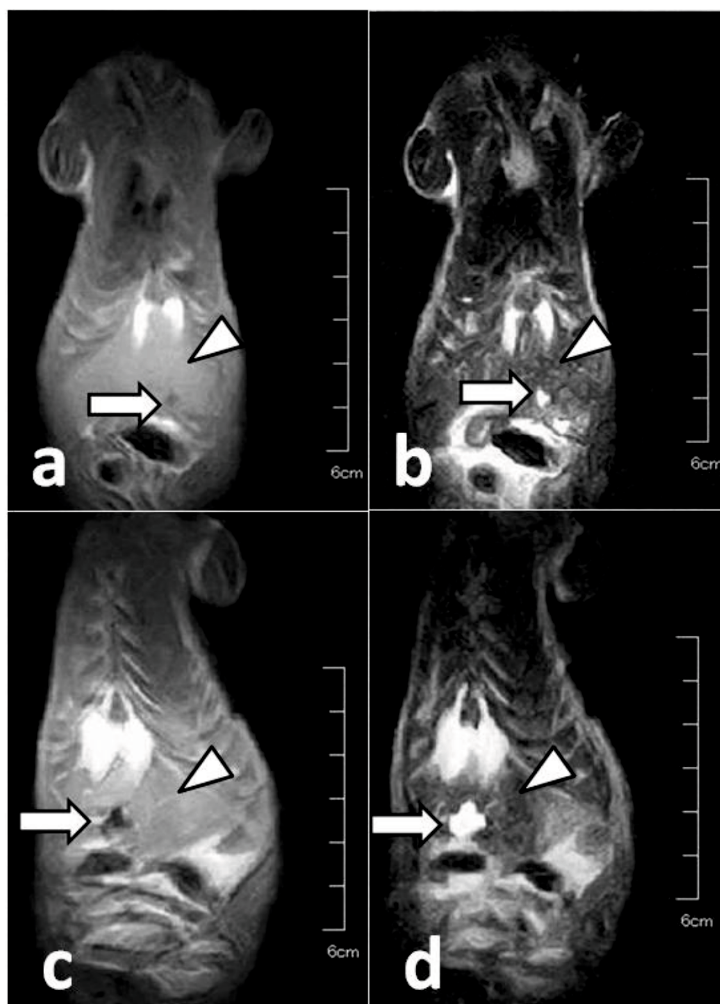

FIGURE 3. The coronal MR imagings in the same individual. T1W (a) and T2W (b) imagings at 4 weeks, and T1W (c) and T2W (d) imagings at 8 weeks after transplantation. The lesions formed by metacestode (arrows) are visualized as hyperintense on T2WI and isointense or hypointense on T1WI with clear boundaries. The size of the lesion is 4.6 × 3.6 mm at 4 weeks and 8.1 × 8.2 mm at 8 weeks. Arrowheads indicate the liver.

Chapter II

Serological validation of an alveolar echinococcosis rat model with a single hepatic lesion.

ABSTRACT

Serology is important for the diagnosis and follow-up of human alveolar echinococcosis (AE). However, patient conditions are highly variable among those with AE, and antibody responses in serological follow-up have not been well-defined. In chapter I, I described a new AE rat model established by implantation of small AE tissue into a single arbitrary location in the liver; no metastasis and dissemination were observed. In the present study, I examined the serological characteristics in my rat model before and after surgical treatment. The results showed that antibody responses against crude antigens were increased at one month after transplantation and similar to those of other model animals. For the antigen Em18, antibody responses were slower in my rat model than in other animal models. After surgical resection,

changes in antibody responses against Em18 were similar to those observed in human patients with AE. Because of the slow growth of lesions, establishment of a single hepatic lesion and patterns of antibody responses, my rat model may be useful for clarifying follow-up serodiagnoses in human AE and determining the mechanisms of multi-organ involvement by primary infection with oncospheres rather than metastasis.

INTRODUCTION

Human alveolar echinococcosis (AE) is caused by the accidental ingestion of eggs of the fox tapeworm, *Echinococcus multilocularis*. AE is a typically neglected zoonotic disease occurring in the northern hemisphere. Recent reports have indicated that *E. multilocularis* infection is spreading among wild animals in Europe, Asia and North America; the increased incidence of AE is becoming a public health concern, in both developing and developed countries, because of the presence of foxes in urban areas in developed countries in Europe, Japan and Canada (Deplazes et al., 2004; Davidson et al., 2012; Liccioli et al.,

2015). Human AE is characterized by hepatic malignant tumors, and if appropriate treatment is not provided, the patient may experience liver failure and even death (Pawlowaki et al., 2001; Nunnari et al., 2012).

In human AE, abdominal imaging to detect occupational lesions in the liver is essential, followed by reliable specific serology, as recommended by the World Health Organization (Pawlowaki et al., 2001; Brunetti et al., 2010). Serology using ezrin–radixin–moesin-like protein and Em10, EmII/3, Em4 or Em18 has been shown to be useful for serological differentiation of hepatic malignant tumors in patients with AE and for the monitoring of AE cases after surgical and medical treatments (Ito et al., 2001). In patients with AE, follow-up serology can vary widely according to patient condition, PNM stage, age, duration of infection, lesion size, and presence or absence of metastasis (Ma et al., 1997; Ishikawa et al., 2009; Tappe et al., 2009). To validate the follow-up serodiagnosis, experimental animal models with lesions of uniform size and location as well as similar durations of infection are urgently needed.

Recently, I developed an AE model in which an alveolar

hydatid vesicle was directly embedded into the liver of rats (Yamashita et al., 2013). This model differs from previous animal models generated by oral inoculation with eggs of *E. multilocularis* and by infusion of a homogenate of alveolar hydatid vesicle (Liance et al., 1984; Matsumoto et al., 1998; Matsumoto et al., 2010), because the AE lesion is localized at an arbitrary point in the liver. Furthermore, recent studies re-evaluated the potential for the metastasis of AE lesions (Aoki et al., 2015; Kvascevicius et al., 2016). Confirmation of disseminated AE lesions in this new model may support the occurrence of metastasis. Moreover, certain lesions may be completely resected because of their localization. Thus, my AE model may be useful for observing host immune responses before and after treatment.

To examine the immune response in my rat model, I used two antigens (crude antigens of a metacestode and recombinant Em18 [RecEm18] antigen). Em18, which can be either native or recombinant, has been shown to be highly useful and reliable for the differential diagnosis of human AE with approximately 90–100% specificity (Pawlowski et al., 2001; Xiao et al., 2003),

whereas crude antigens, which include highly cross-reactive nonspecific components, are expected to be useful for monitoring of the infection itself under experimental conditions. Although crude antigens are not used for differential diagnosis, they may be useful for preliminary screening (Yamano et al., 2009). Furthermore, the antibody levels generated in response to Em18 were found to be dramatically decreased after curative surgical resection of the whole lesion (Tappe et al., 2009) and chemotherapy (Ishikawa et al., 2009). Thus, these antigens were used for follow-up after surgical treatment of AE.

In this study, I examined serological characteristics in my rat model and compared these characteristics with the features of human cases of AE and other model animals inoculated orally with eggs and injected with homogenates of alveolar hydatid vesicles. The present aim was to clarify whether my rat model could be used as an AE model for observing serological findings.

MATERIALS AND METHODS

Model animals: All procedures were carried out in accordance

with the experimental animal institutional guidelines at Tottori University. The model animal was established as described in chapter I. Female Sprague-Dawley rats (4–5 weeks old; SLC; Hamamatsu, Japan; n = 9) were used in this study. The rats were acclimated to an air-conditioned animal room for infected experimental animals (containment room, biosafety level 2) in the Experimental Animal Facility at Tottori University (lights on from 7 a.m. to 7 p.m.; and temperature, 25 ± 3°C). All animals had access to food and water ad libitum. Metacestodes of *E. multilocularis* were isolated from *Myodes rufocanus bedfordiae* in Ebetsu, Hokkaido, and passaged 10 times in BALB/c mice at Asahikawa Medical University. The metacestodes were divided into small pieces, and tiny vesicles were originally isolated under a stereomicroscope. Vesicle measuring 1.0–2.0 mm in diameter was inserted into the livers of six rats and covered with a gauze-type absorbable hemostat (Surgicel; Ethicon, Somerville, NJ, USA). Three control rats were treated in the same manner but without transplantation. Plasma was collected and used to calculate the cut-off value.

Observation by magnetic resonance imaging (MRI) and blood

sampling: After transplantation, blood sampling and MRI were carried out once a month to evaluate serological changes and changes in the sizes of transplanted lesions, respectively. Blood samples were collected from the common jugular vein, mixed with heparin and centrifuged at 1,000 ×*g* for 10 min at 4°C to collect the plasma. The plasma was stored at -80°C until analysis by enzyme-linked immunosorbent assays (ELISAs).

Resection of the AE lesion: After confirmation that the AE lesion proliferated and increased the antibody titer against RecEm18 (>0.2, based on ELISA absorbance), the AE lesion was resected at 366 days after transplantation. Under isoflurane anesthesia, the median line of the abdomen was cut, and the part of the liver containing the cysts was resected by ligation at the proximal part of the cysts. After ensuring that no AE metacestodes remained in the liver or other abdominal organs, the abdominal cavity was closed. After the operation, blood was collected on days 1, 3, 5, 7, 21 and 60. At 60 days after lesion resection, all rats were sacrificed, and the abdominal and thoracic cavities were examined macroscopically.

Antigen preparation: Antigens were prepared as described

previously (Sako et al., 2002, 2009 and 2011; Nakaya et al., 2006). Crude *E. multilocularis* antigen extract was prepared from fresh whole alveolar hydatid vesicle tissue materials. *E. multilocularis* metacestode tissue was obtained from nonobese diabetic severe combined immunodeficiency mice infected by intraperitoneal passage of metacestodes (Nakaya et al., 2006). Microvesicle and protoscolex suspensions were prepared by pressing metacestode tissue through a 300-µm metal mesh with phosphate-buffered saline (PBS). The microvesicles and protoscoleces were washed 5-7 times with PBS and homogenized with three volumes of lysis buffer consisting of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl and 1.0% 3-([3-cholamidopropyl] dimethylammonia)-1- propanes ulfonic acid in the presence of peptidase inhibitors (protease inhibitor cocktail for mammalian tissues; Sigma-Aldrich, St. Louis, MO, USA). After one freeze-thaw cycle and centrifugation at 10,000 ×*g* for 30 min at 4°C, the supernatant (crude antigen) was recovered and stored at -80°C until use (Sako et al., 2011). RecEm18 protein was extracted from *Escherichia coli* harboring a RecEm18-expressing plasmid (Sako et al., 2009). The expressed RecEm18 was purified using a chitin column (New England

Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. Protein concentrations were determined using a BSA Protein Assay Kit (Pierce, Rockford, IL, USA) (Sako et al., 2002 and 2009).

ELISA protocol: ELISA was carried out as described previously (Sako et al., 2011). Briefly, crude antigen and RecEm18 antigen were used to coat microtiter plates (Nunc-Immuno Plate; Thermo Fisher Scientific Inc., Waltham, MA, USA) at a concentration of 100 ng/well. The wells were blocked with 250 µl blocking buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl and 1% casein) at 37°C for 1 hr. After washing the wells with PBS containing 0.1% Tween 20 (PBST), 100 µl of plasma diluted 1:100 in blocking buffer was added to each well, and the plates were then incubated at 37°C for 1 hr. The wells were washed four times with PBST, incubated with 100 µl anti-rat IgG (Jackson ImmunoResearch, West Grove, PA, USA) at 37°C for 1 hr and washed five times with PBST. After incubation with 400 µM (100 µl) of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Sigma-Aldrich) for 30 min at room temperature, absorbance was measured at 405 nm with a

reference wavelength of 490 nm, as described previously (Sako et al., 2011). The cut-off values for each antigen were defined as the average absorbance + standard deviation × 4 of three control rats. The ELISA index of the individual plasma sample was calculated by dividing the sample's absorbance by the cutoff.

MRI: MRI was performed using a 0.3 T AIRIS Vento instrument (HITACHI Medico Inc., Tokyo, Japan) under isoflurane anesthesia. The following parameters were employed: field of view, 180 mm; slice thickness, 2.5 mm; and interval, 3.0 mm. T1-weighted and T2-weighted images were obtained with repetition/echo times of 380/15 and 4,000/100 ms, respectively. Volumes of the AE lesions were calculated as (major axis × minor axis2)/2 for each slice.

Histological examination: The AE lesions in rat Nos. 2, 5 and 6 and the tissues in which the AE lesion exhibited suspected metastasis or dissemination were removed and fixed in neutral-buffered 10% formalin and then embedded in paraffin. Five-micrometer-thick sections of the lesions were stained with hematoxylin and eosin.

RESULTS

After transplantation, the AE lesions were observed by MRI (Figure 1). In all rats, the lesions were localized at a single point in the liver, and no metastasis of metacestodes was observed in the thoracic and abdominal cavities. The lesions were confirmed by MRI beginning 30 days after infection in all six rats, and growth of the AE lesion was observed (Fig. 1). Rapid lesion growth was observed from 60 to 150 days (average of 82 ± 31 days) after transplantation. The standard deviation was not large; however, the subsequent rate of lesion growth differed among individuals. Additionally, even at 360 days after transplantation, a lesion was only detected at one site in the liver.

Blood was sampled immediately before hydatid transplantation and at approximately monthly intervals thereafter. In nearly all individuals, approximately 1 ml blood was sampled at each blood draw, and I was able to measure the antibody titers for each antigen over time. Figure 1 shows the ELISA index against RecEm18 and the crude antigen, which was calculated based on absorbance using cut-off values established in control rats from immediately before transplantation until lesion resection. The

antibody against the crude antigen increased in all individuals; antibody positivity was observed on day 30 after infection. The mean timing of antibody positivity in against RecEm18 was 120 days, and the antibody level continued to increase from the initial response until resection of the lesions.

Figure 2 shows one of the resected lesions. The focus was localized to the tail side of the left lateral lobe. For all rats, the focus could be completely removed by resecting half of the affected lobe. The resected lesion ranged from 9 to 15 mm in diameter. In the histological findings of AE lesions, protoscolices were not found, and germinal cells were poor. After completion of the experiment, I performed gross observations of the rat lungs, liver, and abdominal cavity, and the tissues in which AE lesion showed suspected metastasis or dissemination were evaluated histologically, but no metastasis or dissemination was detected.

I acquired blood samples after treatment every other day (0.5 ml) for 1 week after resection. The antibody against Em18 decreased in all rats after resection. By day 21, the antibody level was reduced by 50% in three rats, while by day 60, the antibody level was reduced by 50% in five rats; however, the antibody

response did not completely disappear in any individual (Fig. 3). The antibody against the crude antigen was decreased after resection; however, the reduction was more gradual than that of the Em18 antibody levels. Similarly to the antibody response against Em18, the antibody did not completely disappear in any individual within 60 days. The rate of change for both antibody levels against Em18 and crude antigen was similar in each individual after lesion resection; however, the ELISA index (estimated at the time of lesion resection) exhibited substantial variations among individuals. In addition, in rat No. 6, the reduction in the antibody response to Em18 was slow and minor, while the antibody response to the crude antigen increased postoperatively. After necropsy, other AE lesions were not macroscopically observed in any rat.

DISCUSSION

After vesicle transplantation, I observed antibody responses and lesion sizes for an extended duration. Based on MRI analysis, the lesions were localized to one part of the liver during the entire

study period. Lesion growth was observed at 81 days after transplantation, and lesion size increased until 360 days. No lesions occupied the entire liver or other tissues or organs. In mouse models in which eggs were ingested or homogenates were injected into the liver or portal vein, lesions increased substantially and occupied the whole liver within 20 weeks (Liance et al., 1984; Matsumoto et al., 1998 and 2010; Ito et al., 2001). Moreover, metastasis or multiple lesions are formed in egg and homogenate models, in contrast to the results of the present model (Liance et al., 1984; Matsumoto et al., 1998 and 2010). Based on the present results and those of previous studies, I concluded that lesion growth in my model was much slower than that in other models. In human AE, there are no suitable tools for detecting early hepatic stage AE with lesions less than 1 or 3 cm in size, except for needle biopsy (Aoki et al., 2015). Additionally, the latent period is very long; it can take several years after infection for patients to be diagnosed (Lightowlers et al., 1993; Yamano et al., 2014). The slow growth and hepatic localization of lesions observed in this model were similar to those in human AE. Using the present rat model, immune responses and lesion sizes

(with the absence of metastasis) were followed for over 1 year. Thus, the present model may be suitable for long-term observations, including observation of metastasis.

In the present study, ELISA indices against crude and RecEm18 antigens showed high variations among individual rats. However, I validated the changes in lesion sizes and antibody responses against crude and Em18 antigens in each rat. After transplantation of AE vesicles, antibody responses against crude antigens were observed on day 30, and those against Em18 were observed on day 120 on average in six rats. Human AE is rarely diagnosed during the early hepatic stage, and the time course of the immune response is unclear. Nearly all early AE cases are accidentally confirmed based on misdiagnosis of metastasis of rectal or stomach cancer (Aoki et al., 2015). In two experimental mouse models of oral inoculation with eggs, antibody responses against crude antigens were initially detected at 4 and 9 weeks (Matsumoto et al., 1998 and 2010), and in an experiment involving homogenate injection, antibody responses were detected at 5–10 weeks (Yamano et al., 2014). Antibody responses against EmII/3, a source of Em18, were detected at 4 weeks after egg

inoculation in a mouse model (Matsumoto et al., 2010). When comparing the current results with these previous reports, the timing of antibody responses against crude antigens was similar, but the response to Em18 occurred later than that in a previous report of egg inoculation. These findings suggest that the changes in antibody responses against Em18 in the present model may be similar to those observed in human AE. In the present model, the antibody response against Em18 antigen was increased; however, the AE lesions had no protoscolices. Protoscolices are rarely formed in the human host (Pawlowski et al., 2001), and the histological finding of the present rat model was similar to human AE. Although the source of Em18 antigen is protoscolices (Ito et al., 1993), Em10, a source of Em18, is also expressed in germinal epithelium of brood capsules (Frosch et al., 1991).

Using my animal model, I generated a lesion in an arbitrary location in the liver, facilitating complete resection. Furthermore, frequent blood sampling after resection was unproblematic in rats with no clinical symptoms. The antibody responses against crude antigens showed minimal reduction until 60 days after the resection of AE lesions. In contrast, those against Em18 were

decreased by approximately 50% in five of six rats until 60 days. In a previous study of human patients with AE, the antibody responses against Em18 and crude antigens were decreased after surgical treatment when observed at 6-month intervals (Ishikawa et al., 2009; Tappe et al., 2009). Moreover, the ELISA indices of crude antigens changed more slowly than those of tests using single recombinant or other affinity-purified antigens (Tappe et al., 2009). Recent studies reported two cases of human AE in which antibody responses against RecEm18 began declining within a few days after resection and were negative within 6 months (Akabane et al., 2012; Ito, 2013). In rat models in which homogenates were injected into the liver, the antibody responses against crude antigens were rarely decreased at 35 weeks after resection of the AE lesion (Yamano et al., 2014). Reduction of the antibody response against crude antigen in the present study was similar to that in previous human cases rather than in animal models. Moreover, the decreased antibody responses against Em18 in the present study were more rapid than those previously observed in human cases at 6-month intervals (Ishikawa et al., 2009; Tappe et al., 2009), but were similar to or slightly slower

than those in other studies of human cases (Akabane et al., 2012; Ito, 2013). Based on these findings, the changes in antibody responses in the current model may resemble those of human AE. In one rat, the levels of antibodies against crude antigen and RecEm18 were not decreased. I evaluated the liver, lungs and intraperitoneal tissues using MRI and gross necropsy to ensure that there was no remaining parasite tissue; however, it is possible that undetectable micrometastases or parasite tissue was overlooked.

As described above, multi-organ AE cases involving the lung and brain have been explained by metastasis (Pawlowski et al., 2001; Ozdol et al., 2011). However, my study provided no evidence of metastasis. Furthermore, recent studies by Aoki et al. (2015) and Kvascevicius et al. (2016) in AE cases and by Sadjjadi et al. (2013) in cases of cystic echinococcosis (CE) suggested that the occurrence of multi-organ echinococcosis, either AE or CE, may be due to invasion by multiple oncospheres in all primary lesions in the liver, lung and brain. This hypothesis was also supported by the findings of Ito et al. (2016). Thus, my animal model is expected to facilitate additional studies of metastastic potential.

In summary, I elucidated the serological characteristics of my rat model. The present findings demonstrated that the rat model resembled the pathological features of AE lesions with slow and localized growth in the liver and serological responses of human AE to a greater extent than other previously reported animal models. Furthermore, my model permitted the long-term monitoring of AE lesions.

FIGURE

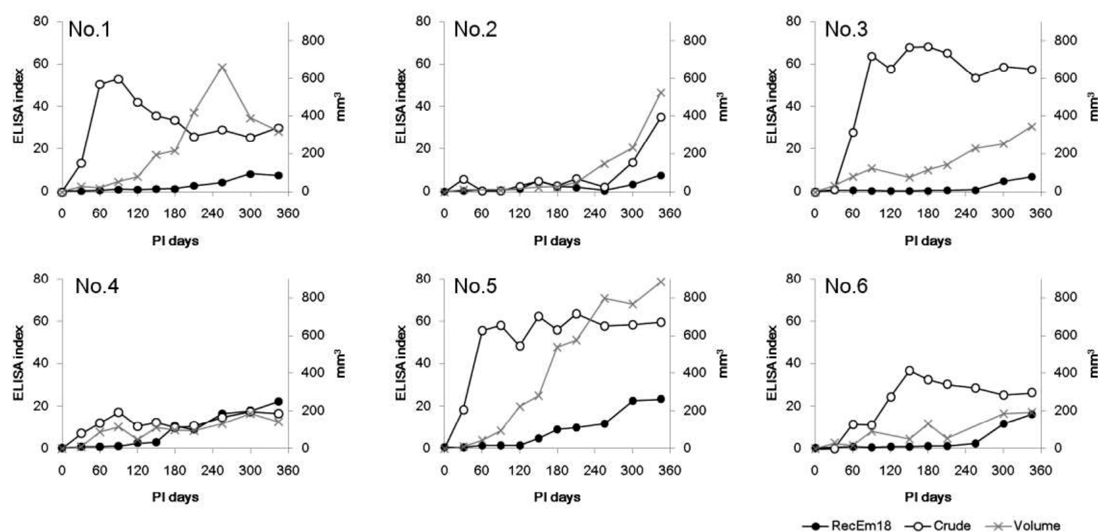

FIGURE 1. Changes in antibody levels against RecEm18 (black circles) and crude antigen (white circles) and changes in the lesion area (x) observed by MRI immediately after transplantation of vesicles into the livers of six rats and every 30 days until lesion resection. The antibody response for each antigen is shown as an ELISA index. The index of the individual plasma sample was calculated by dividing the sample's absorbance by the cutoff. The RecEm18 cut-off value was 0.036, and the crude antigen cut-off value was 0.014. Cut-off = 1 in the plot.

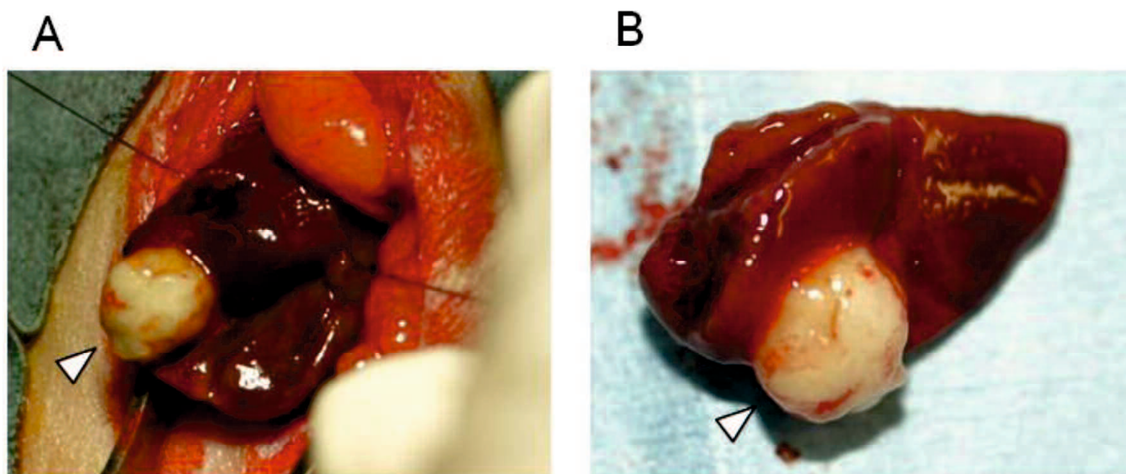

FIGURE 2. A: Resection of AE lesions. B: Resected lesion and liver margin. The arrowheads indicate the AE lesion.

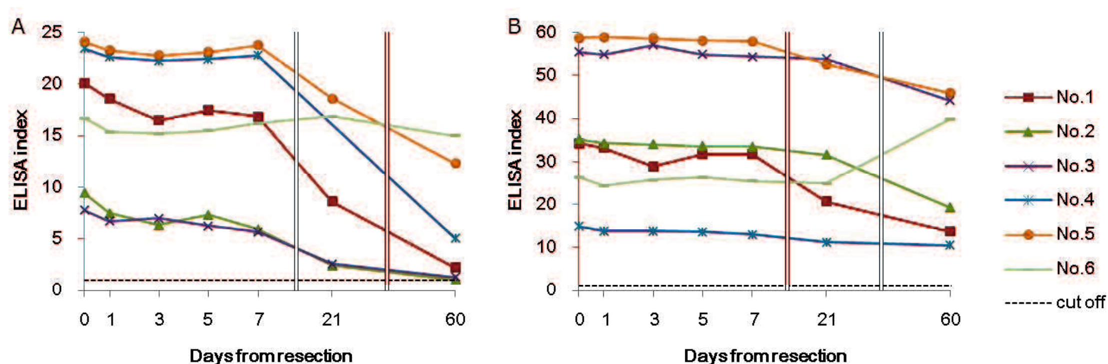

FIGURE 3. Changes in antibody responses against RecEm18 (A) and crude antigen (B) after lesion resection. Blood was sampled on days 0, 1, 3, 5, 7, 21 and 60 after lesion resection to examine the antibody response. The antibody response for each antigen is shown as an ELISA index.

CONCLUSION

The present study describes a new AE animal model and its serological characteristics in the model.

In chapter I, I described a method that yields a new animal model in which an AE lesion is formed in one arbitrary place of the liver without metastasis or abdominal dissemination.

In chapter II, I discuss my attempts to test whether my rat model could be used as an AE model to further investigate the serological responses described earlier. I analyzed the serological monitoring of parasite-specific antibodies in the AE rat model with a single hepatic lesion before and after surgical treatment. The present results showed that the antibody response against crude antigens increased 1 month after transplantation, similar to other animal models. For the antigen Em18, the antibody responses were slower in my rat model than in other animal models. The changes in antibody responses against Em18 after surgical resection were, however, similar to those observed in human patients with AE. Owing to the slow growth of lesions, occurrence of a single hepatic lesion, and patterns of antibody

responses, my rat model could be useful for clarifying follow-up serodiagnoses in human AE. It would also facilitate the investigation and determination of the mechanisms underlying multi-organ involvement by primary infection with oncospheres, rather than metastasis.

In conclusion, I have established a new AE animal model with a lesion that is similar to the human AE lesion. Moreover, I successfully reproduced the host status upon resection of the AE lesion. The present short-span serological observations showed that the antibody response against Em18 reflected the effect of the treatment immediately after surgery, and Em18 is considered to be a useful antigen. My new rat model can also be used to characterize the serological changes occurring after resection against other antigens such as EmII/3 and Em2, and other antibody subtypes such as IgM.

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ACKNOWLEDGMENT

I wish to pay sincere acknowledgement to Professor Tomohiro Imagawa, Department of Veterinary Clinical Medicine, Joint School of Veterinary Medicine, Tottori University, for his supervising and encouragement during the present investigation.

I also wish to thank Professor Hiroshi Sato, Joint Faculty of Veterinary Medicine, Yamaguchi University; Professor Yoshiharu Okamoto, Department of Veterinary Clinical Medicine, Joint School of Veterinary Medicine, Tottori University; Professor Takehito Morita, Joint School of Veterinary Medicine, Tottori University; Associated Professor Takeshi Tsuka, Joint School of Veterinary Medicine, Tottori University; Tomohiro Osaki, Joint School of Veterinary Medicine, Tottori University, for their helpful suggestion as co-supervisor.

I am grateful to Professor Akita Ito, Asahikawa Medical University; Professor Munehiro Okamoto, Kyoto University; Professor Yasuhito Sako, Asahikawa Medical University; Associated Professor Kazuhiro Nakaya, Asahikawa Medical

University; Associated Professor Tetsuya Yanagida, Yamaguchi University.

Finally, I wish to express special thanks to my family, especially to my parents Tomohisa and Sachiko for their hearty supports.

PUBLICATIONS

The contents of this thesis were published in the following journals.

Chapter I

Masamichi Yamashita, Tomohiro Imagawa, Kazuhiro Nakaya, Yasuhito Sako, Yoshiharu Okamoto, Takeshi Tsuka, Tomohiro Osaki, Munehiro Okamoto, and Akira Ito: *Echinococcus multilocularis*: Single hepatic lesion experimentally established without metastasis in rats. Experimental Parasitology, 135: 320-324, 2013

Chapter II

Masamichi Yamashita, Tomohiro Imagawa, Yasuhito Sako, Munehiro Okamoto, Tetsuya Yanagida, Yoshiharu Okamoto, Takeshi Tsuka, Tomohiro Osaki, and Akira Ito: Serological validation of an alveolar echinococcosis rat model with a single hepatic lesion. Journal of Veterinary Medical Science, in press.